Genetics of adaptation to low-water-activity stress in *Escherichia coli*: identification and characterization of insertion mutations conferring NaCl-sensitivity

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CERTIFICATE

The research work presented in this thesis has been carried out at the Centre for Cellular and Molecular Biology, Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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Dedicated to my parents,
without whom none of this
would have been possible.

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ABBREVIATIONS

Abbreviations for standard (SI) units of measurement, and chemical formulae and amino acid three-letter codes are not included in the list below.

A	-	Absorbance
Amp	-	Ampicillin
cAMP	-	Cyclic adenosine-5' monophosphate
bp	-	Base-pairs
CAA	-	Casamino acids
°C	-	Degrees in Centigrade
cfu	-	Colony-forming unit
Cm	-	Chloramphenicol
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylene diamine tetra acetic acid
EG	-	Ethylene glycol
h	-	hour(s)
IPTG	-	Isopropyl-β-D-thiogalactoside
Kan	-	Kanamycin
kb	-	kilobase-pairs
M	-	Molar
MA	-	Minimal A medium supplemented with 0.2% glucose
MIC	-	Minimal inhibitory concentration
min	-	minute
MNNG	-	*N*-Methyl-*N*'-Nitro-*N*-Nitrosoguanidine
MOI	-	Multiplicity of infection
ORF	-	Open reading frame
PCR	-	Polymerase chain reaction
pfu	-	Plaque-forming unit
RNA, mRNA, tRNA	-	Ribonucleic acid, messenger RNA, transfer RNA
rpm	-	Revolutions per minute
RT	-	Room temperature
Spec	-	Spectinomycin
Str	-	Streptomycin
Tet	-	Tetracycline
U	-	Units
UV	-	Ultra-violet
WT	-	wild-type
Xgal	-	5-Bromo 4-Chloro 3-Indolyl-β-D-thiogalactoside

The superscripts 'R' and 'S' represent 'resistance' and 'sensitivity' respectively.

Abstract

Bacterial species are perhaps the most versatile of all living organisms, inhabiting almost every environmental niche known. Amongst other properties, the ability to adapt to changes in the osmolarity of the external environment is one that is very important for the growth and survival of bacteria, and for this purpose they have evolved a number of osmoadaptive strategies. The cellular mechanisms for adaptation to water stress appear to be remarkably similar in the diverse biological kingdoms.

The cytoplasmic membrane of bacteria is permeable to water but forms an effective barrier for most solutes in the medium and metabolites in the cytoplasm. A lowering of the external water activity (hyper-osmotic conditions) leads to a rapid efflux of water and loss of turgor, resulting in plasmolysis (i.e., retraction of the cytoplasmic membrane from the cell wall). Upon hypo-osmotic shock, water flows into the cell and increases the cytoplasmic volume and / or turgor pressure. Osmoregulation refers to the set of physiological processes by which organisms adapt to survive and grow in environments of varying water activity.

The availability of ingenious genetic and biochemical techniques, combined with the ability of *Escherichia coli* to survive in quite a wide range of osmolarities, makes it useful model system to understand the mechanisms of adaptation. Members of *Enterobacteriaceae,* including *E. coli,* accumulate 'compatible solutes' either by increased synthesis and / or by increased active transport when grown in conditions of high osmolarity. Glutamate and trehalose accumulate following increased rate of biosynthesis, whereas potassium ions, proline and glycine betaine accumulation occurs by active transport from the medium. The majority of the studies undertaken earlier by various groups had focused on osmoresponsive genes in bacteria, and much less was known about mutations that confer osmosensitivity phenotype. In *E. coli*, mutations in trehalose biosynthesis (*otsA*) and Na^+/H^+ antiporter (*nhaA*) conferred sensitivity to osmotic and salinity stress, respectively.

The studies reported in this thesis were initiated to understand, by molecular genetic approaches, the mechanisms of cellular adaptational pathway(s) to low-water-activity (hyperosmotic) stress in *E. coli*. The specific aims of the present project were to identify and characterize null insertion mutations in *E. coli* which confer NaCl-sensitivity. The strategy involved generation of a population of *E. coli* carrying transposon insertions at random sites in the genome using λp*lac*Mu55 (which encodes kanamycin-resistance

and is capable of forming promoter-*lac* transcriptional operon fusions). The starting strain used for transposon mutagenesis harbored a mutation in *gltBD* (encoding the glutamate synthase, GOGAT) which conferred partial NaCl-sensitivity to the strain. The populations of transposon insertion mutants were then subjected to successive rounds of ampicillin-selection in medium supplemented with a sufficiently high NaCl concentration, so as to enrich for NaCl-sensitive mutants.

Eighteen NaCl-sensitive mutants were isolated following the above strategy. It was established that the NaCl-sensitivity phenotype in each case was linked 100% to the *lac*-Kan insertion. Inverse polymerase chain reaction strategy was standardized and successfully employed to characterize at the molecular level 16 of the NaCl-sensitive mutants. The junction sequences were used to determine the gene disrupted in each case. The physical data were confirmed by genetic methods (transductional mapping approach). The screen identified new loci which were hitherto not known to be involved in NaCl-sensitivity viz., *apaG, argP, glnE, lysC,* and *thiI*. The screen also yielded mutants harboring null insertions in *proU* and *speC* genes that have previously been implicated in the NaCl-sensitivity phenotype. Isolation of *proU* and *speC* mutations conferring NaCl-sensitivity in the present screen served to validate the rationale of the experimental approach adopted for the screen (Chapter 3).

Some of the genes identified in this study conferred NaCl-sensitivity only in the *gltBD*$^-$ background (*proU, argP* and *glnE*), while others conferred the phenotype even in a *gltBD*$^+$ background (*lysC, apaG, speC* and *thiI*). Based on their differential sensitivity to other dissolved solutes, all the NaCl-sensitive mutants were classified as osmosensitive.

To understand the osmotic stress adaptational pathways in *E. coli*, the various NaCl-sensitive mutants were further characterized (Chapter 4). Two mutants harboring transposon insertions in *proU* locus (encoding the high affinity glycine betaine system) conferred osmosensitivity in a *gltBD* background. The results, which were consistent with earlier reports of the *proU* locus in the osmoadaptation of *E. coli* indicated that *gltBD* and *proU* act independently and additively in conferring NaCl-sensitivity.

The apparent osmosensitivity phenotype of *apaG* mutant was attributed to the failure to express *apaH* (which encodes Ap_4A hydrolase) gene due to polarity effect of the transposon insertion in *apaG*. However, the *apaH* mutant was also more sick than any of the control strains on minimal medium even without osmotic stress, and it was concluded that the apparent osmosensitivity of the *apaH* mutant is merely a manifestation

of the slow-growing nature of the mutants and not a true defect in water stress adaptation. Similar results were observed when the *apaG* mutant was tested, with the difference that it grew slightly better than *apaH* on both non-stressed and osmotically stressed media.

Based on the finding that *apaG* and *apaH* mutants were identified as NaCl-sensitive simply because they were "non-specifically" sick, a set of criteria was evolved for defining true osmosensitivity (involving comparisons of the test strains with a panel of strains of increasing non-specific sickness, including *recA, recB, ruvABC* mutants). When these criteria were applied to the other novel NaCl-sensitive mutants obtained in this study, viz., *argP, glnE, lysC, proU, speC* and *thiI,* all of them were established to be truly osmosensitive.

Null mutations in the *glnE* gene (encoding adenyltransferase, which controls the catalytic activity of glutamine synthetase, GS) in a *gltBD* background were shown to confer osmosensitivity. The growth promotion of *glnE gltBD* strain by aspartate (which is known to increase the internal glutamate pools) on media of low water-activity, and the fact that growth of the *glnE gltBD* strain was crippled on media supplemented with poor nitrogen sources pointed to the probable impairment in the double mutant of ammonia assimilation and glutamate synthesis, both of which are intimately connected in *E. coli.* In the double mutant, because of the absence of the modifying adenyltransferase enzyme, the catalytic activity of the GS enzyme may be constitutive, resulting in an uncontrolled shunting / channeling of glutamate to glutamine in a manner which is insensitive to the intracellular glutamine to 2-ketoglutarate ratio. This might result in impaired NH_4^+ assimilation leading to low intracellular glutamate pools and osmosensitivity.

A loss of function mutation in *lysC* (encoding aspartokinase-III which is one of three isozymes that catalyzes the first step in the common biosynthetic pathway for lysine, methionine and threonine) was obtained which conferred osmosensitive phenotype in *E. coli.* Mutations in *thrA* (which encodes aspartokinase-II) did not confer osmosensitivity, suggesting that it is the LysC function which is essential for cell survival under low water activity conditions. Osmosensitivity of a *lysC* mutant was exacerbated in the presence of exogenous methionine or threonine [which decrease, respectively, AK-I (MetL) and AK-II (ThrA) activities], indicating that at least two AK isoenzymes (one of which should necessarily be LysC) need to be functionally active for a strain to survive under hyperosmolarity conditions. In a *lysC* mutant, some of the intermediates of the biosynthetic pathway may be limiting on a high osmolarity medium (but sufficient enough for growth on non-stressed media), resulting in osmosensitivity phenotype.

Another possibility assumes that AK-III has an additional novel function (apart from its catalytic phosphorylation of Asp for the synthesis of three amino acids), which is essential for *E. coli* to survive under low a_w stress.

Several independent loss-of-function mutations in *thiI* (which is involved in thiamin biosynthesis and thiolation of uracil in tRNAs) were shown to confer an osmosensitivity phenotype in *E. coli*. These mutants, as expected, also exhibited thiamin auxotrophy and had lost the NUV phenotype. Complementation analysis showed that the C-terminal 18 amino acid region of ThiI is essential for its function. Since ThiI in *E. coli* is proposed to contain a C-terminal extension with sequence similarity to rhodanese-like sulfur transferases, these 18 amino acids may form part of such a sulfur-transferase motif. ThiI has previously been shown to interact with IscS (a cysteine desulfurase involved in the mobilization of sulfur) in the biosynthesis of the thiazole moiety and tRNA modification, but it is not known whether ThiI has any role in IscS-catalyzed Fe-S cluster assembly. It is possible that ThiI plays an accessory role in the formation or repair of [4Fe-4S] clusters in some of the hitherto unidentified proteins, one or more of which may specifically be required for survival in low water-activity medium. Another hypothesis envisages tRNAs themselves as having a prominent role in the cellular hyperosmolar adaptational pathway. Thus, in a *thiI* mutant, the absence of thiolated tRNAs may result in the deficiency of gene function(s) needed for water stress adaptation. It is also plausible that in a *thiI* mutant, defective translation of a particular mRNA may lead to the absence of a critical regulatory protein, resulting in NaCl-sensitivity phenotype.

Mutations in *speC* gene (which encodes the enzyme ornithine decarboxylase catalyzing the conversion of ornithine to putrescine) were shown to confer osmosensitivity, which was relieved by supplementation with arginine, citrulline, ornithine, or the polyamines (putrescine or spermidine), as also by introduction of an *argR* mutation. It has been previously reported that under conditions of high osmolarity, one polyamine molecule with two positive charges is extruded for accumulation of two potassium ions, thereby maintaining electrical neutrality even as the intracellular osmolarity is increased for turgor restoration. Based on the results, it is proposed that the cellular polyamine pools are critical for K^+ accumulation during osmoadaptation.

Results were also obtained in this study to implicate *argP* (which encodes a LysR-type of transcriptional regulator) in ammonia assimilation, osmoadaptation and arginine metabolism in *E. coli*. The growth promotion of *argP gltBD* by aspartate on media of low water-activity pointed to the probable impairment in the double mutant of ammonia

assimilation and glutamate synthesis. The fact that the *argP gltBD* mutant is severely compromised for NH_4^+ assimilation even in the absence of osmotic stress provided strong support to the hypothesis that it is an inability to synthesize sufficient glutamate that is responsible for the osmosensitive phenotype of the double mutant.

Null mutations in *argP* conferred extreme sensitivity to canavanine, which is a toxic analog of arginine. Point mutations in *argP* had previously been shown to confer a canavanine resistance phenotype and various gain-of-function *argP* point mutations conferring canavanine-resistance were also obtained in this study following chemical mutagenesis; their molecular characterization revealed that they harbored mutations in different predicted domains of ArgP.

Subsequently, it was demonstrated that null insertions in a hitherto uncharacterized ORF, *yggA*, also conferred canavanine supersensitivity. Analysis of the YggA amino acid sequence predicted that it is an extremely hydrophobic protein and may contain six transmembrane helices. YggA shows homology to members of membrane-associated exporters, which are involved in the efflux of basic amino acids, like arginine and lysine. The results indicated that YggA may be involved in the export of Arg (and related compounds), and accordingly, the name ***argO*** for (Arg outward transport) is proposed for *yggA*.

The *yggA*::*lacZ* expression *in vivo* was shown to be abolished in an *argP* mutant, providing strong evidence that ArgP is the transcriptional activator of *yggA*. This result provided the first *in vivo* evidence for the activator role of the previously predicted transcriptional regulator, ArgP. It was also identified in this study that arginine, citrulline, ornithine and canavanine induced *yggA* expression while lysine repressed it, all via ArgP. The canavanine-resistance phenotype of the various *argP trans*-dominant alleles could be a result of increased efflux of canavanine through YggA, following constitutive transcription of the *yggA* gene in these strains.

Exogenous lysine addition phenocopied the mutation in *argP* for all the three phenotypes tested viz., ammonia assimilation, osmosensitivity, and *yggA* gene expression. Two alternative hypotheses (not mutually exclusive) were considered to explain the physiological role of YggA. (i) One possible role may be to ensure a correct balance between intracellular arginine and lysine pools. When the former goes up, arginine efflux is activated, while when the latter goes up, it is inhibited. Lysine might have a repressing role on *yggA* expression to fine-tune the regulation of levels of basic amino acids (like lysine and arginine) inside the cell. (ii) The other physiological function of YggA protein

may be to protect the cells from the toxic effects of canavanine, which is a natural (plant-derived) anti-bacterial agent. Thus, under conditions of growth in the presence of canavanine, the YggA protein may function to efflux the canavanine molecules which enter the cell through Arg uptake systems.

In order to gain greater insights into the mechanisms by which the various genes identified in the present study might act in conferring low water activity stress adaptation, NaCl-tolerant suppressors of various osmosensitive mutants were sought by a localized P1 mutagenesis approach (Chapter 6). Broadly three categories of suppressors of the various osmosensitive mutants were obtained: one class was the crossing out of the Kan^R insertion with the chloramphenicol-resistance marker-linked WT gene (for *thiI, glnE,* and *lysC*); the second class was the crossing out of Δ*gltBD* with the *gltBD*$^+$ genes (for *argP* and *glnE*); and the third category represented mutations specific to each suppressor (including *argR* for *speC*). These results in one way served to bolster the confidence with which one could implicate these genes in water-stress adaptation. Especially for *thiI* and *lysC*, the fact that the majority of the NaCl-tolerant colonies had swapped the Kan^R insertion for the WT gene suggested the importance of these genes in low water-activity stress adaptation. For genes like *argP* and *glnE*, the fact that almost all the suppressors had become *gltBD*$^+$, indicated a strong synergism between these genes on the one hand and *gltBD* on the other in conferring water-stress sensitivity.

The Appendix describes the genome-wide transcriptional profiling of the wild type strain and osmosensitive mutants of *E. coli* in response to low water activity stress. In the wild type strain, approximately 4% and 2.4% of genes showed >3-fold induction and repression, respectively, under high NaCl growth conditions. Analyses of the results revealed many genes involved in iron uptake and cysteine biosynthesis to be differentially regulated under water-stress conditions. In an *argP* mutant, the levels of genes involved in arginine metabolism and transport were differentially expressed. In the *thiI* mutant, some of the genes encoding proteins that require Fe-S clusters for their activity were downregulated.

In summary, eighteen NaCl-sensitive mutants were isolated following whole genome transposon mutagenesis. Based on their sensitivity to other dissolved solutes, they were all classified as osmosensitive mutants. Molecular characterization of these mutants revealed that at least five novel hitherto unknown genetic loci (*argP, glnE, lysC, speC* and *thiI*) were involved in osmotolerance in *E. coli*. Null mutations in *speC*

(encoding ornithine decarboxylase involved in polyamine biosynthesis) was shown to be osmosensitive, indicating that polyamine pools play an important role in potassium ion accumulation during osmoadaptation. Mutations in *glnE* (encoding adenyltransferase, involved in controlling the catalytic activity of glutamine synthetase) and *argP* (encoding LysR-type transcriptional regulator) were shown to confer osmosensitivity only in *gltBD* mutants (deficient in glutamate synthase), and evidence was obtained that impaired ammonia assimilation and glutamate biosynthesis was responsible for the osmosensitivity phenotype. Null mutations in *thiI* (which is involved in sulfur transfer reactions in thiamin biosynthesis and thiolation of tRNAs) conferred growth inhibition on low-water-activity media, suggesting that either defective iron-sulfur cluster assembly or an inability of the undermodified tRNAs to participate in proper translation or gene regulatory functions may be responsible for osmosensitivity. Loss-of-function mutations in *lysC* (encoding aspartokinase III involved in the biosynthesis of lysine, methionine and threonine) resulted in osmosensitive phenotype either due to limitation of these amino acids or their pathway intermediates. Furthermore, ArgP was shown to activate the transcription of a previously uncharacterized ORF, *yggA* (***argO***), providing the first direct *in vivo* evidence for the activator role of the transcriptional regulator.

Chapter 1

Introduction

1.1 Introduction to water-stress adaptation:

Bacteria have evolved a remarkable repertoire of mechanisms to adapt to environmental changes. The successful occupation by microbes of hostile environments uncongenial to other life forms has been attributed to the presence of complex stress management strategies that have evolved to allow the bacterial cell to sense and respond to changes in its external environment. One such stress encountered by bacteria is biological water-stress, that is, an environmental osmolarity or water activity that is not optimal for growth.

1.1.1 Some definitions:

The terminology commonly employed in the study of biological water-stress adaptation is described in this section (Wood, 1999).

Osmolality: is the osmotic pressure of a solution at a particular temperature, expressed as moles of solute per kilogram of solvent (osm).

Osmolarity: is an approximation for osmolality, expressed as moles of solute per liter of solution (osM). Osmolarity is calculated as the sum of the concentrations of the osmotically active solutes in a solution.

Osmoprotectants: are compounds that stimulate bacterial growth in high-osmolality media.

Compatible solutes: are cytoplasmic solutes whose level can be modulated over a broad range without disrupting cellular functions.

Osmoregulatory response: is a physiological process that mitigates passive adjustments in cell structure caused by changes in the extracellular osmolality.

Osmotolerance: The osmolality range for the media that supports bacterial growth.

Osmosensor: is a device that detects either the changes in extracellular water activity (direct osmosensing) or the resulting changes in cell structure or composition (indirect osmosensing).

Osmotic downshift: is a decrease (over time) in the osmolality of the extracellular environment.

Osmotic upshift: is an increase (over time) in the osmolality of the extracellular environment.

Water activity (a_w): is defined as the ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure at the same temperature of pure water. Values for a_w range from 0 to 1 and are unitless. Pure water has an a_w of 1.00.

The a_w is effectively the concentration (or mole fraction) of water in a solution. It is related to osmotic pressure π as follows:

$$\pi = (RT/V_w) \ln a_w$$

where, R is the gas constant, T is the absolute temperature and V_w is the partial molal volume of water.

1.1.2 Osmoregulation

Adaptability to change in the environment is of crucial importance for the survival of any living organism. These adaptational mechanisms can be divided into three phases: (i) perception of an external signal, (ii) intracellular transmission of this signal, and (iii) the consequent adaptive response.

The biological membrane is readily permeable to water but presents a more effective barrier to most other solutes. When the external concentration of water (water activity, a_w) changes because of increases or decreases in the concentrations of extracellular solutes that are excluded by the membrane, water moves out of or into the cells. Since bacterial cell walls are rigid and can withstand pressures up to 100 atm, hypo-osmotic shock generally results only in minor increases in cell volume but a considerable increase in the turgor pressure (Stock *et al.*, 1977). Hyper-osmotic shock, on the other hand, causes considerable shrinkage of the cytoplasmic volume, leading to plasmolysis of the cells (Koch, 1982).

Osmoregulation refers to the active processes carried out during adaptation to the osmotic strength of the environment. The term osmoadaptation describes both the physiological and genetic manifestations of adaptation to low and high a_w environments (Galinski, 1995). There are remarkable similarities between bacteria and plants in their cellular responses to osmotic stress, because organisms from both kingdoms accumulate the same set of cytoplasmic solutes upon exposure to conditions of hyperosmolarity. Thus, it is likely that there will be close parallels in the mechanisms that these organisms employ to regulate responses to osmotic stress. In bacteria, two strategies of osmoadaptation have evolved to cope with elevated osmolarity: (i) the 'salt in cytoplasm' type and (ii) the organic osmolyte type (compatible solute accumulation) (Galinski and

Truper, 1982). Adaptation to hypo-osmotic shock, on the other hand, involves a combination of both specific (secondary transport) and non-specific (stretch-activated channel) solute effluxes followed by aquaporin-mediated water efflux. The physiological

Phase	Structural Change	Approximate Duration	Physiological Change
III	Cell Wall and Nucleoid Remodeled DNA/Protein Synthesis Resume Cell Growth and Division Resume Co-solvent Composition Adjusted	1 or more hours	Osmoresponsive Genes Expressed (e.g. *proP*, *proU*, *kdpFABC*, *betT*) Compatible Solute Uptake/Efflux Cycle Established
II	Nucleic Acid Counterions Replaced Rehydration Begins	20 to 60 minutes	Putrescine Extruded K^+ Glutamate and Compatible Solutes Accumulate Respiration Resumed (Reduced Rate) $\Delta\tilde{\mu}_{H^+}$ Restored ATP Level Restored
I	Cell Dehydrates, Shrinks Cytoplasmic a_w Decreased Cytoplasmic Crowding Increased Wall/Membrane Strain Altered	1 to 2 minutes	Respiration and Most Transport Cease; Trk/ProP Activate ApH Increased Transiently ATP Level Increased Transiently
Shift	**Upshift: $\Delta\Pi$ decreased, $\Delta\Pi < \Delta P$, $\Delta\mu_w < 0$** ↑ **Time 0: $\Delta\Pi = \Delta P$, $\Delta\mu_w = 0$** ↓ **Downshift: $\Delta\Pi$ increased, $\Delta\Pi > \Delta P$, $\Delta\mu_w > 0$**		
I	Cell Hydrates, Swells Cytoplasmic a_w Elevated Cytoplasmic Crowding Decreased Wall/Membrane Strain Is Altered	< 1 minute	Channels Open
II	Cell Shrinks Cytoplasmic Crowding Increased	1 to 2 minutes	Co-solvents and Water Extruded $\Delta\tilde{\mu}_{H^+}$ Collapsed?
III	?	10 to 20 minutes	Channels Close $\Delta\tilde{\mu}_{H^+}$ Restored Co-solvents Re-accumulate

Fig. 1.1 Phases of the osmotic stress response in *E. coli* K-12. Structural and physiological responses triggered by osmotic shifts (up or down) imposed at time zero proceed in parallel along the indicated, approximate time-scales. (Adapted from Wood, 1999).

responses triggered by osmotic shifts (up or down) during osmotic stress adaptation in *E. coli* are depicted in Fig. 1.1.

1.1.3 Salt in cytoplasm - the halobacterial solution:

This mechanism, which is typical of members of the *Halobacteriaceae* (Martin *et al.*, 1999), achieves osmotic equilibrium by maintaining a high cytoplasmic salt concentration (KCl) similar to that of the surrounding solution. As a consequence, the cytoplasm is exposed to high ionic strength (up to 7 M KCl) in species of *Halobacterium* (Lanyi, 1974). In comparison with their eubacterial counterparts, halobacterial proteins exhibit extensive amino acid changes, involving enrichment in aspartyl, glutamyl and weakly hydrophobic residues that appear to have been selected in evolution to confer salt tolerance in the latter. Organisms exhibiting the 'salt in cytoplasm' mechanism of osmoadaptation (the *Halobacteriaceae* as well as obligately halophilic eubacterial anaerobes such as *Halobacteroides acetoehylicus*) (Rengpipat *et al.*, 1988) are thus strictly confined to environments of elevated osmolarity.

1.1.4 Compatible solute accumulation:

The decrease in the internal water content under high hyper-osmotic conditions brings about a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. Most organisms including eubacteria and plants respond to hyperosmotic stress by increasing the concentration of K^+ ions and other oraganic osmolytes called compatible solutes. The compatible solute answer to the problem of elevated osmolarity appears to involve a biphasic response viz., short-term / initial and long-term response.

1.1.4.1 Initial response:

The initial response of enteric bacteria to an osmotic upshock involves the uptake of K^+ via Kdp and Trk, the main transport systems for K^+ (Epstein, 1986). To maintain electroneutrality, the accumulation of K^+ is accompanied by decrease in cellular putrescine (Put) pools by efflux and increase in the Glu pools by *de novo* synthesis during growth at high osmolarity (Cayley *et al.*, 1991; McLaggan *et al.*, 1994). It is a transient response, leading to an accumulation of K^+ and Glu. Concomitantly with K^+ uptake, Glu synthesis is strongly induced to counterbalance the accumulated positive charges (Dinnbier *et al.*, 1988). In *E. coli*, Glu accumulation is dependent on K^+, and it is not observed in medium lacking K^+ (McLaggan *et al.*, 1994). However, in *Salmonella*

enterica (formerly *Salmonella typhimurium*), it has been shown that Glu is not required for the accumulation of K^+ ions in the initial response (Yan *et al.*, 1996). K^+ and Glu accumulation is discussed in the Sections 1.1.5 and 1.2.

1.1.4.2 Long-term response (osmoprotectant accumulation):

Given an upper limit of ~400 mM for K^+-Glu accumulation (Dinnbier, 1988; Tempest *et al.*, 1970), the cut-off point for the initial response appears set at ~0.5 M NaCl, at least in the Gram-negative bacteria (Galinski, 1995). Increases in the salt concentration above this level triggers the long-term response, i.e., accumulation of neutral osmoprotectants, which in contrast to the ionic osmolytes of the primary response, can be accumulated to high intracellular concentrations without adversely affecting cellular processes (Brown, 1976; Yancey, 1982). Compatible solutes are so called because they are accumulated to very high intracellular concentrations without being inhibitory to cellular processes. They cannot cross the cell membranes rapidly without the aid of transport systems, do not carry a net electrical charge near pH 7.0, and can be accumulated by bacteria by *de novo* synthesis or by transport from the culture medium. The common compatible solutes are the amino acids Glu, Gln, Pro, γ-aminobutyrate and alanine; the quaternary amines, glycine betaine (Bet) and related fully *N*-methylated amino acid derivatives; and the sugars, sucrose, trehalose, and glucosylglycerol (Flowers *et al.*, 1977; Yancey *et al.*, 1982; Imhoff, 1986; Reed *et al.*, 1986).

In addition to their role as osmotic balancers, compatible solutes function as effective stabilizers of enzyme function, providing protection against salinity, high temperature, freeze-thaw treatment and even drying (Brown, 1976; Galinski and Truper, 1982; Yancey *et al.*, 1982; Lippert and Galinski, 1992; Welsh, 2000). The osmoprotecting efficacy of solutes apparently depends not only on their ability to replace K^+ and Glu but also on other factors, such as their interactions with macromolecules (Yancey *et al.*, 1982).

Bet and proline betaine are synthesized by cyanobacteria or algae and are found in fresh or salt water environments as a result of excretion or leakage from the producing organisms (Mason and Blunden, 1989; Oren, 1990). Because they are abundant plant metabolites, Pro, Bet and proline betaine are present in the diet of many animals and are thus available to intestinal bacteria. Bet, which is used for the maintenance of the osmotic balance of kidney cells, is synthesized in cells of the renal medulla and excreted in urine where it can be taken up by uropathogenic bacteria (Chambers *et al.*, 1987; Chambers and

Kunin, 1987). The various osmoregulatory systems and osmoresponsive genes in *E. coli* are summarized in Tables 1.1 and 1.2. In the following sections, the physiology and mechanisms of solute accumulation and efflux will be discussed.

Table 1.1 Osmoregulatory systems in *E. coli* (from Wood, 1999)

System	Encoding genes	Function	Genetic regulators in *E. coli* K-12
TrkAG(H)E	*trkA, trkG, trkH, sapD*	K^+ uptake	Nil
KdpFABC	*kdpFABC*	K^+ uptake	KdpDE
ProP	*proP*	Osmoprotectant uptake	RpoS, CRP, Fis
ProU	*proVWX*	Osmoprotectant uptake	TopA, GyrAB, IHF, HU, H-NS
BetTBA	*betTBA*	Choline uptake and oxidation	BetI, ArcA
OtsBA	*otsBA*	Trehalose synthesis	RpoS
BetU	*betU*	Betaine uptake	Unknown
MscL	*mscL*	Solute efflux (postulated)	Unknown
MscS	*mscS*	Solute efflux (postulated)	Unknown

Table 1.2 Osmoresponsive genes and operons in *E. coli* (from Wood, 1999)

Gene or operon	Encoded function	Locus-specific transcriptional regulator(s)
aqpZ	Aquaporin	
betTIBA	Choline uptake and oxidation	BetT
kdpABCDE	Potassium transport and transcriptional regulation	KdpD/KdpE
mdo	MDO synthesis	
ompF	Porin	EnvZ / OmpR
ompC	Porin	EnvZ / OmpR
osmB	Putative lipoprotein	
osmC	Putative lipoprotein	
osmE	Unknown	
osmY	Unknown	
otsAB	Cytoplasmic trehalose synthesis	
proP	Compatible-solute transport	
proU	Compatible-solute transport	
stpA	Nucleoid protein	
treA	Periplasmic trehalose hydrolysis	

1.1.5 K^+ ions:

K^+ ions are the most prevalent cations in the cytoplasm of bacteria (Christian and Waltho, 1961) and consequently, they serve as one of the major intracellular osmolytes that maintain turgor. The intracellular concentration of K^+ in a wide assortment of bacterial species has been found to be nearly proportional to the osmolarity of the growth medium (Epstein and Schultz, 1965; Measures, 1975; Reed and Stewart, 1985) and there is a positive correlation between the intracellular content of this cation and the ability of bacteria to tolerate conditions of high osmolarity (Christian and Waltho, 1961). Epstein and Schultz (1965) had found that in exponentially-growing *E. coli*, the steady-state intracellular concentration of K^+ increased from 0.15 to 0.55 M as the osmolarity of the medium was increased from 0.1 to 1.2 osM. Increased accumulation of K^+ was elicited only by high concentrations of impermeable solutes but not by glycerol, which is freely permeable across the cell membrane. It was suggested that the signal for enhanced K^+ accumulation is not the decrease in the intracellular water activity *per se*, but the loss of turgor or possibly the reduction in cytoplasmic volume.

1.1.5.1 K^+ uptake:

K^+ uptake has been well characterized in Gram-negative bacteria. *E. coli* possesses four constitutive low-affinity K^+ transport systems: TrkG, TrkH, Kup (formerly TrkD) and TrkF, as well as one inducible high-affinity system, Kdp. Osmotically-induced accumulation of K^+, representing the primary or initial phase of osmoadaptation, is mediated by rapid activation of low- and high- affinity systems in both Gram-positive and Gram-negative bacteria.

1.1.5.2 TrkG and TrkH:

In media containing > 1 mM K^+, the predominant uptake system is Trk. Encoded by constitutively expressed genes dispersed on the chromosome, K^+ uptake, previously attributed to TrkA, is now known to be mediated by two integral membrane-bound proteins, TrkG and TrkH (Dosch *et al.*, 1991). TrkA is believed to regulate *trkG/H*, mediating activation by ATP, or by acting as a protein kinase (Bossemeyer *et al.*, 1989b). In addition to TrkA, *trkE* (now called *sapD*) represents a further regulatory domain which, when disrupted, eliminates and impairs K^+ transport via TrkH and TrkG, respectively (Parra-Lopez *et al.*, 1993, 1994). K_m values for K^+ uptake via TrkG and

TrkH are 0.3-1 mM and 2.2-3.0 mM. As with Kdp (see below, Section 1.1.5.4), in addition to contributing to the salt stress response, Trk also plays a role in K^+ homeostasis. Osmotic regulation of Trk is mainly at the level of transport activity.

1.1.5.3 Kup:

The Kup system (formerly called TrkD) represents a low affinity K^+ uptake system. Distinguished from the other systems by its ability to transport cesium (Bossemeyer *et al.*, 1989a), Kup exhibits a K_m of 0.3-0.4 mM. It has a modest uptake rate and affinity for K^+ (Rhoads and Epstein, 1978). Osmotic upshock stimulates K^+ influx approximately two-fold via Kup.

1.1.5.4 Kdp:

Under conditions of K^+ limitation or severe osmotic upshock (which results in loss of turgor), the high affinity Kdp transport system becomes functionally active. The membrane-associated Kdp-ATPase (KdpFABC) is encoded by the *kdpFABC* operon.

The Kdp system is highly specific for K^+, exhibiting a K_m of 2 μM and a V_{max} of 150 μmol min^{-1} g^{-1} cells (Rhoads *et al.*, 1976; Epstein *et al.*, 1978). A member of the P-type ATPases, the driving force for K^+ uptake via Kdp comes from ATP hydrolysis. A complex consisting of the KdpA, B and C proteins has been shown to have a K^+-dependent ATPase activity (Siebers and Altendorf, 1988). This complex has been purified and the hydrolysis of ATP has been shown to involve the phosphorylation of the KdpB protein (Siebers and Altendorf, 1989).

Osmotic regulation of the Kdp system occurs at the levels of both transcription and enzyme activity. Located at the promoter-distal end of the *kdpFABC* operon, the *kdpDE* regulatory genes encode respectively, the sensor kinase KdpD and soluble transcriptional activator KdpE that constitute the two-component regulatory system, KdpDE for activation of the *kdpFABC* transcription. The *kdpDE* genes are expressed as an operon from a promoter located within *kdpC*; however read-through from the upstream *kdp* promoter has also been observed (Polarek *et al.*, 1992; Voelkner *et al.*, 1993). Kdp thus serves as an osmotically-inducible system scavenging K^+ when the ion is present at low concentrations. Although involved in protecting the cell from the detrimental effects of elevated osmolarity, the primary function of the Kdp system is the maintenance of K^+ homeostasis particularly at limiting K^+ concentration.

Three alternative hypotheses have been proposed for the signal that controls activation of *kdpFABC* expression via KdpDE viz., (i) the cell turgor (Laimins *et al.*, 1981), (ii) the rate of K^+ influx (Asha and Gowrishankar, 1993), and (iii) the size of a postulated special pool of cytoplasmic K^+ (Gowrishankar, 1987), but the question of the environmental signal for *kdp* regulation has not been conclusively answered so far.

It has been shown that the K^+ concentration inside the cell is also regulated by the K^+ efflux systems. Bakker *et al.* (1987) have provided evidence for the presence of at least three efflux systems, two of which (KefB and KefC) have been genetically characterized. Mutations in both *kefB* and *kefC* do not affect Bet-induced K^+ efflux under high osmolarity conditions and therefore, the authors have postulated the presence of a third K^+-efflux system whose activity is supposedly modulated by turgor pressure.

1.1.6 Pro and Bet:

The imino acid Pro and the quaternary amine Bet (*N, N, N*-trimethyl glycine) are the most commonly accumulated osmoprotectants in a wide variety of bacteria and plants (Measures, 1975; Yancey *et al.*, 1982; Le Rudulier *et al.*, 1984; Csonka and Hanson, 1991). Both *E. coli* and *S. enterica*, when subjected to osmotic stress, accumulate Pro and Bet through enhanced transport (Le Rudulier and Bouillard, 1983; Dunlap and Csonka, 1985; Strom *et al.*, 1986). For example, growth of *E. coli* in high osmolarity media (>500 mOsm) is promoted, and the upper limit of osmotolerance is increased (up to 0.8 or 0.9 M NaCl), in the presence of submillimolar concentrations of either proline or Bet in the medium.

Pro has been shown to be an osmoprotectant for several bacteria that are capable of producing it for protein synthesis (Csonka, 1989). Proline-overproducing mutants that acquired increased tolerance of osmotic stress have been isolated in *S. enterica* (Csonka, 1981), suggesting that these organsisms could make their own proline in sufficient amounts to overcome osmotic inhibition. But, in wild-type *Enterobacteriaceae*, neither the synthesis (Brady and Csonka, 1988; Hernandez *et al.*, 1983) nor the catabolism of proline (Csonka, 1988) is subject to osmotic control, and these organisms depend on exogenous proline for an osmoprotectant.

Bet can be accumulated to very high concentrations (exceeding 800 mM) in the cytoplasm of osmotically stressed cells by active transport (Larsen *et al.*, 1987). Unlike Pro, which can both be synthesized *de novo* for use in protein synthesis and be metabolized to serve as sole C and N sources, Bet can only be synthesized from choline,

provided the latter is available in the culture medium (see Section 1.1.8) and it cannot be metabolized.

There are three transport systems, namely, PutP, ProP, and ProU for the uptake of proline in *E. coli* and *S. enterica* (Wood, 1988). However, only ProP- and ProU- mediated proline uptake results in osmoprotection (Menzel and Roth, 1980; Csonka, 1982; Gowrishankar, 1985; Gowrishankar, 1986). These two transport systems are also involved in the uptake of Bet and therefore, play important roles in osmotic adaptation (Perroud and Le Rudulier, 1985).

1.1.6.1 PutP:

The major proline permease, PutP (encoded by the *putP* gene), is required for growth of cells on Pro as the sole carbon and / or nitrogen source (Milner *et al.*, 1987). It is a proline / Na^+ symport system (Cairney *et al.*, 1984) with an affinity of 2 μM. Its function in proline catabolism is subject to catabolite repression and is induced by growth in medium containing proline (Ratzkin *et al.*, 1978). Catabolism of proline requires the product of the adjacent gene, *putA*, which codes for a bifunctional enzyme (PutA) having the activities of both proline dehydrogenase and Δ^1-pyrroline-5-carboxylate dehydrogenase. In addition, PutA acts as the repressor of its own synthesis as well as that of PutP (Hahn *et al.*, 1988), and this regulation is unaffected by the medium osmolarity.

1.1.6.2 ProP:

ProP (encoded by *proP*) was initially identified as a minor proline permease in mutants lacking the PutP transport system (Menzel and Roth, 1980; Stalmach *et al.*, 1984), and was subsequently shown also to transport Bet, Pro and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) with similar affinities (Cairney *et al.*, 1985a; Wood, 1998). ProP, which catalyzes the H^+ / osmoprotectant symport, comprises of a single polypeptide (Culham *et al.*, 1993) belonging to an extended superfamily of integral membrane-associated transporters driven by proton motive force. It has a K_m of 300 μM for Pro and 44 μM for Bet under high osmolar conditions, leading to an increase in Bet uptake and reduction in Pro uptake under these conditions when both substrates are present in the medium (Cairney *et al.*, 1985a). On the other hand, ProP has been shown to be the major contributor to osmoprotection by proline (Csonka, 1982).

Transport via ProP is enhanced by a combination of transcriptional induction (two to five-fold) and a five-fold stimulation of the activity of the ProP protein in response to osmotic upshock (Dunlap and Csonka, 1985; Cairney *et al.*, 1985a, Gowrishankar, 1986).

1.1.6.3 ProU:

The existence of a third proline permease, ProU (encoded by the *proU* locus), was inferred from the observation that proline was able to osmoprotect *putP proP* double mutants (Csonka, 1988). Subsequently, it was shown that ProU transports both proline and Bet under high osmolarity conditions (Cairney *et al.*, 1985b). With the aid of *lac* and *phoA* reporter gene-fusion studies, the *proU* locus was identified in both *E. coli* and *S. enterica* as an osmoresponsive locus in several independent studies, whose transcription is induced more than 100-fold in response to an increase in osmolarity of the medium (Cairney *et al.*, 1985b; Dunlap and Csonka, 1985; Gowrishankar, 1985; May *et al.*, 1986). Iso-osmolar concentrations of both ionic and non-ionic impermeable solutes induce *proU* to equivalent degrees. On the other hand, glycerol, ethanol and methanol (which are freely permeable across the cell membrane) do not induce its expression, indicating that the signal for *proU* is truly osmotic and not merely a decrease in water activity of the growth medium (Cairney *et al.*, 1985b; Dunlap and Csonka, 1985; Gowrishankar, 1985; Barron *et al.*, 1986; Gutierrez *et al.*, 1987).

ProU is a multi-component-binding protein-dependent transporter that belongs to a superfamily of prokaryotic and eukaryotic ATP-binding cassette (ABC) transporters (Higgins, 1992). The components of the ProU system are encoded by an operon containing three cistrons: *proV, proW* and *proX*, encoding two cytoplasmic membrane-bound proteins, ProV and ProW, and the periplasmic binding protein ProX (Barron *et al.*, 1987; Higgins *et al.*, 1987; Dattananda and Gowrishankar, 1989; Gowrishankar, 1989; Overdier *et al.*, 1989). Two promoters upstream of *proU* have been identified in *E. coli*: an osmoresponsive promoter P_2 recognized by the σ^{70}-RNA polymerase holoenzyme, situated 190 bp downstream of a second σ^S-dependent promoter P_1 (Dattananda *et al.*, 1991; Manna and Gowrishankar, 1994). In addition, it has been shown that there exists a transcriptional activator site ~200 bp upstream of the P_2 promoter (Lucht and Bremer, 1991, 1994), together with a *cis*-acting negative regulatory sequence within *proV* (Dattananda *et al.*, 1991; Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992). Recent findings suggest that the σ^S-dependent P_1 promoter may be involved in activating *proU* expression during low-temperature growth (Rajkumari and Gowrishankar, 2001).

Maximal Bet uptake by ProU at elevated osmolarities is achieved by a combination of transcriptional induction (>100-fold) and stimulation of transporter activity (Cairney *et al.*, 1985b; reviewed in Gowrishankar and Manna, 1996). Unlike the ProP system, ProU transports Bet with a much higher affinity than proline and exhibits a K_m value for Bet of 1.3 μM.

Transcriptional regulation of *proU*:

As mentioned above, intracellular [K^+] was originally proposed as a possible signal for osmotic activation of *in vivo proU* expression by Sutherland *et al.* (1986); subsequently, Ramirez *et al.* (1989) reported that expression of the operon *in vitro* is increased in proportion to the K^+-glutamate concentration in the assay buffer. Other workers have disputed this proposal based on experiments showing that failure to achieve glutamate (Glu) accumulation in osmotically stressed cells interferes with concomitant K^+ accumulation (Yan *et al.*, 1996) but not with *proU* transcriptional induction (Csonka *et al.*, 1994). Since multiple cellular processes are stimulated by [K^+], it was suggested that the previously observed dependence of *proU* transcription on [K^+] might be a reflection of the general stimulatory effect of the ion on enzymatic reactions, rather than evidence for a specific osmoregulatory signal (Csonka *et al.*, 1994).

DNA supercoiling has also been suggested to function as a regulator of *proU* expression (Higgins *et al.*, 1988; Bhriain *et al.*, 1989). Mutations in *topA* (encoding topoisomerase I) were shown to increase *proU* expression at low osmolarity, while inhibition of DNA gyrase reduced expression of the operon at high osmolarity in *S. enterica* (Higgins *et al.*, 1988). Here too, other workers have suggested that the effects of DNA supercoiling on the expression of *proU* may be the indirect consequence of the pleiotropic effects of supercoiling on transcription, rather than proof of supercoiling as a specific osmoregulatory signal (Pruss and Drlica, 1989).

Mutations in *hns* (*osmZ*) encoding the DNA-binding protein H-NS, resulted in a moderately elevated expression of *proU* at all osmolarities. H-NS binds both to the negative regulatory sequence within *proV* and to the region 200 bp upstream of P_2, and deletion of the former was shown to increase expression of *proU* by up to 25-fold in low osmolarity medium (Dattananda *et al.*, 1991). Mutations in the gene for IHF (integration host factor) on the other hand, decreased the induced level of *proU* expression two-fold (Lucht and Bremer, 1994), while mutations in the genes for the two subunits of HU-B

(another histone-like protein) were shown to reduce both basal and induced levels of *proU* expression (Manna and Gowrishankar, 1994). Since expression of *proU* continues to remain osmotically controlled in strains mutated in H-NS, IHF or HU-B, it was suggested that these proteins function as modulators rather than absolute regulators of *proU* expression (Kempf and Bremer, 1998).

1.1.7 Trehalose:

Trehalose, a non-reducing disaccharide of glucose, has been observed to accumulate and contribute up to 20% of cytoplasmic osmolarity in *E. coli* cells grown in media of high osmolarity (Larsen *et al.*, 1987). This sugar has been postulated to provide stability to the membrane under dehydrating conditions (Crowe *et al.*, 1984). It is synthesized in a two-step pathway catalyzed by enzymes encoded by the σ^s-dependent *otsAB* operon, that is induced 5- to 10-fold at high osmolarity (Hengge-Aronis *et al.*, 1991). The *otsA* and *otsB* genes encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase respectively, and mutations in either of these genes confers an osmosensitive phenotype (Giaever *et al.*, 1988). The activities of both enzymes are stimulated at high osmolarity. Furthermore, the *otsA*-encoded enzyme has been shown to be stimulated *in vitro* by 0.1 to 0.6 M K-Glu, suggesting that K-Glu regulates its activity (Giaever *et al.*, 1988).

E. coli can utilize exogenously-supplied trehalose as the sole carbon and energy source at both low and high osmolarity by two separate pathways (Boos *et al.*, 1987; Rimmele and Boos, 1994), but it is not taken up uncleaved for use as an osmotic solute under the latter conditions. The first pathway operates only during growth under low-osmolarity conditions, and is induced by the presence of trehalose in the medium. It involves the trehalose-specific PTS-mediated transport (which requires the product of the *treB* gene), and further degradation inside the cell requires the activities of a phosphatase (encoded by *treE*) and an amylotrehalase (encoded by *treC*). This pathway is repressed under high-osmolarity conditions, thereby ensuring that the internally synthesized trehalose under these conditions is not catabolized. However, there is a second pathway which is specifically induced in media of high osmolarity, and which requires the *treA* gene product, trehalase. Trehalase, which is present in the periplasmic space, catabolizes trehalose into glucose, and the glucose thus formed is transported into the cell via the PTS-mediated transport. Thus, the internally synthesized trehalose is effectively

employed as a compatible solute while the external trehalose (when available) is used for metabolic purposes (Gutierrez *et al.*, 1989; Styrvold and Strom, 1991). Thus, studies on trehalose synthesis, transport and catabolism reveal how the same metabolite can be distinctly used for two different functions at the same time.

1.1.8 Choline:

Choline can also act as an osmoprotectant, but through an indirect mechanism. Although incapable of *de novo* Bet synthesis, *E. coli* can take up exogenously provided choline (through the high-affinity transport system BetT, encoded by the *betT* gene, and the low-affinity system, ProU) (Lamark *et al.*, 1991, 1992) and convert it into Bet in a two-step oxidation reaction. Choline is first oxidized to glycine betaine aldehyde by the enzyme choline dehydrogenase (BetA), which is subsequently oxidized to Bet by the catalytic activity of glycine betaine aldehyde dehydrogenase (BetB) (Landfald and Strom, 1986; Styrvold *et al.*, 1986; Andresen *et al.*, 1988). The *betA, betB* and *betI* (which encodes the choline sensing repressor protein, BetI) are arranged in an operon (*betIBA*) located downstream of *betT* on the chromosome. Both gene systems are transcribed divergently under the control of separate, though partially overlapping, promoters (Lamark *et al.*, 1991). *betA, betB* and *betT* genes are all transcriptionally induced (7 to 10-fold) by an increase in the osmolarity of the medium (Eshoo, 1988). Addition of choline (in the absence of Bet) during osmotic stress results in a further induction of *betT* and *betA* by reducing BetI-mediated repression at the promoter region (Rokenes *et al.*, 1996). *betA* and *betB* genes are located very close to the *lac* operon (at 7.5 min on the *E. coli* chromosome), and several Δ*lac* strains of *E. coli* (including MC4100 and its derivatives used in this study) are also deleted for these genes. *S. enterica*, which is naturally deleted for the *lac* locus, also does not have *bet*-locus genes and therefore, cannot utilize choline as an osmoprotectant. However, transfer of *bet* genes from *E. coli* confers choline-mediated osmoprotection in *S. enterica*.

1.1.9 Osmotic responses not involving compatible solute accumulation:

1.1.9.1 Outer membrane porins: OmpC and OmpF:

Porins are outer membrane proteins, which exist as trimers and form fairly nonspecific channels in the outer membrane for the diffusion of hydrophilic molecules of less than 600 kDa. The porins include OmpF, OmpC, LamB and PhoE.

Reciprocal variations in the levels of the porins OmpF and OmpC of *E. coli* are controlled by EnvZ / OmpR two-component regulatory system (Stock *et al.*, 1989; Mizuno *et al.*, 1988; Igo *et al.*, 1989; Mizuno and Mizushima, 1990). Expression of *ompF* is depressed while that of *ompC* is enhanced at elevated osmolarity (Csonka, 1989). MicF, a 174 nucleotide antisense RNA acts as a negative regulator of *ompF* expression at the post-transcriptional stage (Pratt *et al.*, 1996). Although *ompF* and *ompC* are osmoresponsive genes, their role in the physiology of osmoregulation is not clearly yet established clearly. The levels of OmpC and OmpF appear also to respond to a variety of other environmental parameters, including temperature, carbon source, oxygen availability and pH of the medium (Csonka and Hanson, 1991).

1.1.9.2 Aquaporins:

Aquaporins are a large family of membrane channels believed to be involved in osmoregulation. They were first identified in mammalian cells and have now been shown to exist in bacteria. The discovery of aquaporin AqpZ in *E. coli* (encoded by the monocistronic gene *aqpZ*) and other bacteria challenged the view that water enters and leaves bacteria primarily via the phospholipid bilayer (Calamita *et al.*, 1995, 1997, 1998). In *E. coli*, the channel formed by AqpZ is composed of monomers with six membrane-spanning α-helices and has been shown to mediate large water fluxes in response to sudden changes in extracellular osmolarity (Delamarche *et al.*, 1999).

The disruption of *aqpZ* was not lethal for *E. coli* but *aqpZ*-deficient bacteria formed smaller colonies on LB medium and survived less well in liquid medium at 39°C or at low osmolarity than did $aqpZ^+$ bacteria. *aqpZ::lacZ* expression peaked in mid-logarithmic phase and was suppressed up to 30-fold when the bacteria were cultivated in high-osmolality media (Calamita *et al.*, 1998).

1.1.9.3 Membrane-derived oligosaccharides (MDOs):

The periplasmic space of Gram-negative bacteria contains highly anionic polysaccharides, which in *E. coli* are referred to as MDOs (Kennedy, 1982, 1987). Encoded by constitutively-expressed genes, *mdoA* and *mdoB*, these anionic polymers (containing between six and twelve glucose units with an average charge of –5) generate a Donnan potential across the outer membrane, resulting in the accumulation of cations to a higher concentration in the periplasm than in the medium. Their levels decrease with

increasing osmolarity (Miller *et al.*, 1986). Blocking MDO synthesis did not inhibit growth of *E. coli* in media of either high or low osmolarity (Fiedler and Rotering, 1988).

1.1.9.4 Mechanosensitive channels and volume-activated channels:

Solute release or uptake in response to an applied osmotic gradient is often mediated through gated transmembrane channels that open when there is a pressure differential. Two types of these channels exist in bacteria and eukaryotes. Mechanosensitive ion channels (Msc) are gated by membrane tension and are thought to be primary biosensors for osmoregulation in bacteria (Blount and Moe, 1999). Such channels could lead to internalization of K^+ upon hyperosmotic shock. Mechanosensitive or stretch-activated channels (of which MscL, MscM and MscS of *E. coli* are the best characterized members) are, as their name suggests, activated by membrane stretch (Martinac *et al.*, 1990). MscL has been implicated in the release of both K^+ and small proteins such as thioredoxin during osmotic down-shock (Blount *et al.*, 1997; Ajouz *et al.*, 1998).

Volume-activated channels (VACs) share characteristics of anion channels and are likely to play a critical role in the hypo-osmotic response. When cells swell because of a hypo-osmotic shock, the release of cytoplasmic solutes can induce an efflux of water and a return to the original cell volume. VACs, the class of channels that respond to these volume / pressure differentials (Perlman and Goldstein, 1999), appear to serve as the conduit for expulsion of a wide variety of osmolytes (e.g., amino acids, polyols).

1.1.10 Osmosensing:

While much information is available concerning the genetic and physiological responses of bacteria to environmental osmolarity, considerably less is known about the signals regulating these responses. Unlike other biological responses, osmoregulation differs in that the information from the environment is not a specific molecule but a physical parameter.

The possible osmosensing mechanisms include:

(i) Internal hydrostatic pressure

(ii) Membrane pressure differential

(iii) Internal osmolarity

(iv) External osmolarity or water activity

(v) Cytoplasmic membrane area

1.1.11 Osmostress and virulence:

In view of the variety of stresses encountered by pathogenic bacteria during the course of infection, pathogens also might possess additional classes of proteins or contributory factors that are involved in the complex stress management strategies for successful infection.

Gowrishankar and Manna (1996) proposed that *proU* may function as a virulence gene in the pathogenic enterobacteria. Deletion of *proP* dramatically reduced the ability of the pathogenic *E. coli* strains (which cause urinary tract infections and pyelonephritis) to colonize mouse bladders (Culham *et al.*, 1998). Elimination of OpuC (which is a transporter of choline) significantly reduced the ability of *Listeria monocytogenes* strains following peroral administration, to colonize the upper small intestine in mice (Sleator *et al.*, 2001).

Mutations in *ompR* dramatically reduced virulence of both *Shigella flexneri* and *S. enterica*, suggesting a major role for this locus in these pathogens (Dorman *et al.*, 1989; Bernardini *et al.*, 1990). *ompC ompF* double mutants were also severely attenuated for virulence when administered via the oral route but not when administered intravenously. OmpR has been shown to regulate the SsrA-SsrB two-component system in the *Salmonella* pathogenicity island, SPI2, which in turn regulates a type III secretion system required for both murine infection and replication within macrophages (Lee, 2000). It has been suggested that EnvZ, sensing both the low pH and osmolarity of the phagosome, activates OmpR, which in turn stimulates rapid expression of *ssrA* and *ssrB*. The SsrA-SsrB two-component system then detects another signal (possibly mediated by PhoP-PhoQ) and in turn activates expression of the SPI2 type III secretion system (Lee, 2000; Deiwick, 1999).

1.2 Role of Glu in osmoregulation in *E. coli*:

1.2.1 Glu as a compatible solute:

The cytoplasmic levels of Glu increase in most prokaryotes after exposure to media of high osmolarity (Tempest *et al.*, 1970; Brown and Stanley, 1972; Measures, 1975; Yap and Lim, 1983; Botsford, 1984). In Gram-negative bacteria, osmotic stress can elicit greater than 10-fold increase in the levels of Glu, so that this amino acid can account for more than 90% of the free amino acids in the organisms grown in media of

elevated osmotic strength (Tempest *et al.*, 1970). The levels of Gln also increase in response to osmotic stress in Gram-negative bacteria (Tempest *et al.*, 1970; Brown and Stanley, 1972; Csonka, 1981; Le Rudulier *et al.*, 1982), but because Gln is present at much lower levels than Glu, it is probably not important for the maintenance of osmotic balance between cytoplasm and external medium. Since Gln is a precursor of Glu in most bacteria (Tempest *et al.*, 1970), its accumulation in response to osmotic stress may be a consequence of the increased need for the synthesis of Glu.

At low osmolarity, Glu accounts for only about one-fourth of the small anions that are associated with free K^+. At high osmolarity by contrast (when free K^+ levels are themselves elevated), Glu is the major small anion and accounts for between 70 and 90% of free K^+. The rest of the free K^+ is presumably balanced by the pool of anionic intermediates, whose concentrations are not likely to be grossly affected by osmolarity.

The only exception to the general rule of Glu in balancing free K^+ at high osmolarity comes from studies of growth at high osmolarity under nitrogen-limiting conditions, where high pools of K^+ were found but Glu pools remained low (Welsh *et al.*, 1991). These results suggest that the cells use some other anionic osmolyte, perhaps one not containing nitrogen, since nitrogen is the limiting nutrient.

Glu accumulation in high osmolarity-grown cells occurs through increased synthesis. Botsford *et al.* (1994) and McLaggan *et al.* (1994) have argued that the increase in Glu pools in cultures grown at elevated osmolarity represent a very small, and possibly insignificant, load in comparison to the total flux through the biosynthetic pathway.

1.2.2 Ammonia assimilation and Glu biosynthesis:

In *E. coli*, Glu biosynthesis is intimately connected to ammonia assimilation. Ammonia is the preferred source of nitrogen for the growth of enteric bacteria in a defined minimal medium with glucose as the source of carbon. All cellular nitrogen for the synthesis of macromolecules in the enteric bacteria is derived from the amido group of Gln, the amido group of Glu or directly from incorporation of ammonia. About 90% of all nitrogen used by the cell passes through Glu, which provides nitrogen for the synthesis of purines, pyrimidines, amino sugars, His, Trp, Asn, NAD and β-aminobenzoate.

Pathway 1

NH_3 + 2-ketoglutarate + NADPH $\xrightarrow{\text{GDH}}$ Glutamate + $NADP^+$

Pathway 2

NH_3 + glutamate + ATP $\xrightarrow[Mg^{+2}]{\text{GS}}$ Glutamine + ADP + Pi

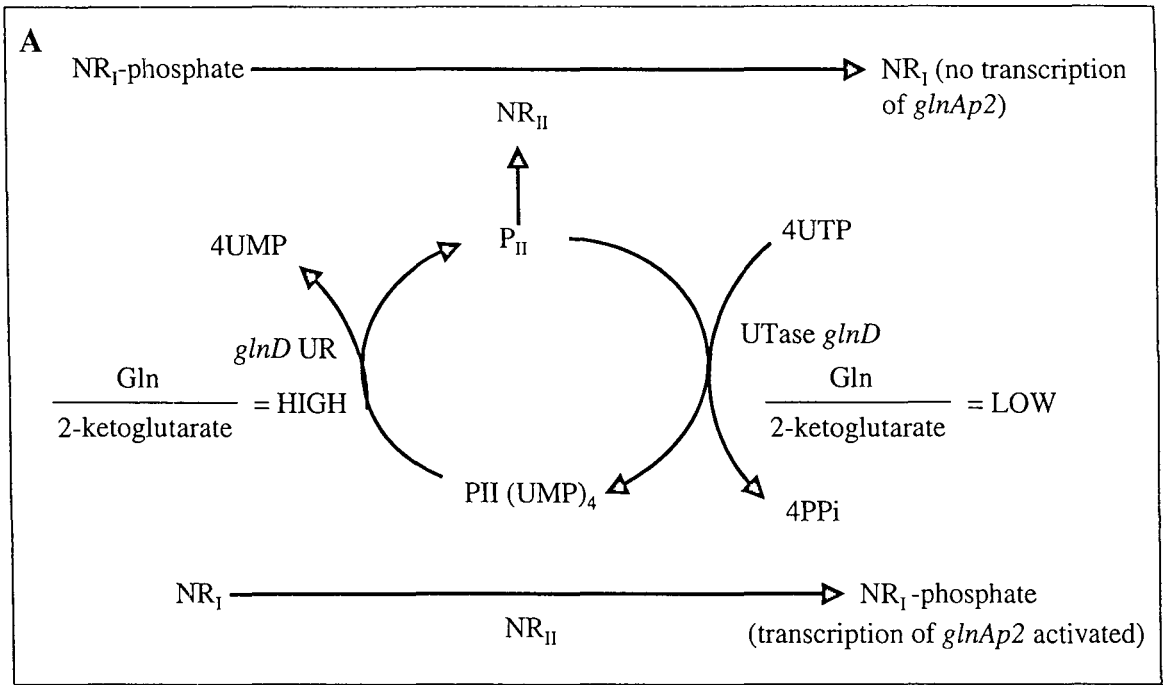
glutamine + 2-ketoglutarate + NADPH $\xrightarrow{\text{GOGAT}}$ 2 Glutamate + $NADP^+$

Net reaction:

NH_3 + 2-ketoglutarate + ATP + NADPH $\xrightarrow{\text{GS-GOGAT}}$ Glutamate + $NADP^+$ + ADP + Pi

Fig. 1.2 Ammonia assimilation and glutamate biosynthetic pathways in *E. coli*.

There are two routes of ammonia assimilation, both of which result in the condensation of NH_3 with 2-ketoglutarate to give Gln (Fig. 1.2). For the first pathway, glutamate dehydrogenase (GDH, encoded by *gdhA* gene) assimilates ammonia and synthesizes Glu. For the second pathway, glutamine synthetase (GS, encoded by *glnA*) along with glutamate synthase [Glutamine:2-oxoglutarate aminotransferase (GOGAT) encoded by *gltBD*] assimilates ammonia and synthesizes Glu. The most important difference between the two pathways appears to be that the former does not consume ATP, while the latter does. The K_m of GDH for ammonia is ~20-fold higher than that for GS. The GDH pathway is associated with the presence of ammonia, and the GS-GOGAT pathway with low ammonia levels or growth with a nitrogen source other than ammonia. Based on these features, it is obvious that at low ammonia levels or with poor nitrogen sources, GS-GOGAT pathway is the only route for Glu synthesis. On the other hand, at high NH_4^+ (when both pathways can operate), it appears that the choice of pathways is determined by the availability of energy to the cells. Helling (1994, 1998) has suggested that the GDH pathway is physiologically advantageous during carbon- and energy-limited growth while the GS-GOGAT pathway is used whenever energy is readily available. Ammonia assimilation involves the regulation of these three enzymes (Fig. 1.3). Each of them is described below in detail.



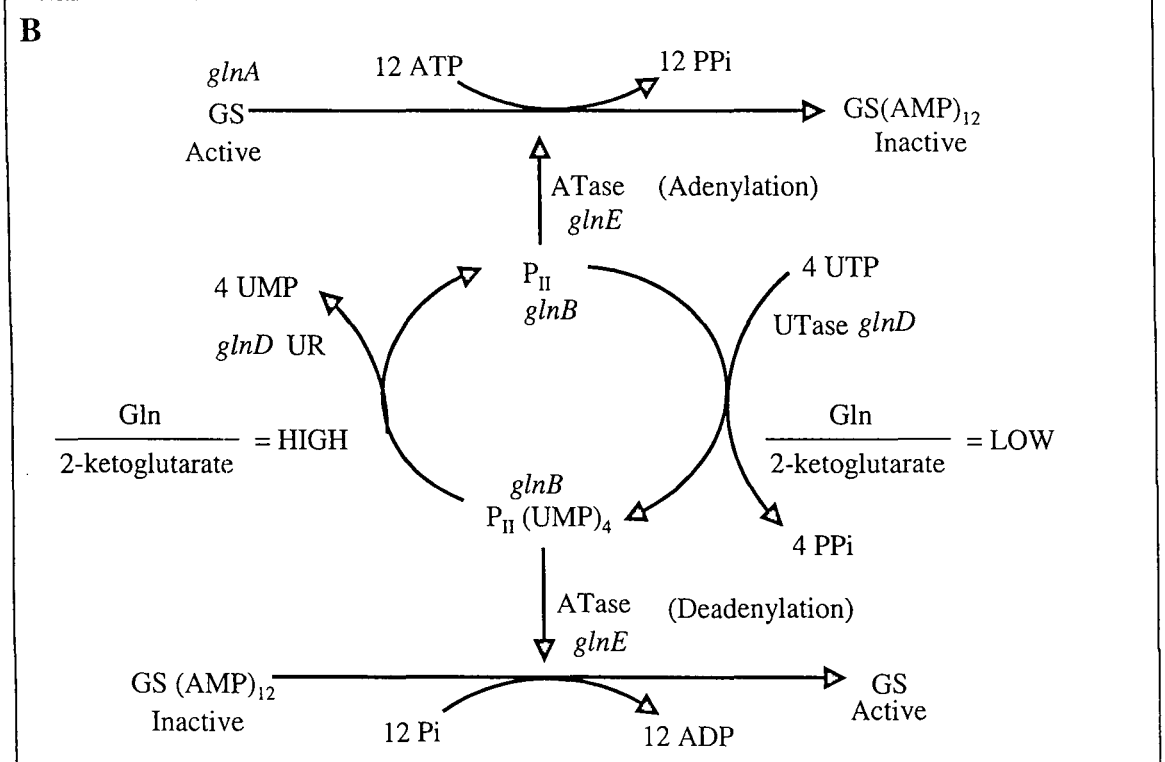


Fig. 1.3A Covalent modification of NRI, the regulator of *glnA* transcription. UR / UTase, uridyl-removing enzyme / uridyltransferase.

Fig. 1.3B Cyclic adenylation and regulation of GS activity. A high ratio of Gln to 2-ketoglutarate triggers adenylation of GS, whereas a low ratio triggers deadenylation. Adenylated GS is inactive, while deadenylated GS is enzymatically active.

1.2.2.1 GDH:

Not much is known about the regulation of GDH activity or synthesis in *E. coli*. Its activity *in vitro* is stimulated by K^+ (Measures, 1975), a finding which has been interpreted as the possible basis of increased Glu levels in high osmolarity-grown cultures. On the other hand, Ohyama *et al.* (1992) have shown that Glu accumulation at elevated osmolarity is unaffected even in cells that fail to accumulate K^+.

1.2.2.2 GS:

GS catalyzes the ATP-dependent synthesis of Gln from NH_3 and Glu, which is the only known biosynthetic route for Gln. When cells are grown in NH_3-containing medium, both GDH and GS-GOGAT synthesize Glu, whereas for cells in medium with a growth-rate-limiting source of NH_3, GDH is not involved in NH_3 assimilation and Glu formation; instead GS-GOGAT is the only active NH_3-assimilating enzyme combination. Therefore, GS has two functions: the synthesis of Gln and (in combination with GOGAT) the assimilation of NH_3, particularly when the growth of the cell is limited by the availability of NH_3 (Reitzer, 1996).

The catalytic activity of GS is regulated by the covalent addition of an AMP group to a tyrosine residue in each of its subunits. The major function of adenylation is to downregulate the activity of GS. When GS is highly adenylated, it is just active enough to supply Gln but not to supply Glu. Adenylation of GS also serves to prevent the depletion of intracellular Glu during the transition to a nitrogen-rich environment (Kustu *et al.*, 1984).

1.2.2.3 Regulation of GS synthesis and catalytic activity in response to nitrogen availability:

The state of the cell with regard to excess or deficiency of nitrogen is reflected in the intracellular ratio of Gln to 2-ketoglutarate. The ready availability of NH_3 increases the rate of synthesis of Gln by GS and the conversion of 2-ketoglutarate to Glu by GOGAT and GDH. Conversely, a deficiency of NH_3 decreases the rate of Gln synthesis and the rate of 2-ketoglutarate utilization. Therefore, growth in a minimal medium with an excess of NH_3 results in a high Gln-to-2-ketoglutarate ratio; and growth in a medium deficient in NH_3 results in a low Gln-to-2-ketoglutarate ratio and a high intracellular concentration of unmodified GS (Senior, 1975).

Enteric bacteria use a complex regulatory system to transmit the information regarding N availability to the systems responsible for the appropriate adjustment of GS activity and the rate of its synthesis. The function of GS is controlled at two levels: (i) transcription and (ii) catalytic activity. Each of these is described in detail below.

A. Regulation of GS synthesis through the nitrogen regulation (Ntr) system:

The regulation of *glnA* gene transcription in response to the availability of N results from the action of four proteins (Fig. 1.3A): (i) uridyltransferase (UTase) / uridyl-removing (UR) enzyme (encoded by *glnD*, it is a bifunctional enzyme which catalyzes the covalent uridylation and deuridylation of P_{II}); (ii) P_{II} (encoded by *glnB*, it is a regulator protein); (iii) nitrogen-regulator I (NR_I, encoded by *glnG / ntrC*, it is the response regulator of the Ntr system); and (iv) nitrogen-regulator II (NR_{II}, encoded by *glnL / ntrB*, it is the histidine kinase of the Ntr regulon), and requires core RNA polymerase and σ^{54} (σ^N) (Backman *et al.*, 1981; Chen *et al.*, 1982; Magasanik, 1982; Pahel *et al.*, 1982; Bueno *et al.*, 1985; Hirschman *et al.*, 1985; Hunt and Magasanik, 1985).

The Ntr-dependent adaptive response to nitrogen starvation involves an increase in the level of GS to enhance the ability of the cells to use low concentrations of ammonia and then the induction of Ntr operon products such as histidase and nitrogenase to provide ammonia from sources of organic N or from atmospheric nitrogen. *glnALG* operon (also known as *glnA ntrBC* operon) plays a central role in the adaptive response of *E. coli* to nitrogen starvation. *glnALG* operon encodes GS (the *glnA* gene product), NR_{II} and NR_I. Starvation for N results in an increase in the transcription of the *glnALG* operon from the *glnAp2* promoter, which requires both NR_I and RNA polymerase containing σ^{54} (encoded by *rpoN / glnF / ntrA* gene).

When the intracellular concentration of 2-ketoglutarate is high, UTase converts P_{II} to P_{II}-UMP, and the consequent reduction in the levels of P_{II} results in NR_{II} catalyzing the conversion of NR_I to NR_I-phosphate (Ninfa and Magasanik, 1986). This phosphorylated protein then activates the initiation of *glnA* transcription at the σ^{54}-dependent promoter *glnAp2*. On the other hand, when the intracellular concentration of Gln is high, UR-enzyme converts P_{II}-UMP to P_{II}; P_{II} causes NR_{II} to remove the phosphate group from NR_I-phosphate and thus, stops the initiation of transcription at *glnAp2* (Ninfa and Magasanik, 1986).

B. Regulation of GS activity: the role of ATase:

The regulation of GS enzymatic activity is accomplished by the action of three proteins (Fig. 1.3B): (i) UTase / UR enzyme; (ii) P_{II}; and (iii) adenyltransferase (ATase). The first two are also common to the mechanism of GS synthesis regulation and have been described in the preceding section. The third, ATase is encoded by *glnE*, and is a bifunctional enzyme which catalyzes the adenylation and deadenylation of GS). The *glnE* gene is located at 68.9 min on the *E. coli* chromosome. *glnE* gene was isolated in *K. aerogenes* as a second site prototrophic revertant of *glnB* mutant. Mutants with lesions in *glnB*, which encodes the regulatory protein P_{II}, are Gln auxotrophs and contain repressed levels of highly adenylated GS. Prototrophic pseudorevertants of a *glnB* mutant acquired an additional mutation in *glnE* (Janssen and Magasanik, 1977). Later, the *glnE* gene was identified in *S. enterica* as a gene whose product affected the covalent modification of GS (Bancroft *et al.*, 1978). *glnE* encodes a 945 amino acid-long polypeptide, with a molecular weight of about 115 kDa (Caban and Ginsburg, 1976). ATase catalyzes the ATP-dependent addition of AMP to a subunit of GS with the release of PPi.

$$GS + nATP \rightarrow GS\text{-}AMPn + nPPi$$

Each subunit of GS can be adenylated so that GS can have twelve adenyl groups. ATase also catalyzes the phosphate-dependent removal of AMP from each subunit of GS.

$$GS\text{-}AMPn + nPi \rightarrow GS + nADP$$

Purified ATase can catalyze both adenylation and deadenylation of GS without the regulatory proteins, P_{II} and UTase / UR (Ginsberg and Stadtman, 1973). The sequence analysis of GlnE indicates that its N-terminal part is highly similar to its C-terminal part (van Heeswijk *et al.*, 1993). Biochemical and genetic evidence indicates that the two activities of ATase are at separate sites on the same polypeptide (Reuveny *et al.*, 1981). Subsequently, Jaggi *et al.* (1997) have shown that its antagonistic activities (adenylation and deadenylation) are catalyzed by different active sites: the deadenylation activity is mediated by the N-terminal part (amino acids 1 to 423) and the adenylation activity is mediated by the C-terminal part (amino acids 425 to 945). The tyrosine residue at position 398 is the target site of reversible adenylation and deadenylation of GS.

The role of P_{II} in regulation of GS activity is different from its role in regulation of GS synthesis (described above). A high intracellular concentration of Gln activates UR, which causes the deuridylation of the regulatory protein, P_{II}-UMP. The interaction of unmodified P_{II} with ATase results in adenylation of GS. At a high intracellular

concentration of 2-ketoglutarate, when P_{II} is in the form of P_{II}-UMP, the latter interacts with ATase which, in turn catalyzes the removal of AMP from GS.

Adenylated GS is inactive whereas unadenylated GS is active. When neither P_{II} nor P_{II}-UMP is available (for e.g., in *glnB* mutants), Gln stimulates ATase-dependent adenylation and phosphate stimulates ATase-dependent deadenylation of GS (Ebner *et al.*, 1970; Anderson and Stadtman, 1971). Genetic evidence also confirmed that UTase / UR and P_{II} control the activity of ATase *in vivo*.

The entire adenylation system is called a bicyclic cascade, because two reversible modifications, uridylation-deuridylation and adenylation-deadenylation, control the activity of GS. It was suggested that the advantage of such a bicyclic cascade (as opposed to regulation by a single reaction cycle) was that the response is faster and more sensitive to changes in the Gln / 2-ketoglutarate ratio (enhanced sensitivity has been termed 'signal amplification').

In *K. aerogenes*, it was suggested that adenylated GS inhibits the transcription of *glnA*, which codes for GS (Foor *et al.*, 1975). Thus, in a *glnE* strain, there was constitutively high production of GS.

That *glnE* mutants have a lowered ratio of Glu to Gln has been shown in both *Streptococcus coelicolor* and *S. enterica*. In the latter, *glnE* mutants were shown to drain their Glu pool into Gln when they were shifted from nitrogen-limiting to NH_4^+-excess conditions (Yan *et al.*, 1996). Loss of Glu (to ~10% of the level in an isogenic *glnE*$^+$ strain) was accompanied by loss of K^+ (~50% that of *glnE*$^+$ strain) and a profound growth defect. The loss of K^+ occurred despite the fact that there was no decrease in the ATP pool and no change in external osmolarity. The results indicated that Glu is required to maintain the steady-state K^+ pool and that K-glutamate is required for optimal growth in high osmolarity medium. It was also suggested that a major function of adenylation of bacterial GS was to protect the cellular Glu pool upon shift to NH_4^+-excess conditions.

1.2.2.4 GOGAT:

GOGAT converts one mole Gln + two moles of 2-ketoglutarate to two moles of Glu in the presence of NADPH and is encoded by the *gltBDF* operon located at 72.3 min on the *E. coli* chromosome and equivalent positions on the *Klebsiella aerogenes* and *S. enterica* chromosomes. Its level is high in ammonia-containing minimal medium and Glu or Glu-generating nitrogen sources, such as Arg, Asp, His and proline. The *gltB* and *gltD* genes specify the large glutaminase subunit and the small transaminase subunit,

respectively (Covarrubias *et al.*, 1980; Madonna *et al.*, 1985). *gltB* or *gltD* mutants grow slowly on poor nitrogen sources because they are starved for Glu. The discovery of a third gene downstream of the *gltBD* operon, *gltF*, led to an alternative explanation that the *gltF* product is essential for nitrogen regulation and that *gltBD* mutations are polar on *gltF* expression (Castano *et al.*, 1988, 1992). However, it was subsequently shown, based on the results obtained employing nonpolar *gltB* and *gltD* mutations and *gltF* deletion strains, that mutations in *gltB* and *gltD* are not polar over *gltF* and that *gltF* is not involved in nitrogen-regulation (Grassl *et al.*, 1999). The leucine-responsive regulatory protein (Lrp) controls the synthesis of GOGAT (Ernsting *et al.*, 1992).

Accumulation of K^+ ions and Glu plays a primary role in maintaining osmotic balance in *E. coli*, as illustrated by the high concentrations of these ions present in cells grown in medium of high osmolarity (McLaggan, 1990). Yan *et al.* (1996) had shown that *gltB* and *gltD* mutant strains of *S. enterica* had a low Glu pool when limited for NH_4^+ as the nitrogen source, and they were unable to maintain a normal K^+ pool under these conditions, particularly at high external osmolarities. But, when challenged by hyperosmotic shock, they increased K^+ levels transiently to the same level as that found in glt^+, indicating that Glu is not required for the large initial response. This was consistent with the earlier conclusions of McLaggan *et al.* (1994) wherein it was shown that upon hyperosmotic shock, *E. coli* grown in medium lacking NH_4^+ was unable to accumulate Glu but showed a normal transient increase in K^+. Most of the K^+ present in an osmotically-active state in the cells is charge-balanced by Glu with minor contributions from glutathione, γ-glutamylglutamine and probably other anions present in low concentrations.

Csonka *et al.* (1994) have shown that in GOGAT-defective mutants of *S. enterica* grown under ammonia-limiting conditions, there is an inverse relationship between growth rate and the osmolarity of the culture media, providing indirect genetic evidence that increased Glu synthesis is necessary for optimal growth under hyperosmotic stress.

In an earlier work reported from this laboratory, an *E. coli* double mutant in *gltBD* and *fnr* (encoding the regulator protein FNR for anaerobic gene expression) was shown to be osmosensitive even on high ammonia medium (Saroja and Gowrishankar, 1996). The results obtained, employing dominant-negative *fnr* gene, suggested the requirement for monomeric FNR during aerobic growth of GOGAT-deficient *E. coli* in high osmolarity media, presumably for Glu accumulation via the GOGAT-independent pathway catalyzed

by GDH. Mulitple copies of *spoT* [encoding guanosine 3',5'-bispyrophosphate (ppGpp) synthetase II / ppGpp-3' pyrophospho-hydrolase] overcomes the defect in ammonia assimilation associated with GOGAT deficiency, and thereby suppresses osmosensitivity in *gltBD fnr* strains. It was shown that the *gltBD* single mutant was moderately osmosensitive, while the *fnr* single mutant was osmotolerant.

1.3 Arginine metabolism and transport:

In the present study, it was observed that mutations in genes involved in arginine (Arg) and polyamine metabolism (biosynthesis and transport) and/or regulation conferred NaCl-sensitivity in *E. coli*. Accordingly, a brief introduction about these processes is described below.

1.3.1 Arg and polyamine biosythesis:

The shared biosynthetic pathway for Arg and polyamines is depicted schematically in Fig. 1.4. The *argA*-encoded acetylglutamate synthase reaction represents the first committed step in the pathway, and ornithine (Orn) represents a branch-point intermediate which can be channeled either into polyamines via the activity of the enzyme ornithine decarboxylase (encoded by *speC*) or into Arg via its reaction with carbamoyl phosphate to form citrulline (Cit). Carbamoyl phosphate is also required for biosynthesis of the pyrimidines, and synthesis of carbamoyl phosphate therefore, is regulated independently and additively by the cytoplasmic concentrations of the respective end products, Arg and pyrimidines. The *arg* biosynthetic genes constitute a regulon under repression control (in the presence of Arg) of the *argR* gene, and acetylglutamate synthase is subject also to feedback inhibition by Arg (Glansdorff, 1996).

The diamine putrescine (Put) can be made either directly by *speC*-mediated decarboxylation of Orn (as mentioned above) or indirectly by decarboxylation of Arg into agmatine followed by hydrolysis of agmatine into Put and urea by an agmatine ureohydrolase (encoded by *speB*). The triamine spermidine is synthesized in a reaction catalyzed by spermidine synthase, encoded by *speE*, from Put by transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine. The latter in turn, is synthesized by decarboxylation from *S*-adenosylmethionine and the reaction is catalyzed by *S*-adenosylmethionine decarboxylase, encoded by *speD*.

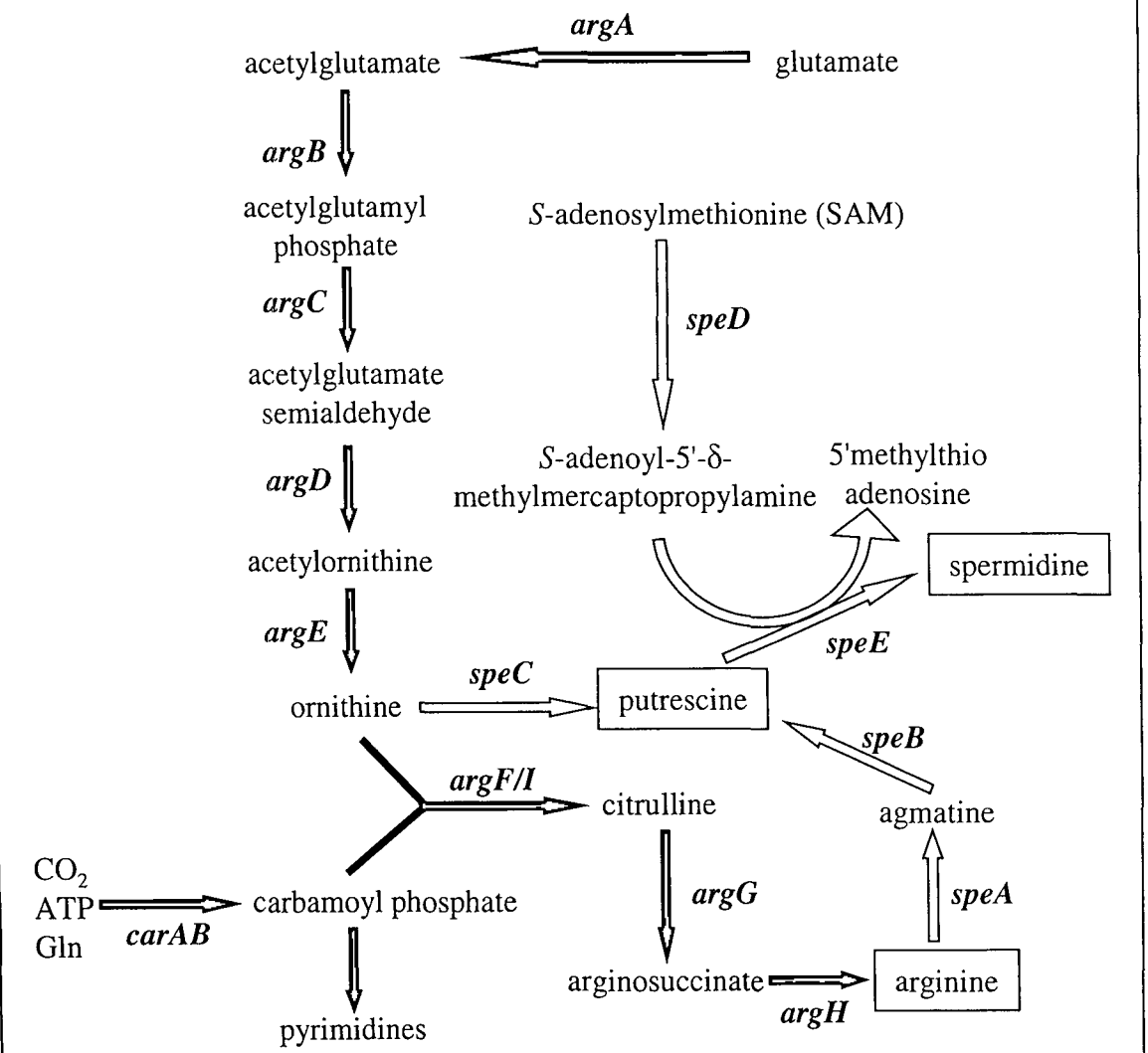


Fig. 1.4 Biosynthesis of arginine (solid arrows) and polyamines (open arrows). [From Glansdorff, 1987.]

speC codes for ornithine decarboxylase involved in the conversion of Orn to Put in the Arg / polyamine biosynthetic pathway in *E. coli*. *speC* mutants were isolated as derivatives of a *speA* strain requiring Put or spermidine (Spd) in the absence of Arg; the fact that *speC speA* double mutants are auxotrophic for Put, Spd or spermine established that the polyamines are essential for cell growth. Together with Mg^{2+}, they are among the major polycations in the cell. The polyamines and Mg^{2+} (which are present in higher free concentrations than Ca^{2+}) can bind to intracellular polyanions such as nucleic acids and ATP to modulate their function. The polyamine content of cells is regulated by biosynthesis, degradation and transport.

1.3.2 Polyamines and osmotic stress:

In *E. coli*, it has been shown that there is a rapid loss of intracellular Put following a sudden increase in the osmotic strength of the medium imposed with NaCl, $MgCl_2$, KCl or sucrose (but not with glycerol). The reduction in intracellular Put levels is mediated by a process of Put efflux that accompanies K^+ uptake in response to osmotic upshifts. The rapid excretion of cellular Put was not accompanied by decrease in Spd and was dependent on the presence of K^+ ions in the medium. It has been suggested that this decrease may provide a mechanism for the electroneutral exchange of two K^+ ions for one Put, enabling cells to increase their osmolarity without disturbing the ionic balance. Put is also rapidly reaccumulated when cells are transferred to a low-osmolarity medium (Munro *et al.*, 1972; Yamamoto, 1989).

1.3.3 Arg transport and the role of ArgP:

1.3.3.1 Previously described role of *argP* in Arg transport in *E. coli*:

The transport of Arg in *E. coli* is mediated by three kinetically distinct transport systems. The first is the LAO system, which is well characterized and represents one of the prototypes of the binding-protein-dependent uptake system (Ames, 1986). The binding protein-dependent systems are also called ABC (ATP-binding cassette)-transporters (Higgins, 1992).This is a high affinity system which is common to Lys, Arg, and Orn. It is reported that the expression of proteins constituting the high affinity system is repressible only by Lys (Celis, 1977a,b). The expression of LAO system in *S. enterica* is σ^{54}-dependent; it is comprised of two periplasmic-binding proteins (HisJ and LAO protein) and the membranous components (HisQ and HisM) and the membrane-associated ATPase (HisP) (Higgins and Ames, 1981; Higgins *et al.*, 1982). The substrates of the binding proteins are histidine (for the HisJ-binding protein), and Lys, Arg and Orn (for the LAO protein). The corresponding genes are organized into two adjacent transcriptional units i.e., *argT* (encoding LAO) and *hisJQMP* (encoding HisJQMP proteins). A similar system also exists in *E. coli*, and the genes are located at 52.3 min on the *E. coli* chromosome, but the system is less well characterized as compared to that in *S. enterica* (Nonet *et al.*, 1987; Kraft and Leinwand, 1987). The LAO protein encoded by *argT* binds Lys, Arg and Orn with high efficiency.

The second Arg uptake system was identified by its binding protein, the Arg-binding protein II (Arg-BP II) (Rosen, 1973a). It is identical to the Arg-Orn (AO)-binding

protein described by Celis (1977a,b; 1981) and its expression is σ^{54}-independent (Reitzer and Schneider, 2001). The binding-protein was isolated and characterized, and the gene locus for the structural gene (*abpS*) was mapped roughly at 63.5 min of the *E. coli* chromosome. Expression of the proteins constituting the AO transport system is believed to be sensitive to repression by either Arg or Orn (Rosen, 1973a; Celis, 1982, 1984).

The presence of a third Arg-specific transport system was suggested by Rosen (1973a) and Celis (1977a,b), based on the identification of an additional Arg-specific binding protein (Arg-binding protein I or ArgBP I) of lower content and affinity. The third uptake system was analyzed and characterized at gene and protein levels (Wissenbach *et al.*, 1995). This third system is encoded by five adjacent genes, *artPIQMJ* (*art* standing for Arg transport), which was organized in two transcriptional units (*artPIQM* and *artJ*) and located at 19.4 min in *E. coli*. ArtI and ArtJ are periplasmic-binding proteins with sequence similarity to HisJ and LAO transport-binding proteins (which themselves share sequence similarity with one another). The ArtQ, ArtM and ArtP proteins are similar to the transmembrane proteins and the ATPase of binding protein-dependent carriers. ArtJ specifically binds Arg with high affinity and its overexpression stimulates Arg uptake by the bacteria. The substrate for ArtI is not known, and the isolated protein does not bind common amino acids, various basic uncommon amino acids such as Orn or citrulline, or amines, such as putrescine.

It has been previously reported that a mutation in the *argP* locus of *E. coli* [isolated as a Canavanine (Can)-resistant strain] results in reduced levels of transport activities of AO and LAO systems (Celis, 1973; Rosen, 1973b). Can, a naturally occurring structural analog of Arg, inhibits the growth of *E. coli* and is believed to be transported into the cell by AO and LAO transport systems. Can competes with Arg for protein synthesis and tRNA-charging. It also binds to CarAB protein (carbamoyl phosphate synthase), involved in the synthesis of carbamoyl phosphate which is a common intermediate for pyrimidine and Arg biosynthetic pathways; and to ArgR protein (a negative regulator of Arg biosynthetic pathway genes), thus repressing Arg biosynthesis. Intracellular Arg to Can ratios determine the sensitivity or tolerance of the strain to Can, and the antimetabolite is therefore extensively used to study Arg metabolism and transport in *E. coli*. The *argP* mutant showed a reduction of 90% in Arg transport and was also affected in the transport of Lys and Orn. Thus, it was hypothesized that reduced transport of Can in the *argP* mutant was responsible for its Can^R phenotype.

Subsequently, a mutant defective in phosphorylation of AO and LAO periplasmic proteins was isolated as a Can^R strain, and it was claimed that the mutant harbored a mutation in the *argK* gene, located at 66 min on the *E. coli* chromosome. ArgK was suggested to be an ATPase that is necessary for ATP hydrolysis coupled to the transport of amino acids. ArgP was suggested to transcriptionally activate, in the absence of Arg, *argK* expression. Data from *in vitro* transcription experiments were interpreted to suggest that ArgP, in the presence of Arg, fails to activate *argK* expression and that it also represses *argP* transcription itself (a phenomenon called autoregulation). Thus, the *argP* locus was proposed to encode a protein that regulates Arg transport in *E. coli* in two ways; by regulating (i) the level of the LAO and AO binding proteins, and (ii) the expression of the *argK* gene (Celis, 1999).

Analysis of the *argP* sequence unexpectedly revealed that it is identical with *iciA*, an *E. coli* gene that was previously described as encoding an inhibitor of chromosome initiation of replication. The protein displays sequence similarity with a large group of prokaryotic transcriptional regulatory proteins of the LysR family. Like the other members of the family, the ArgP sequence includes a helix-turn-helix DNA-binding motif in its N-terminal domain. These members also contain a central co-inducer recognition / response domain and a conserved C-terminal domain.

The following section describes the data previously obtained from ArgP / IciA protein as an inhibitor of chromosome replication initiation. For the sake of clarity, the protein is referred to as IciA only in this section. In the rest of this thesis, the same protein is referred to as ArgP.

1.3.3.2 Previously described role of *iciA* in inhibition of chromosome replication initiation:

E. coli chromosomal replication is initiated by the binding of DnaA proteins to the DnaA boxes (or 9-mers) within the origin (*oriC*) sequence. The IciA protein was first identified in Arthur Kornberg's laboratory as a sequence-specific DNA-binding protein which inhibits the initiation of *E. coli* chromosomal replication *in vitro* by bindingan array of three AT-rich 13-mers within *oriC*, and preventing the action of DnaA initiator protein (Hwang and Kornberg, 1990). Subsequently, several other *in vitro* activities of IciA have been described, including its role in transcriptional regulation of *dnaA*.

Transcription from promoter 1P of *dnaA in vivo* was specifically enhanced by the overexpression of IciA, but the mechanism whereby IciA protein was able to activate

dnaA promoter 1P was not elucidated. *In vitro*, IciA protein was able to activate transcription from *dnaA* promoter 1P in the presence of limiting amounts of RNA polymerase (Lee *et al.*, 1996, 1997; Lee and Hwang, 1997). When the level of RNA polymerase saturated the reaction, the activation activity of IciA on promoter 1P was abolished. These results suggested not only that the IciA protein may assist binding of RNA polymerase to the promoter 1P, but also that IciA is not necessary for *dnaA* transcription. It was hypothesized that IciA blocks the formation of the open complex for RNA primer synthesis during initiation of chromosome replication from *oriC*, and that IciA has no effect on the subsequent stages of replication (Hwang and Kornberg, 1990). However, the *iciA* null mutant had no obvious growth phenotype (Messer and Weigel, 1997).

Several other apparently disconnected observations have been reported with respect to IciA. The protein was shown to bind the promoter of *nrd* (which encodes ribonucleotide reductase), and its overexpression led to five-fold increased expression of the gene, suggesting that IciA may function as a transcriptional activator of the *nrd* gene (Han *et al.*, 1998). It was also reported that *E. coli* cells in which IciA was overexpressed exhibited a pronounced lag upon transfer to fresh medium (Thony *et al.*, 1991). It was shown that IciA binds to DNA apparently in a sequence-nonspecific fashion but shows preference to curved DNA (Azam and Ishihama, 1999). Ali Azam and Ishihama (1999) have shown that about 800 molecules (or 400 dimers) of IciA exist in exponentially growing cells of *E. coli*, and that their level decreased to about 500 molecules (or 250 dimers) per cell in the early stationary phase. However, Hwang *et al.* (1992) have reported that the IciA levels increase four-fold at the stationary phase. The disagreement between these two measurements remains unresolved. The expression of *iciA*::*lacZ* fusion increased under phosphate-depleted conditions and was dependent on the presence of the PhoB protein (the transcriptional activator of the *pho* regulon; Han *et al.*, 1999).

1.4 Objectives of the present work:

Very little is known about muations that confer an osmosensitivity phenotype in bacteria. In a limited number of cases, differential sensitivities of various cellular processes to osmotic stress have been observed. For example, nitrogen fixation in *Klebsiella pneumoniae* (Le Rudulier *et al.*, 1982) and the conjugal transfer of F plasmid in *E. coli* (Singleton, 1984) are more sensitive to hyperosmotic stress than overall cell

growth. Mutations that block the pathway for trehalose synthesis were shown to result in sensitivity to osmotic stress (Giaever *et al.*, 1988, Rod *et al.*, 1988). It has been reported that mutations affecting membrane structure, which confer sensitivity to acriflavine, also resulted in sensitivity to hyperosmotic media (Nakamura, 1977). *E. coli* mutants defective in K^+ uptake are also sensitive to hyperosmotic stress (Gowrishankar, 1985). Mutations that inactivate the Na^+/H^+ antiporter confer sensitivity to Na^+ and Li^+ ions (Goldberg *et al.*, 1987), but this sensitivity is not associated with a generalized sensitivity to osmotic stress.

A transposon-mutagenesis approach has been employed to identify genes involved in osmotolerance in *Listeria monocytogenes* (Garden *et al.*, 2003) and *Caulobacter crescentus* (Zuleha *et al.*, 2003). Transposon insertions in genes that encode putative transporters conferred sensitivity to salt and alkaline stresses in *L. monocytogenes*. In *Caulobacter crescentus*, mutations in genes involved in lipopolysaccharide biosynthesis, in *nhaA* (which encodes a Na^+ / H^+ antiporter) and in *ppiD* (which encodes a peptidyl-prolyl *cis-trans* isomerase) were shown to confer NaCl-sensitivity.

In the present study, it was proposed to use a transposon-mutagenesis approach to obtain NaCl-sensitive mutants, in order to gain a better understanding of the mechanisms involved in water-stress adaptation in *E. coli*. The transposon employed was λp*lac*Mu55, which has the advantages that (i) null (loss-of-function) insertions can be isolated, (ii) strains containing promoter-*lac* transcriptional (operon) fusions can be isolated in a single step, and (iii) the presence of the Kanamycin-resistance gene permits cells containing insertions of these phages to be selected independently of their Lac phenotype. Accordingly, the specific objectives of the present work were:

(i) the isolation, identification and characterization of null insertion mutations that confer NaCl-sensitivity, and

(ii) to interpret the data obtained in terms of the possible cellular adaptational mechanism(s) to biological water-stress.

Chapter 2

Materials and Methods

MATERIALS

2.1 Bacterial strains: All the bacterial strains that were used in this study are derivatives of *Escherichia coli* K-12 and their genotypes are listed in Table 2.1. Bacterial strains were routinely stored on solid agar plates at 4°C and also as thick suspensions in 20% glycerol at –70°C.

Table 2.1 Strains of *Escherichia coli*[a]

Strain	Genotype[b]
160-37	ATCC9637 *E. coli* W *argE*
CAG12017	MG1655 *zba-3054*::Tn*10*
CAG12072	MG1655 *zha-203*::Tn*10*
CAG12095	MG1655 *zab-3051*::Tn*10*
CAG12148	MG1655 *tsx-247*::Tn*10*
CAG18425	MG1655 *thr-3091*::Tn*10*Kan
CAG18430	MG1655 *mdoB*::Tet
CAG18442	MG1655 *thr-34*::Tn*10*
CAG18462	MG1655 *zdh-603*::Tn*10*
CAG18472	MG1655 *nupG511*::Tn*10*
CAG18475	MG1655 *metC162*::Tn*10*
CSH57	*purE trp his argG met ilv leu thi ara lacY gal malA xyl mtl rpsL*
CSH117	*ara* Δ(*gpt-lac*) *mutY*::Tn*10*
CSH119	HfrC *car-96*::Tn*10* Δ(*gpt-lac*)*5 cysG303 relA? spoT1? metB1*
CSH120	Hfr P4X *relA1 spoT1 metB1 zje-2005*::Tn*10*
CSH121	HfrH *ara ilvJ? zae-502*::Tn*10* Δ(*gpt-lac*)*5*
CSH122	Hfr KL16 *zed-977*::Tn*10 relA1 spoT1 thi-1*
CSH123	Hfr KL14 *relA1 spoT1 thi-1 thiA*::Tn*10*
CSH124	Hfr PK191 Δ(*gpt-lac*)*5 nupG-511*::Tn*10*

DH5α	Δ(*argF-lac*)*U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (ϕ80 *lacZ*ΔM15)
Gif 106M1	*thrA1101 glnV44*(AS) *rpsL9 malT1*(LamR) *xylA7 mtlA2 ilvA296 metL1000 thi-1 lysC1001 arg-1000*
HB117 *nuvC*	*nuvC*
JM2017	*leu-0* Δ *lac-8888 his- galP77*::Tn*10 ilvA0 mglP0*
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*
MA1030	*purF1 thi-1 argR64*
MA1034	*purF1 thi-1 argP55*
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flb5301 deoC1 ptsF25*
MG1655	Wild-type
MJF451	*rha thi gal lacZ* Δ*yggB*
MJF455	*rha thi gal lacZ* Δ*mscL*::*Cm* Δ*yggB*
PA340	*leuB6*(Am) *fhuA2 lacY1 glnV44*(AS) *gal-6 gdhA1 hisG1*(Fs) *rfbD1 galP63* Δ(*gltB-gltF*)*500 rpsL9 malT1 xylA7 mtlA2* Δ*argH1 thi-1*
PAL12103K	Δ(*lac-pro*) *gyrA rpoB metB argE*(Am) *ara supF ksgA*::Kan
PAL12103G	Δ(*lac-pro*) *gyrA rpoB metB argE*(Am) *ara supF apaG*::Kan
PAL12103A2	Δ(*lac-pro*) *gyrA rpoB metB argE*(Am) *ara supF apaH*::Kan
QC1556	Δ*soxRS4*::Cm
RI179	*araD139* Δ(*araABC-leu*)*7679 galU galK* Δ(*lac*)*X74 rpsL thi phoR* Δ*ara714 leu*$^+$ Δ*dsbC*::Cm
VJS2890	F *araD139* D(*argF-lac*)*U169 flhD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 thiI456*::Kan
GJ46	MC4100 *zfi-900* Tn*10*
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)*461*
GJ157	MC4100 Δ*putPA101 proP221 proX224*::*lac* Δ(*pyr-76*::Tn*10*)*462*
GJ184	Δ*putPA101* Δ(*pyr-76*::Tn*10*) *proP228*::Mu d1(*lac* Amp)

GJ216	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*Tn*10*)461 *recA56 srl-300*::Tn*10*
GJ629	MC4100 *zhc-904*::Tn*10*
GJ901	MC4100 *fnr-266 zha-900*::Tn*10*dTet
GJ966	MC4100 Δ*fnr*::Ω(Str^R Spec^R) Δ*gltBDF500 zha-6*::Tn*10*
GJ980	MC4100 *gdhA*::Kan
GJ1293	MC4100 *speC3*
GJ1380	MC4100 Δ(*speC-glc*)*63*
GJ1921	MG1655 *lon-104*::IS*186*::Tn*10*dCm
GJ2259	*ara zbh-900*::Tn*10d*Kan(Ts)*1 lacZ4525*::Tn*10d*Kan *uup-351*::Tn*10*dTet*1 recA56 srl*::Tn*10 zgb-910*::Tn*10*dCm
GJ2269	*ara zbh-900*::Tn*10d*Kan(Ts)*1 lacZ4526*::Tn*10*dTet
GJ2529	MC4100 *gltBDF500 zha-6*::Tn*10*
GJ2530	Δ*fnr*::Ω(Str^R Spec^R) *gltBDF500 zha-6*::Tn*10*
GJ2533	GJ2530 *thiI51*::Kan / pHYD909
GJ2534	GJ2530 *proV610*:Kan / pHYD909
GJ2535	GJ2530 *thiI52*::Kan / pHYD909
GJ2536	GJ2530 *argP202*::Kan / pHYD909
GJ2537	GJ2530 *lysC82*::Kan / pHYD909
GJ2539	GJ2530 *thiI53*::Kan / pHYD909
GJ2540	GJ2530 *argP203*::Kan / pHYD909
GJ2541	GJ2530 *apaG11*::Kan / pHYD909
GJ2542	GJ2530 *speC100*::Kan *thiI57*::Kan / pHYD909
GJ2543	GJ2530 *thiI54*::Kan / pHYD909
GJ2544	GJ2530 *proU611*::Kan / pHYD909
GJ2545	GJ2530 *glnE463*::Kan / pHYD909
GJ2546	GJ2530 *thiI54*::Kan / pHYD909
GJ2547	GJ2530 *glnE464*::Kan / pHYD909
GJ2549	GJ2530 *glnE465*::Kan / pHYD909
GJ2550	GJ2530 *thiI55*::Kan / pHYD909
GJ2552	GJ2530 *thiI56*::Kan / pHYD909
GJ2560	GJ2529 *proV610*::Kan
GJ2561	GJ2529 *thiI51*::Kan

GJ2562	GJ2529 *thiI55*::Kan
GJ2563	GJ2529 *thiI56*::Kan
GJ2564	GJ2529 *argP202*::Kan
GJ2565	GJ2529 *argP203*::Kan
GJ2566	GJ2529 *glnE463*::Kan
GJ2567	GJ2529 *glnE464*::Kan
GJ2568	GJ2529 *apa11*::Kan
GJ2569	GJ2529 *speC100*::Kan
GJ2570	GJ2529 *lysC82*::Kan
GJ3533	GJ2530 *thiI51*::Kan / pHYD909
GJ3534	GJ2530 *proV610*:Kan / pHYD909
GJ3535	GJ2530 *thiI52*::Kan / pHYD909
GJ3536	GJ2530 *argP202*::Kan / pHYD909
GJ3537	GJ2530 *lysC82*::Kan / pHYD909
GJ3539	GJ2530 *thiI53*::Kan / pHYD909
GJ3540	GJ2530 *argP203*::Kan / pHYD909
GJ3541	GJ2530 *apaG11*::Kan / pHYD909
GJ3542	GJ2530 *speC100*::Kan / pHYD909
GJ3543	GJ2530 *thiI54*::Kan / pHYD909
GJ3544	GJ2530 *proU611*::Kan / pHYD909
GJ3545	GJ2530 *glnE463*::Kan / pHYD909
GJ3546	GJ2530 *thiI54*::Kan / pHYD909
GJ3547	GJ2530 *glnE464*::Kan / pHYD909
GJ3549	GJ2530 *glnE465*::Kan / pHYD909
GJ3550	GJ2530 *thiI55*::Kan / pHYD909
GJ3552	GJ2530 *thiI56*::Kan / pHYD909
GJ3553	GJ2530 *thiI57*::Kan / pHYD909
GJ4533	MC4100 *thiI51*::Kan
GJ4534	MC4100 *proV610*:Kan
GJ4535	MC4100 *thiI52*::Kan
GJ4536	MC4100 *argP202*::Kan
GJ4537	MC4100 *lysC82*::Kan
GJ4539	MC4100 *thiI53*::Kan
GJ4540	MC4100 *argP203*::Kan

GJ4541	MC4100 *apaG11*::Kan
GJ4542	MC4100 *speC100*::Kan
GJ4543	MC4100 *thiI54*::Kan
GJ4544	MC4100 *proU611*::Kan
GJ4545	MC4100 *glnE463*::Kan
GJ4546	MC4100 *thiI54*::Kan
GJ4547	MC4100 *glnE464*::Kan
GJ4549	MC4100 *glnE465*::Kan
GJ4550	MC4100 *thiI55*::Kan
GJ4552	MC4100 *thiI56*::Kan
GJ4553	MC4100 *thiI57*::Kan
GJ4559	MA1030 *zha-900*::Tn*10*dTet
GJ4560	GJ4542 *argR64 zha-900*::Tn*10*dTet
GJ4564	MG1655 *thiI51*::Kan
GJ4571	GJ1380 *argP202*::Kan
GJ4572	GJ4553 *tsx-247*::Tn*10*
GJ4578	GJ4572 *argP202*::Kan
GJ4584	MC4100 *recA56 srl-300*::Tn*10*
GJ4593	MC4100 *apaG*::Kan
GJ4594	MC4100 *apaH*::Kan
GJ4595	MC4100 *ksgA*::Kan
GJ4596	GJ2529 *apaG*::Kan
GJ4597	GJ2529 *apaH*::Kan
GJ4612	GJ2564 *recA56 srl-300*::Tn*10 zgb-910*::Tn*10*dCm
GJ4613	GJ2566 *recA56 srl-300*::Tn*10 zgb-910*::Tn*10*dCm
GJ4614	GJ2567 *recA56 srl-300*::Tn*10 zgb-910*::Tn*10*dCm
GJ4617	GJ4536 *argR64 zha-900*::Tn*10*dTet
GJ4618	GJ4540 *argR64 zha-900*::Tn*10*dTet
GJ4631	MC4100 *argR64 zha-900*::Tn*10*dTet
GJ4652	MC4100 Δ*gltBDF500*
GJ4654	GJ4652 *argP202*::Kan
GJ4655	GJ4652 *argP203*::Kan
GJ4659	GJ4652 *thiI51*::Kan

GJ4660	GJ4652 *lysC82*::Kan
GJ4661	GJ4652 *apaG11*::Kan
GJ4662	GJ4652 *speC100*::Kan
GJ4663	GJ4652 *glnE463*::Kan
GJ4674	MG1655 *zbh-900*::Tn*10*dKan(Ts)*1* *lacZ4525*::Tn*10*dKan *lon-103*::IS*186* *serA*
GJ4676[c]	GJ4674 *serA*$^+$ *argO204*::Tn*10*dTet
GJ4677	GJ4674 *serA*$^+$ *argO205*::Tn*10*dTet
GJ4678	GJ4674 *serA*$^+$ *argO206*::Tn*10*dTet
GJ4679	GJ4674 *serA*$^+$ *argO207*::Tn*10*dTet
GJ4680	GJ4674 *serA*$^+$ *argO208*::Tn*10*dTet
GJ4681	GJ4674 *serA*$^+$ *argO209*::Tn*10*dTet
GJ4682	GJ4674 *serA*$^+$ *argO210*::Tn*10*dTet
GJ4683	GJ4674 *serA*$^+$ *argO211*::Tn*10*dTet
GJ4684	GJ4674 *serA*$^+$ *argO209*::Tn*10*dTet
GJ4691	GJ4652 *argO204*::Tn*10*dTet
GJ4692	GJ4652 *argO205*::Tn*10*dTet
GJ4693	GJ4652 *argO206*::Tn*10*dTet
GJ4694	GJ4652 *argO207*::Tn*10*dTet
GJ4695	GJ4652 *argO208*::Tn*10*dTet
GJ4696	GJ4652 *argO209*::Tn*10*dTet
GJ4697	GJ4652 *argO210*::Tn*10*dTet
GJ4698	GJ4652 *argO211*::Tn*10*dTet
GJ4699	GJ4652 *argO209*::Tn*10*dTet
GJ4706	GJ4536 *argOA204*::Tn*10*dTet
GJ4707	GJ4536 *argO205*::Tn*10*dTet
GJ4723	GJ4659 *sti-1* linked 85% to *zhb-915*::Tn*10*dCm
GJ4736	GJ4537 *zjb-913*::Tn*10*dCm
GJ4737	GJ4663 *lysC82*::Kan *zjb-913*::Tn*10*dCm
GJ4738	GJ4654 *lysC82*::Kan *zjb-913*::Tn*10*dCm
GJ4739	GJ4659 *sti-47* linked 90% to *zhb-915*::Tn*10*dCm
GJ4740	GJ4659 *sti-49* linked 80% to *zhb-915*::Tn*10*dCm
GJ4741	MC4100 *thiI*::Kan
GJ4742	GJ4652 *thiI*::Kan

GJ4748	MC4100 *argR64 zhb-914*::Tn*10*dCm
GJ4749	GJ4748 *argO204*::Tn*10*dTet
GJ4750	GJ4748 *argO205*::Tn*10*dTet
GJ4752	HB117 *nuvC* (*thiI*) *tsx-247*::Tn*10*
GJ4754	MC4100 *nuvC (thiI) tsx-247*::Tn*10*
GJ4756	GJ4652 *nuvC (thiI) tsx-247*::Tn*10*
GJ4757	Gif 106M1 *thiA*::Tn*10*
GJ4758	Gif 106M1 *mdoB*::Tn*10*
GJ4759	GJ4652 *lysC1001 thiA*::Tn*10*
GJ4760	GJ4652 *thrA1101 mdoB*::Tn*10*
GJ4790	GJ4749 *argP202*::Kan
GJ4791	GJ4750 *argP202*::Kan
GJ4820	MC4100 Δ*gltBDF500 argP202*::Kan *lacZ*::Tn*10*dTet
GJ4822	MC4100 *argO204*::Tn*10*dTet
GJ4823	MC4100 *argO205*::Tn*10*dTet
GJ4828	MC4100 *apaH*::Kan
GJ4831	MC4100 *thr-3091*::Tn*10*Kan
GJ4832	GJ4652 *thr-3091*::Tn*10*Kan
GJ4833	MC4100 *thr-34*::Tn*10*
GJ4834	GJ4652 *thr-34*::Tn*10*
GJ4835	MC4100 Δ*putPA101 proP222*
GJ4836	MC4100 Δ*putPA101 proP221 proX224*::*lac*
GJ4837	GJ4835 *proV610*::Kan
GJ4838	GJ4836 *proV610*::Kan
GJ4839	MC4100 *recB268*::Tn*10*
GJ4840	GJ4652 *recB268*::Tn*10*
GJ4841	MC4100 Δ*ruvABC*::Cm
GJ4842	GJ4652 Δ*ruvABC*::Cm
GJ4843	GJ4652 *recA56 srl-300*::Tn*10*
GJ4873	GJ4835 *argP202*::Kan *lacZ*::Tn*10*dTet
GJ4890	RI179 *argP202*::Kan
GJ4891	GJ4663 *argP202*::Kan Δ*dsb*::Cm
GJ4892	MC4100 *argP202*::Kan *lacZ*::Tn*10*dTet

GJ4894	GJ4748 *argO204*::Tn*10*dTet
GJ4895	GJ4748 *argP202*::Kan *lacZ*::Tn*10*dTet
GJ4898	GJ4663 *sge-18* linked 22% to Tn*10*dCm
GJ4899	GJ4660 *slc-12* linked 10% to Tn*10*dCm

[a] 160-37 is *E.coli* W. All other strains are K-12 derivatives.

[b] Genotype designations are as in Berlyn (1998). All strains are F⁻ unless indicated. Allele numbers are given wherever known.

[c] *argO* is allelic to *yggA*.

2.2 Bacteriophages: The bacteriophage P1*kc* was from the laboratory collection and is referred to as P1 throughout this thesis. Other bacteriophages that were used in this study included the following:

(i) λNK1323 [a phage for generating transpositions of mini-Tn*10* transposon having the tetracycline-resistance marker, Tet^R, and with an Altered Target Specificity (ATS) transposase].

(ii) λNK1324 [that is similar to λNK1323, but carries a mini-Tn*10* transposon Tn*10*dCm with chloramphenicol-resistance determinant, Cm^R instead of Tet^R].

(iii) λp*lac*Mu55 [a specialized phage used for generating random transpositions. It carries the kanamycin resistance marker, Kan^R and the promoterless *lacZY* genes within the Mu *c* and Mu *S* ends, and lacks transposase gene].

(iv) λpMu507 [a helper phage which provides the transposase enzyme in *trans*].

The above lambda phage vectors were used to make random insertions in the chromosome either for the purpose of insertional mutagenesis or for tagging antibiotic resistance markers to point mutations.

The ordered λ phage library of the *E.coli* genome constructed by Kohara *et al.* (1987) was obtained from Dr. Isono and whenever required, the phages were amplified and used.

2.3 Plasmids:

1. pBR322 is a medium-copy-number ColE1 plasmid which carries Amp^R and Tet^R as selectable markers (Bolivar *et al.*, 1977).

2. pBR329 is a medium-copy-number ColE1 plasmid which carries Amp^R, Tet^R, and, Cm^R as selectable markers (Covarrubias and Bolivar, 1982).

3. pBluescriptII KS (pBKS) is a high-copy-number ColE1 based vector with Amp^R as selectable marker and also carries a multiple-cloning site (MCS) region in *lacZ*α fragment, so that it can be employed in blue-white screening of recombinant clones (obtained from Stratagene).

4. pCL1920 is a low-copy-number vector with pSC101 replicon, that carries streptomycin/spectinomycin marker (encoded by *aadA*) and also carries a MCS region in the *lacZ*α gene (Lerner and Inouye, 1990).

5. pCL1921 is the same as pCL1920, but the MCS is reversed.

6. pMU575 is an IncW-based single-copy promoter-probe vector carrying *lacZYA* reporter genes, with trimethoprim resistance marker (Yang and Pittard, 1987). It has an MCS region placed upstream of a promoterless *galK'-lacZ* gene fusion. The *galK'-lacZ* fusion consists of the first 58 codons of *galK'* fused in-frame to the 8^{th} codon of *lacZ*. Translation of the hybrid gene is controlled by the ribosome-binding site of *galK*. There are stop codons in all three reading frames in the region of DNA between the MCS and the initiation codon of *galK*, so that there is no interference caused by translational readthrough from inserts cloned into the MCS region. A strong *pheR* terminator located upstream of the MCS prevents readthrough from vector-based promoters into the *lac* genes.

Plasmid DNA preparations were routinely prepared from *recA* strains such as DH5α and were stored in 10 mM Tris-Cl (pH 8.0) plus 1 mM EDTA at −20°C.

2.4 Sources of chemicals:

Bacterial media components were obtained from Hi-Media or Difco; antibiotics, amino acids, sugars and other supplements from Sigma Co. Restriction enzymes and other enzymes, and buffers used for molecular genetic work were procured from New England Biolabs, Promega, Perkin-Elmer or Bangalore Genei. Primers were synthesized either at Eurogentec, France; Microsynth, Switzerland; or at the CDFD oligo-synthesis facility. Perkin-Elmer sequencing kits were used throughout this study.

2.5 Media and buffers:

All the media and buffers were sterilised by autoclaving for 15 minutes at 121°C (15 pounds per square inch).

Minimal A medium (MA)

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate $2H_2O$	0.5 g
Water to	1000 ml

After autoclaving, the following solutions were added.

$MgSO_4$ (1M)	1 ml
Thiamine (0.1%)	0.5 ml
Glucose (20%)	10 ml

Amino acids and uracil, when required, were added to a final concentration of 40 μg/ml and vitamins to 4 μg/ml. Sugars and Casamino acids were added to a final concentration of 0.2%. MA agar was prepared by mixing equal volumes of 2 X MA medium with 4% bactoagar.

W-salts medium

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
Water to	1000 ml

After autoclaving, the following solutions were added.

$MgSO_4$ (1M)	1 ml
Thiamine (0.1%)	0.5 ml
Glucose (20%)	10 ml

LB medium

Bactotryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g
Water to	1000 ml

pH adjusted to 7 with NaOH.

LB Agar

LB medium	1000 ml
Bacto-agar	15 g

LB Soft agar

LB medium	100 ml
Bacto-agar	0.75 g

Z broth

LB medium	100 ml
$CaCl_2$ (0.5 M)	0.5 ml

Z agar

Z broth	100 ml
Bacto-agar	0.75 g

MacConkey agar

MacConkey Agar (Difco)	51.5 g
Water to	1000 ml

Citrate buffer

Citric acid (0.1 M)	4.7 volumes
Sodium citrate (0.1 M)	15.4 volumes

SM buffer

NaCl	5.8 g
$MgSO_4.7H_2O$	2.0 g
1 M Tris-Cl (pH 7.5)	50 ml
Gelatin (2%)	5 ml

TB buffer

PIPES (free acid)	10 mM
$CaCl_2.2H_2O$	15 mM

KCl	250 mM
$MnCl_2.4H_2O$	55 mM

pH adjusted to 6.7 with 1 N KOH.

. PIPES, $CaCl_2$ and KCl were dissolved at a pH of 6.7 with 1 N KOH (the salts go into solution only after the pH reaches 6.7). $MnCl_2$ was dissolved separately in MilliQ water and added drop by drop while stirring. The pH was adjusted to 6.8, filter-sterilized, and stored at –20 °C.

TE buffer

Tris.Cl (pH 8.0)	10 mM
EDTA	1 mM

TBE buffer

Tris-Borate	90 mM
EDTA (pH 8.0)	2 mM

Prepared as 10 X stock solution and diluted with distilled water.

TAE buffer

Tris-Acetate	40 mM
EDTA (pH 8.0)	2 mM

Prepared as 50 X stock solution and diluted with distilled water.

Z buffer

$Na_2HPO_4.7H_2O$	16.1 g
$NaH_2PO_4.H_2O$	5.5 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.246 g
β-mercaptoethanol	2.7 ml

pH adjusted to 7.0 and stored at 4°C. (This buffer was not autoclaved.)

Phage buffer

Tris.Cl (pH 7.4)	20 mM

$MgSO_4$	10 mM
NaCl	100 mM

2.6 Antibiotics:

Antibiotics were used at the following final concentrations (μg/ml) in various media.

Antibiotic	LB	Minimal A
Ampicillin (for plasmids)	100	50
Ampicillin (chromosome)	30	30
Chloramphenicol (for plasmids)	50	30
Chloramphenicol (chromosome)	25	25
Kanamycin	50	25
Tetracycline	15	8
Trimethoprim	60	30
Streptomycin [chromosome (*rpsL*)]	100	200
Streptomycin [for plasmids (*aadA*)]	50	100
Spectinomycin	50	50

2.7 Oligonucleotide primers:

The primers used in this study are listed below.

Table 2.2 Primers used for PCR amplification and sequencing of various regions in *E. coli*.

Primer ID	Sequence (5' → 3')
MuC1[a]	TGCGTTTTTCTTCAGGTAATG
MuC2[b]	TCCCGAATAATCCAATGTCCTCCCG
TetF[c]	TGGTCACCAACGCTTTTCCCGAG
TetR[c]	CTGTTGACAAAGGGAATCATAG
AH1[d]	TACCAAAATCATTAGGGGATTCATC
AH2[d]	TAAGTTAAGGTGGATACACATCTT
YGGBF[a]	TCCAGGAATTCAACGCGATCGA
YGGAR[a]	ACCTCTGGATCCAAGCTTAG

ARGP1[a]	GGGCGCGAACTCGCTGAGCGA
ARGP2[a]	GAGCAAGTTGTACGAACGCTT

[a] this study
[b] Roy *et al.*, 1995
[c] Saisree *et al.*, 2000
[d] Higashitani *et al.*, 1994

METHODS

A. Genetic techniques:

2.8 Phage P1 techniques:

2.8.1 Phage P1 lysate preparation

0.3 ml of overnight culture of the donor strain in Z-broth was mixed with 10^7 plaque-forming units (pfu) of a stock P1 lysate prepared on strain MG1655. Adsorption was allowed to occur at 37°C for 20 min and the lysate was prepared in one of the following ways:

A. Plate method: 0.1 ml aliquots of the infection mix were dispensed in 2.5 ml of LB soft agar and poured onto freshly prepared Z-agar plates. A control plate with 0.1 ml of uninfected cells in a lawn was also similarly prepared. After a period of 8-12 h incubation at 37°C when a 'mottled' lawn was visible on the test plates (compared to an opaque lawn on the control), 2 ml of Z-broth was added to each plate and incubation was continued for another 2 h. The Z-broth and soft agar layers were transferred into a test tube and 5 drops of chloroform were added, followed by vigorous vortexing for 30 seconds. After centrifuging down the debris, the clear supernatant was removed into a sterile test tube, treated again with chloroform (0.3 ml/10 ml) and stored at 4°C.

B. Broth method: To 0.3 ml of infection mixture, 10 ml of Z-broth was added and incubated at 37°C with slow shaking until growth followed by the visible lysis of the culture occurred (in ~ 4 to 5 h). The lysate was treated with 0.3 ml of chloroform, centrifuged and the clear lysate was stored at 4°C with chloroform.

Preparation of P1 lysates on *recA* mutant strains was done similarly, but with a higher multiplicity of infection (i.e., 10^8 starter P1 phage), and in the plate method, the plates were incubated up to 12 h before adding Z-broth.

To quantitate the P1 phage lysate preparations, titration was done using a P1-sensitive indicator strain, such as MG1655. 100 µl each of serial dilutions of the phage (typically 10^{-5}, 10^{-6}) were mixed with 0.1 ml of the fresh culture grown in Z-broth. After 15 min adsorption at 37°C without shaking, each mixture was added in a soft agar overlay to Z agar plates, and incubated overnight at 37°C.

2.8.2 Phage P1 transduction: 10^8 PFU of P1 phage grown on the donor strain was added to 2 ml of a freshly grown overnight culture of the recipient strain and phage adsorption was allowed to occur at 37°C for 15 min. The infection mixture was centrifuged at 3,000 rpm for 5 min in a bench-top centrifuge and the pellet was resuspended in 4 ml LB containing 25 mM sodium citrate, and incubated at 37°C for 30 min to allow expression of the selectable antibiotic marker (this expression step was omitted when selection involved a prototrophic marker). The transduction mix was re-centrifuged at 3,000 rpm for 5 min and the cells were resuspended in 0.4 ml of citrate buffer of which 100 µl aliquots were spread (at neat and 10^{-1} dilutions) on the selection plates supplemented with 5 mM sodium citrate.

2.8.3 Mutagenesis of P1 phage preparation: This procedure involves two steps namely the preparation and concentration of a large-scale P1 lysate, and its subsequent mutagenesis.

(a) Preparation of a large scale P1 lysate: 2 ml of an overnight culture of the strain grown in LB was mixed with 10^8 PFU of P1 phage and 5 mM $CaCl_2$ in a 500 ml Erlenmeyer flask. Adsorption was allowed to occur at 37°C for 20 min, after which 100 ml LB and 5 mM $CaCl_2$ were added and incubated at 37°C with slow shaking until lysis occurred. Growth usually occurred for the first 2 h, followed by visible lysis within the next 2.5 - 3 h. The lysate was treated with chloroform, centrifuged and stored over chloroform at 4°C. This method yielded a titre of at least 1 x 10^9 PFU / ml.

The lysate was concentrated to one-hundredth of the original volume by ultracentrifugation at 100,000 x *g* (37,500 rpm) for 90 min at 4°C using polyallomer tubes in a Beckman L8-M Ultracentrifuge, Rotor Type 60 Ti.

(b) Mutagenesis with hydroxylamine: A 0.4 M solution of hydroxylamine hydrochloride ($NH_2OH.HCl$) containing 2 mM EDTA and 10 mM $CaCl_2$ was freshly prepared. A pilot experiment was first done to determine the time of incubation with the

mutagen. A small volume of the concentrated lysate was mixed with 9 volumes of the NH_2OH solution and incubated at 37°C without shaking. Small aliquots were withdrawn every three hours for 36 h, diluted and the titre determined. There was an exponential drop in titre with time (Fig. 2.1). The time point at 15 h of exposure to the mutagen was considered ideal, since there was drop in titre of two log orders (i.e, 99% killing).

In the main experiment, the remaining concentrated lysate was exposed to 9 volumes of the NH_2OH solution at 37°C for 15 h, diluted with approximately 5 volumes of fresh LB and ultracentrifuged again (as described above) to eliminate the mutagen. This concentrated lysate now possessed a titre of ~1 x 10^9 PFU/ml, and was used in the same manner for transduction as P1 lysates that were routinely prepared.

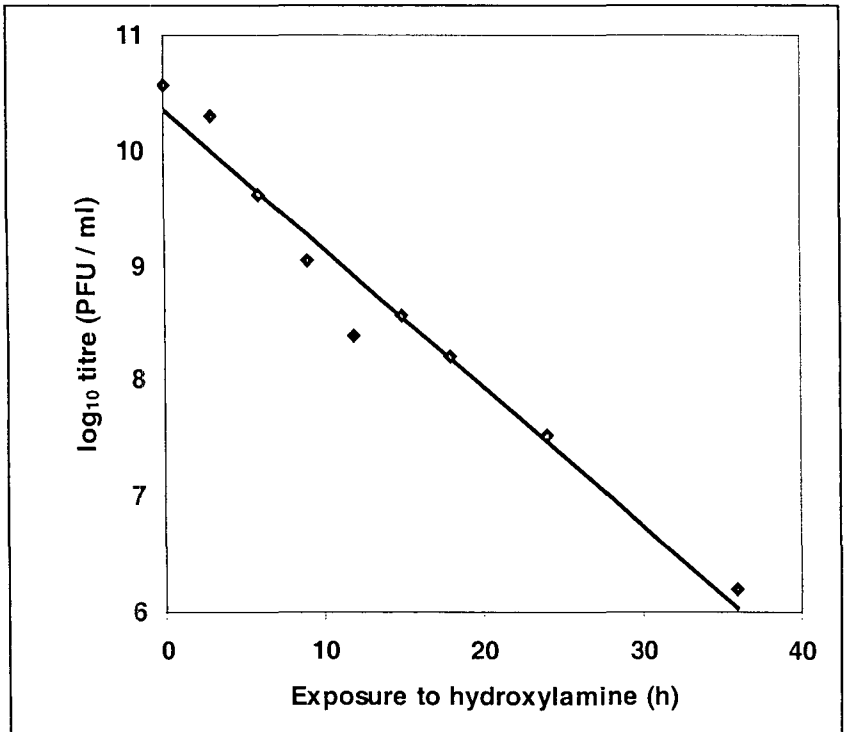


Fig. 2.1 Titration of mutagenized P1 phage

2.9 Phage lambda (λ) techniques:

2.9.1 Preparation of lambda lysates:

The λ-sensitive cells used for infection were grown overnight in LB broth containing 0.4% maltose, and 0.3 ml of this culture was infected with around 3 x 10^7 PFU of λ phage in the presence of 10 mM $MgSO_4$. After adsorption at 37°C for 20 min, 10 ml of fresh LB containing 10 mM $MgSO_4$ was added, and incubated at 37°C with shaking

(200 rpm) until growth followed by visible lysis of the culture occurred (in ~5 to 6 h). The lysate was treated with 0.3 ml chloroform, centrifuged, and the clear lysate was stored at 4°C with chloroform.

2.9.1.1 Clonal purification of λ lysates: A single plaque of λ contains approximately 10^5 PFU. The method of propagation of λ from a single plaque was as follows. The contents of a single isolated plaque were drawn into a 1-ml pipette tip and dispensed into 0.5 ml of SM buffer. After addition of a drop of chloroform, the contents were vortexed and centrifuged. 50 µl of the clear supernatant was mixed with 50 µl of λ-sensitive cells (grown overnight in LB + 0.4% maltose) and incubated for 20 min at room temperature for adsorption. 5 ml of Z-broth supplemented with 5 mM $MgSO_4$ was then added to the infection mixture, and incubated at 37°C with shaking until lysis. The lysate thus obtained usually contained 10^9 pfu/ml.

2.9.1.2 Preparation of large scale λ lysates: A λ-sensitive host was first grown overnight in 10 ml of LB broth containing 0.4% maltose. To 1 ml of this culture, 0.1 ml of phage lysate (titre value 10^9 pfu/ml) was added in the presence of 5 mM $MgSO_4$ and after 20 min infection, the mixture was diluted with 50-100 ml of Z-broth containing 5 mM $MgSO_4$. This was grown with shaking until lysis occurred after ~ 6-8 h. The lysate was treated with chloroform, centrifuged and the supernatant was stored at 4°C.

A similar method was employed for amplifying phages from the ordered λ library of Kohara *et al.* (1987).

2.9.2 Generation of random transpositions into the *E. coli* genome using a λ-vehicle transposon: The λ-vehicle transposons are derivatives of Tn*10* that contain an antibiotic marker between Tn*10*-derived inverted repeats. The transposase gene is outside the Tn*10* element and is under the control of the *tac* promoter, requiring IPTG for induction in strains that carry a functional *lac* operon (Way *et al.* 1984).

To 2 ml of an overnight culture of the recipient strain, the λ lysate was added at an MOI of 0.1 with 10 mM $MgSO_4$ and 0.5 mM IPTG. Phage adsorption was allowed to occur for 20 min at 37°C. The cells were then pelleted, suspended in 10 ml LB with 2.5 mM sodium pyrophosphate and incubated at 37°C for 30 min. The cells were centrifuged and resuspended in 5 ml of LB with 2.5 mM sodium pyrophosphate, and one-hundredth

of the mix was plated on the appropriate antibiotic plate to determine the frequency of transposition. Once the frequency of transposition was estimated, appropriate volumes were plated to obtain approximately 3000 colonies per plate (for a total of 20,000-40,000 colonies, ensuring that the entire *E. coli* genome is represented.

The λ lysates used for the transposition experiments carry amber mutations and were propagated on *supE* strains, such as LE392. By titrating the resulting lysate on *supE* (LE392) and *sup*$^+$ (MG1655) strains, it was ensured that the ratio of amber-revertant phages in the lysate was less than 10^{-3}.

2.9.3 Preparation of λ p*lac*Mu55 and λ Mu507 lysates and generation of λ*lac*Mu transpositions into the chromosome: λ p*lac*Mu55 is a plaque-forming λ phage carrying the two ends of phage Mu but is defective for transposition of the latter as it lacks entirely the Mu *B* gene and carries an amber mutation in the *A* gene. Its transposition can be facilitated by Mu *A* and *B* functions provided in *trans* by a helper phage λpMu507. λ p*lac*Mu55 lysate was prepared on MC4100, while the helper phage λpMu507 (which carries S_{am} mutation and therefore is incapable of replicating in a WT strain) was propagated on a *supF* strain like LE392.

Insertional mutagenesis of *E. coli* using λp*lac*Mu55 was performed in two independent experiments as described below. For creating transpositions into the chromosome, 2 ml (around 2 x 10^9) of recipient cells grown overnight in LB + 0.4% maltose was simultaneously infected with λ p*lac*Mu55 (at MOI of 0.1) and λ Mu507 (at MOI of 0.5) in the presence of 10 mM $MgSO_4$. After an infection step of 20 min at 37°C, the cells were washed with citrate buffer, resuspended in 5 ml of fresh LB containing 25 mM sodium pyrophosphate, and incubated at 37°C for 30 min. Cells were pelleted and resuspended in 1 ml citrate buffer, and 100 µl of 10^{-1} and 10^{-2} dilutions were plated on LB Kan selection plates to determine the frequency of transposition; the rest of the transposition mix was incubated in 50 ml LB containing kanamycin and 25 mM sodium pyrophosphate.

2.10 Conjugation: Conjugation was performed in broth as described by Miller (1992). Both donor and recipient strains were inoculated from overnight cultures 1:100 into fresh LB and incubated at 37°C in order to obtain cultures with an A_{600} of 0.2. They were mixed at a ratio of 1:5 (donor : recipient) and incubated with slow shaking (40 – 50 rpm)

for 90 min at 37°C, and the mixture was then plated on the appropriate selection medium at different dilutions.

2.11 Transformation: For routine plasmid transformation, when very high efficiencies were not essential, the following method was used, which is a modification of the procedure described by Cohen *et al.* (1972). An overnight culture of the recipient strain was subcultured in fresh LB broth and grown at 37°C until an $O.D_{600}$ of 0.5 was reached. Cells were chilled on ice for 15 min. 1.5 ml of the culture was washed with 0.5 ml of ice-cold 0.1 M $CaCl_2$, re-suspended in 100 µl of ice-cold 0.1 M $CaCl_2$ and kept on ice for 20 min. 0.1 to 0.5 µg of plasmid DNA was added, incubated on ice for a further 30 min and given a heat shock for 90 sec at 42°C. The cultures were rapidly chilled on ice, mixed with 0.9 ml LB, incubated at 37°C for 45 min, and plated on the appropriate selection medium at various dilutions.

For transformation of ligation mixes, competent cells were prepared by the method of Inoue *et al.*, 1990. Briefly, an overnight culture of the strain (usually DH5α) was subcultured 1:100 in fresh LB broth and grown at 18°C to an OD of ~0.6. The cells were harvested by centrifuging at 4°C for 10 min at 2500 x *g*, suspended in 0.4 volumes of TB buffer and incubated on ice for 10 min. The cells were centrifuged again and resuspended finally in 0.08 volumes of TB buffer. DMSO (tissue culture grade) was added to a final concentration of 7%. After 10 min on ice, the cells were aliquoted in 100 µl volumes, snap frozen in liquid nitrogen and stored at –70°C. The expected efficiency of these cells is $1 \times 10^8 - 1 \times 10^9$ transformants per µg DNA. Transformation was then performed as described above.

2.12 Obtaining transposon insertions near gene of interest:

Obtaining transpositions near a gene of interest was achieved in a two-step procedure. The population (pool) of cells carrying random transpositions at different places on the chromosome was used to prepare a P1 phage lysate. This lysate was then used to infect a suitable recipient strain and transductants were sought in a simultaneous (double) selection for two markers namely, the antibiotic marker on the Tn element and a chromosomal marker in the vicinity of the region in which the insertion of transposon is desired. The transductants so isolated were purified and further P1 phage preparations were made on these individual clones. By retransducing with these lysates into the same

recipient cells and observing the segregation of phenotypes after selection for the transposon marker, the cotransduction values were obtained for the transposon insertion with the gene of interest.

2.13 Chemical mutagenesis of plasmid DNA with MNNG: The method for MNNG mutagenesis described by Miller (1992) was followed. An overnight culture of a Rec^+ strain, carrying the target gene to be mutagenized on a plasmid, was diluted 50-fold in LB and incubated till A_{600} was 0.6-0.8. 5 ml of cells were centrifuged, washed twice with an equal volume of 0.1 M citrate buffer, and resuspended in the same volume of the buffer. MNNG was prepared fresh as a 1 mg/ml stock solution in citrate buffer and added to cells at a final concentration of 50 μg/ml. Incubation was continued for 30 min at 37°C, followed by washing twice with 0.1 M phosphate buffer (pH 7.4). The cells were finally resuspended and grown overnight at 37°C in 20 ml of LB broth supplemented with the appropriate antibiotic to maintain the plasmid.

The surviving fraction of the cells after MNNG treatment was measured and determined to be about 1-10%. Plasmid DNA was isolated from the overnight amplified culture for further analysis. The glassware and materials used for the treatment of cells with MNNG were deactivated by 1 N NaOH as described by Miller (1992).

2.14 Ampicillin (Amp) selection for enrichment of NaCl-sensitive colonies: The rationale for ampicillin selection is that only dividing cells are killed by Amp (which acts by inhibiting the peptidoglycan formation during the cell wall biosynthesis), whereas nondividing cells are not affected by it. Thus, when a mutagenized pool of Kan^R colonies is allowed to grow in the presence of sufficiently high concentration of NaCl, all the NaCl-tolerant colonies would divide while the NaCl-sensitive colonies are growth-inhibited. To such a culture, when Amp is added, all the NaCl-tolerant (therefore, actively dividing) Kan^R cells would be killed whereas NaCl-sensitive (growth-inhibited, nondividing) Kan^R cells would not be affected. Thus, Amp treatment is expected to enrich for NaCl-sensitive clones starting with a large population of Kan^R cells.

The Kan^R colonies obtained in each experiment following λ*plac*Mu55 transpositions into *E. coli* were amplified overnight by incubating them in 10 ml LB containing Kan and 25 mM sodium pyrophosphate at 37°C. ~10^7 Kan^R cells were added to 10 ml MA + 10 mM sodium pyrophosphate + Kan containing 0.7 M NaCl with 1 mM

Bet, incubated with shaking at 37°C until an A_{600} of 0.1 was reached. At this stage, Amp was added at 100 μg /ml and shaking was continued. By this time, all the Kan^R colonies which were NaCl-tolerant are expected to start actively dividing, and thus would be killed by Amp. By 4-5 h, cell debris of the actively growing NaCl-tolerant cells was visible. The culture was filtered using 0.45 μ Millipore filter, and washed twice with sterile MilliQ (deionized) water to remove Amp and NaCl. The filter containing cells which escaped killing by Amp treatment was transferred aseptically into 10 ml MA+ Kan + 10 mM sodium pyrophosphate and allowed to grow overnight at 37°C. This step of incubating the filter of the first round of Amp treatment would result in the recovery of the growth-arrested nondividing cells which escaped killing by Amp. These cells would now be able to grow and multiply in MA medium in the absence of any NaCl-selection pressure. This step would enable the elimination of all the *lac*-Kan insertion mutants unable to grow in MA medium (for example, auxotrophic mutants, non-specific growth-retarding mutants, etc) and which have nothing to do with NaCl-sensitivity phenotype. 10^7 cells from this culture were then subjected to a second cycle of Amp-enrichment in the presence of NaCl and recovery as described above. Isolated single colonies were purified from the resulting culture and tested for NaCl-sensitivity.

2.15 Scoring for other phenotypes:

2.15.1 UV-sensitivity: The colonies were streaked on duplicate LB plates, one of which was exposed to a 15 W germicidal UV lamp at a distance of 75 cm for 20 s. Both plates were wrapped immediately in aluminium foil, incubated overnight and scored for growth. This test was performed routinely to determine qualitatively the uv sensitivity of various mutant strains.

2.15.2 NaCl-sensitivity testing: The colonies to be tested were streaked on the surface of MA plates containing either 0.6-0.7 M NaCl with 1 mM Bet or 0.4-0.5 M NaCl without Bet, and incubated at 37°C. NaCl-tolerant strains grew to form single colonies in 42-60 h whereas NaCl-sensitive ones did not. As controls, MC4100 (WT) and other NaCl-sensitive mutants previously identified, like *proU, proP* and *otsA*, were routinely streaked for comparison.

2.15.3 Test for *gltBD* phenotype: *gltBDF* is an operon encoding the A and B subunits of glutamate synthase (GOGAT) and is involved in ammonia assimilation and glutamate

biosynthesis. Growth of *gltBD* mutants is either abolished or drastically reduced on medium with poor nitrogen sources. Strains were screened for *gltBD*$^-$ phenotype by streaking on W-Salts medium supplemented with <1mM $(NH_4)_2SO_4$ or Arg or Pro or Ala as nitrogen source (and with 5 mM $(NH_4)_2SO_4$, as positive control). To confirm that the non-growing strains indeed harbor the *gltBD* mutation, they were transduced to *gdhA*::kan using the lysate made on GJ980. *gdhA* encodes glutamate dehydrogenase (GDH), which is involved in the second pathway of glutamate biosynthesis. *gltBD gdhA* double mutants are Asp auxotrophs. Those strains that yielded Kan^R transductants Asp auxotrophs were taken to be *gltBD* mutants.

2.15.4 Test for Canavanine sensitivity: Canavanine (Can) is a toxic analog of Arg and is an inhibitor of growth. Strains were tested for their sensitivity / tolerance to Can by streaking them on MA glucose plates without and with Can at 40 μg/ml (or other concentrations, as indicated).

2.15.5 Test for ArgR$^{+/-}$ phenotype: For testing ArgR$^{+/-}$ phenotype, the colonies were streaked on MA plates containing uracil (40 μg/ml) and Can (65 μg/ml). Uracil was added to the medium to sensitize an *argR*$^+$ strain to Can. An *argR*$^+$ strain is inhibited at 65 μg/ml Can on a uracil-containing plate, whereas on a plate without uracil, *argR*$^+$ would grow even at 700-800 μg/ml Can. Uracil represses the *carAB* transcription, which encodes the carbamoyl phosphate synthase enzyme (CarAB). This results in reduced amounts of carbamoyl phosphate, which is the common intermediate between pyrimidine and Arg biosynthetic pathways. Reduced carbamoyl phosphate levels would result in decreased flux through the Arg biosynthetic pathways. This in turn would result in decrease in Arg pools inside the cell. An *argR* mutant would be derepressed for the Arg biosynthetic pathway and is resistant even to 300 μg/ml Can in a uracil-containing plate.

2.15.6 *argR* cross-feeding phenotype: The principle behind this experiment is that an *argR* strain derepressed for Arg biosynthesis would cross-feed an Arg auxotroph that is seeded in the agar medium. The strain 160-37, an *argE*$^-$ mutant derivative of ATCC9637 *E. coli* W (requiring Arg for growth on defined MA medium) was used as a recipient in the cross-feeding experiments (Bollon and Vogel, 1973). The overnight culture of the test strain was diluted in LB and appropriate dilutions corresponding to 10^4 to 10^5 cells per

plate were poured in 20 ml of MA agar (without Arg), plus 1 μg/ml tetrazolium chloride in a petri plate. Tetrazolium chloride was used as an indicator of growth (Miller, 1992) of the microcolonies of 160-37, since it would turn from colorless to red within the cells. The test strains were then patched or spotted on the surface of such a plate and incubated for 48-60 h. The presence of a red 'halo' around a patched colony indicates the cross-feeding (therefore, Arg-excreting) ability, and the diameter of the red halo would represent the extent of cross-feeding by the test strain.

2.15.7 Measurement of growth delay (NUV phenotype): WT and *thiI* mutants (in a *relA*$^+$ background) were grown in MA medium at 37°C to late exponential phase. 5 ml of each culture was transferred to glass petri dishes and irradiated in a UV cross-linker (Amersham Life Sciences) for 40 to 60 min. After irradiation, the culture was diluted 20-fold into fresh MA medium (pre-heated to 37°C). Growth continued at 37°C and was monitored by measuring absorbance at 600 nm. Unirradiated controls were processed as above, but were covered with cardboard to prevent exposure to UV.

B. Recombinant DNA techniques:

2.16 Isolation of plasmid DNA: Cells from 3 ml of an overnight culture (grown with the appropriate antibiotic selection) were pelleted and suspended in 100 μl of ice-cold Solution I [50 mM Glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0)] with vigorous vortexing. 200 μl of freshly prepared Solution II (0.2 % NaOH, 1% SDS) was added and mixed by inverting. 150 μl of Solution III (prepared by mixing 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml water) was added and the mixture left on ice for 5 min. After centrifuging at 12,000 rpm for 15 min at 4°C in a microfuge, the supernatant containing the plasmid DNA was recovered. The DNA was then precipitated by adding an equal volume of iso-propanol and centrifuging at 12,000 rpm for 30 min at RT. The pellet was washed once with 70% ethanol, air-dried and suspended in 40 μl TE (Sambrook *et al.*, 1989).

2.17 Isolation of chromosomal DNA: Chromosomal DNA isolation was carried out by the method of Silhavy *et al.* (1984). Cells from 5 ml of an overnight grown culture were pelleted and suspended in 250 μl of a solution containing 50 mM Tris.Cl (pH 8.0) and 50

mM EDTA. 250 μg lysozyme was added and the cells were incubated on ice for 60 min. 50 μl of a solution containing 0.5% SDS, 50 mM Tris.Cl (pH 8.0), 50 mM EDTA was then added, followed by 50 μg of proteinase K and incubation at 50°C for at least 90 min. An equal volume (i.e., 300 μl) of phenol : chloroform : isoamyl alcohol (25: 24 : 1) was added and the aqueous phase containing the DNA was extracted. The aqueous phase was then extracted twice with equal volumes of chloroform : isoamyl alcohol (24 : 1). One-tenth the volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol were added, and the DNA was allowed to precipitate. Since DNA recovery by spooling was often difficult and resulted in shearing, it was pelleted by centrifugation at 7,000 rpm in a microfuge. The resulting DNA pellet was washed twice with 70% ethanol, air-dried and dissolved in 50 μl TE. This procedure yielded approximately 5 μg DNA.

2.18 Isolation of phage λ DNA: To 100 ml of the clear supernatant obtained after low-speed centrifugation of the phage λ lysate, pancreatic DNase and RNase were each added to a final concentration of 1 μg/ml and digestion was carried out for 30 min at 37°C. This was followed by the addition of 5.8 g of solid NaCl and 9.3 g of PEG 8000 and mixing with gentle agitation. The mixture was incubated on ice for 1 h following which the precipitated phage particles were recovered by centrifugation (in an SS-34 rotor, Sorvall RC5B centrifuge) at 10,000 rpm for 20 min at 4°C. The pellet was resuspended in 10 ml of phage buffer (20 mM Tris-Cl pH 7.4, 100 mM NaCl, and 10 mM $MgSO_4$) and centrifuged at 8,000 rpm for 2 min to remove the particulate debris. The supernatant was transferred to a fresh tube and successively extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a fresh tube and the phage DNA was precipitated with an equal volume of isopropanol. The DNA was recovered by centrifugation at 10,000 rpm at 4°C. The pellet was washed twice with 70% ethanol, dried and resuspended in TE buffer. The DNA thus obtained was suitable for restriction digestion experiments.

2.19 Isolation of total cellular RNA from bacterial cells

The method described by Aiba *et al.* (1981) was followed for RNA isolation. The concerned strain was grown overnight in LB medium, subcultured in 100 ml of the same medium and grown till late log phase (A_{600} of ~0.8 to1.0). The culture was chilled

suddenly by adding solid ice into the flask, and the cells were then harvested and washed with ice-cold solution of 20 mM sodium acetate, pH 5.5. The cell pellet was suspended in 3 ml of solution containing 20 mM sodium acetate pH 5.5, 0.5% SDS and 1 mM EDTA. To this suspension, 3 ml of acid phenol (phenol equilibrated with 20 mM sodium acetate, pH 5.5) was added and the mixture was incubated at 60°C for 5 min with gentle shaking, followed by a centrifugation step at 10000 rpm at 20°C. The aqueous phase was again extracted with acid-equilibrated phenol followed by centrifugation. The aqueous phase was subjected again to an extraction with chloroform:isoamyl alcohol mixture (24:1). The RNA was recovered by precipitation of the supernatant with the addition of 2.5 volumes of cold ethanol.

The precipitate obtained was dissolved in 3 ml of solution containing 20 mM sodium acetate, pH 5.5, 0.5% SDS and 1 mM EDTA. It was reprecipitated with 2.5 volumes of cold ethanol; the pellet was washed with 70% ethanol (containing 0.1 M sodium acetate), dried and dissolved in water. An aliquot of the dissolved RNA preparation was quantitated by electrophoresis and the quality of the preparation was estimated by measuring the absorbance at both 260 nm and 280 nm, as described.

2.20 Isolation of tRNA: The isolation of tRNA was based on the method of Hou and Schimmel (1988). Cells from 100 ml of an overnight culture were harvested by centrifugation for 30 min at 6,000 x *g* (4,000 rpm in an SS-34 rotor, Sorvall RC5B centrifuge). The supernatant was decanted and the pellet was resuspended in 10 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$. Using a vortex mixer, the cells were vigorously agitated for 1 min with water-saturated phenol (10 ml) and the mixture was subjected to centrifugation for 30 min at 8000 x *g* (7000 rpm in an SS-34 rotor Sorvall RC5B centrifuge). The aqueous phase was stored and the cells were resuspended, extracted and subjected to centrifugation, as just described. The two aqueous phases were combined (13 ml final volume). To precipitate the tRNA, 830 µl 5 mM sodium acetate buffer, pH 5.2 (0.3 M final concentration) was added, followed by addition of 40 ml absolute ethanol and incubation for a minimum of 1 h at –20°C.

The tRNA was pelleted by centrifugation for 30 min at 8000 x *g* and this pellet (2 g) was redissolved in 8 ml 50 mM Tris-HCl buffer, pH 7.6. The tRNA was then passed over a Sephadex G-25 column equilibrated in the same buffer. The final tRNA solution was ~0.2 mM.

2.21 Restriction digestion: 0.5 to 1 μg of DNA was routinely digested in a 20 μl volume with 2-4 U of the enzyme and the appropriate 10 X buffer supplied by the manufacturer for 5-6 h at temperatures specified by the manufacturer. The digested samples were run on 0.8-1% agarose gels to visualize the DNA bands (as described below).

2.22 Agarose gel electrophoresis: DNA samples were mixed with the appropriate volumes of the 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) and subjected to electrophoresis through 0.8% to 1% agarose gels in 1 X TBE or 1 X TAE buffers at 5 V/cm for 2-4 hours. The gel was stained in 1 μg/ml of ethidium bromide for 30 min at room temperature and the bands were visualized by fluorescence over UV-light. Commercially available molecular weight markers were also run to facilitate estimation of the sizes and concentrations of the bands in the DNA sample.

2.23 Gel extraction of DNA fragments: DNA fragments of interest used for ligation or sequencing were extracted from 1% TAE or TBE agarose gels using the QiaQuick Gel Extraction Kit (Qiagen) or Gene-Clean kit according to the instructions specified by the manufacturer.

2.24 Ligation: A 1:1 or 1:3 ratio of vector to insert was used. 100-200 ng of DNA was incubated with 1 U of T4 DNA ligase at 16°C for 14-16 h.

2.25 PCR: For initial standardization of the PCR conditions for a particular pair of primers, 200 to 800 ng of DNA was used as template. Subsequently, the colony PCR approach (Rosenberg *et al.*, 1994) was adopted for obtaining amplified products with the primer pair from a large number of strains. Cells from an overnight culture of the strain grown in LB broth were washed with and suspended in the same volume of sterile MilliQ water, and 10 μl (corresponding to ~10^7 cells) was used as the source of template DNA. All PCR reactions were carried out in 50 μl volumes. The reaction mixture contained, apart from 10 μl of the template preparation, 200 μM of the dNTP mix (Promega or Perkin Elmer), 1.5 U of Taq Polymerase (Bangalore Genei), 5 μl of the 10 X PCR buffer,

supplied by the manufacturer (Bangalore Genei) and 10 pmoles of each of the two primers.

The region flanking the site of transposon insertion in each mutant was amplified with primers MuC1 and MuC2, using the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 1 min and extension at 72°C for 2 min.

The region flanking the site of Tn*10*dTet insertion in the Can^{SS} mutant was amplified with primers AH1 and AH2, using the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 47°C for 1 min and extension at 72°C for 2 min.

The region flanking the site of transposon insertion in *yggA* mutants was amplified with primers TETF, TETR, YGGAR and YGGBF, using the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 1 min and extension at 72°C for 2 min.

Typically, all reactions began with a preliminary denaturation step ("hot start") of 5 min at 94°C and ended with a final extension step of 5 min at 72°C. The hot start also served to lyse the cells when the colony PCR approach was followed.

2.26 Purification of PCR products: PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen) as per the manufacturer's instructions.

2.27 Sequencing: Cycle sequencing was performed on the ABI Prism Perkin Elmer 377 or the ABI Prism Perkin Elmer 3700 (capillary type) machines, using the PE Big-Dye Terminator Kit, as per the manufacturer's instructions.

C. β-Galactosidase assay: β-Galactosidase assays were done, as recommended by Miller (1992). Overnight cultures of the strains were subcultured in fresh medium and grown until an A_{600} of 0.6 to 0.8 was reached. To 0.1-0.5 ml of the culture, Z buffer was added to a final volume of 1 ml, and the cells were lysed by adding 2 drops of chloroform and 1 drop of 1% SDS. 200 µl of ONPG (from a 4 mg / ml stock made in Z-buffer) was added and the initial time noted. The tubes were left at room temperature; when the solution turned yellow, the reaction was terminated by adding 0.5 ml of 1 M Na_2CO_3, and the final time noted. The absorbances of the reactions were taken at 420 nm and 550 nm,

and of the cultures at 600 nm. The enzyme specific activity (expressed in Miller Units) was calculated by the formula:

$$\text{Enzyme specific activity} = \frac{1000 \text{ x } [A_{420nm} - (1.75 \text{ x } A_{550nm})]}{t \text{ x } v \text{ x } A_{600nm}}$$

where t = time of the reaction in min and

v = culture volume.

Chapter 3

Isolation, genetic and molecular characterization of insertion (transposon-mediated) mutations conferring NaCl-sensitivity in *Escherichia coli*

3.1 Introduction:

As described in Chapter 1, there are no reports of a comprehensive screen in *E. coli* for genes involved in low a_w stress adaptation. Earlier reports of genes involved in water-stress adaptation were based mainly on screens aimed at identifying genes which were differentially regulated under water stress conditions (Gutierrez *et al.*, 1987; Gowrishankar, 1985). In order to comprehensively screen the *E. coli* genome for loci involved in conferring water stress adaptation, a whole genome-mutagenesis approach was taken in the present study. This chapter describes the isolation of NaCl-sensitive mutants following transposon-mediated mutagenesis and Amp enrichment for the NaCl-sensitive mutants. Results are presented, which describe the genetic and molecular characterization of mutations conferring NaCl-sensitivity. Further, this chapter seeks to classify the various mutants into categories, (i) depending on the requirement of the *gltBD* locus for the observed NaCl-sensitive phenotype and (ii) based on their differential sensitivity to other dissolved solutes.

3.2 Results:

3.2.1 Strategy for the isolation of NaCl-sensitive mutants:

The NaCl-sensitive mutants were isolated following λp*lac*Mu55-mediated whole-genome insertional mutagenesis of *gltBD* strain (GJ2529) of *E. coli*. The rationale for using *gltBD* mutant (deficient in the enzyme glutamate synthase involved in NH_4^+-assimilation) as the starting strain for mutagenesis was the data previously reported from this lab and others that the glutamate pools are low in *gltBD* mutants of *E. coli* and *S. enterica*, and that decreased glutamate pools make the strain more sensitive than a WT strain to osmotic stress (McLaggan *et al.*, 1994; Csonka *et al.*, 1994; Saroja and Gowrishankar, 1996). Thus, the *gltBD* mutation was expected to sensitize the strain even to any additional alterations / gene disruptions which might otherwise result only in moderate deficiency in adaptation to water-stress. Thus, in a *gltBD* mutant background, the second null insertion mutation would confer NaCl-sensitivity in a synergistic or additive fashion. The screen was also expected to yield insertion mutations which confer NaCl-sensitivity irrespective of the status of the *gltBD* locus.

3.2.2 Construction of GJ2529:

gltBD is an operon situated at 69.8 min on the *E. coli* chromosome, encoding the glutamate synthase A and B subunits that are involved in ammonia assimilation and glutamate biosynthesis. The Δ*gltBD* mutation was transduced into MC4100 from the strain GJ966 by phage P1 transduction. Strain GJ966 carries the Δ*gltBD* mutation and a Tn*10* insertion encoding the tetracycline resistance marker (Tet^R; *zha-6*::Tn*10*) that is 99% linked to the *gltBD* locus in transduction. GJ966 also carries the $Spec^R$ marker in the *fnr* gene (Ω*fnr*::Spec), which encodes fumarate nitrate reductase. A P1 lysate prepared on GJ966 was used to infect MC4100, and Tet^R transductants were selected. Individual Tet^R colonies were then screened for $GltBD^-$ phenotype by streaking on W-Salts supplemented with 0.5 mM ammonium sulfate. Growth of *gltBD* mutants is either abolished or drastically reduced on W-Salts medium with <1 mM NH_4^+ source. None of the eight Tet^R colonies tested grew on W-Salts with <1mM NH_4^+, suggesting that all of them may have inherited the *gltBD* mutation and that Tet^R marker is tightly linked to the *gltBD* locus.

To confirm that these strains indeed harbor the *gltBD* mutation, two colonies were transduced to Kan^R using the lysate made on GJ990. Strain GJ990 harbors the Kan^R insertion in the gene *gdhA* (represented by *gdhA*::Kan), which encodes glutamate dehydrogenase, involved in glutamate biosynthesis and ammonia assimilation. *gdhA gltBD* double mutants were earlier reported to be Asp auxotrophs (Reitzer and Magasanik, 1987). If the above donor strains carry *gltBD* mutation, then all the *gdhA*::Kan colonies would be Asp auxotrophs. When 8-10 Kan^R transductants from the two donor strains were tested for Asp auxotrophy, they were all found to be Asp auxotrophs, indicating that the two donor strains indeed carry Δ*gltBD* mutation. One such donor strain carrying the *gltBD* mutation linked 99% to the Tn*10* marker was designated GJ2529.

3.2.3 Construction of GJ2530 / pHYD909:

It was previously reported from the lab that a strain mutated in *gltBD* and *fnr* (encoding the regulator protein, FNR, required for anaerobic gene expression) loci becomes osmosensitive (Saroja and Gowrishankar, 1996) and it was also shown by genetic approaches that monomeric FNR has a role to play in aerobic ammonia assimilation and osmoregulation. All known transcriptional targets of FNR are regulated by the dimeric form, but there were no reports of monomeric FNR being involved in gene

regulation. Therefore, the present screen for NaCl-sensitive mutants of *E. coli* was so devised that it would be possible to isolate new gene(s) which are regulated by monomeric FNR and which are involved in osmoadaptation in aerobic conditions.

The strategy involved the construction of chromosomal Δ*gltBD* Δ*fnr* strain and the introduction of N-terminal deletion mutant of FNR on a plasmid. This mutant FNR has lost the redox-based dimerization capability due to the deletion of 28 amino acids at the N-terminus. Into such a strain, random insertion mutagenesis would be carried out employing a Mu-based transposon (λp*lac*Mu55), which could form promoter-*lac* operon-fusions and simultaneously create a null (insertion) mutation. Such insertion mutants would then be tested for NaCl-sensitivity phenotype, followed by examination of the effect of FNR on such a fusion. If a gene is involved in conferring NaCl-tolerance and is regulated by monomeric FNR under aerobic conditions, then insertions in that gene would make the strain NaCl-sensitive and the *lacZ* gene fusion (reporter) would be modulated by monomeric FNR. The second possibility in the above case would be that the *lac*-Kan insertion in a gene abolishes its function fully (null) but is integrated in the opposite orientation to its transcription. Then such a strain would still be NaCl-sensitive but would not exhibit any monomeric FNR-mediated gene regulation. The third possibility is that *lac*-Kan insertion would result in an NaCl-sensitive strain, irrespective of the monomeric FNR status of the cell. A fourth possibility is that some insertions would confer NaCl-sensitivity irrespective of *gltBD* status of the cell, whereas other insertions would require *gltBD* to show NaCl-sensitivity phenotype.

In order to construct a strain which is deleted for *gltBD* and *fnr* on the chromosome but which carries a monomeric FNR-encoding gene on a plasmid, the following approach was taken. GJ2529 (Δ*gltBD zha*::Tn*10*) was used as recipient in the next step to transduce Δ*fnr*::Spec by phage P1 transduction experiment. A P1 lysate made on GJ966 (Δ*fnr*::Spec^R) was used to infect GJ2529 and Spec^R colonies were selected for. One such Spec^R colony was designated GJ2530 (Δ*gltBD zha*::Tn*10* Δ*fnr*::Spec).

Plasmid pHYD909 (pACYC184 plasmid carrying the N-terminal deletion mutant of *fnr*) was constructed by cloning the 1.6 kb *Bam*HI-*Hin*dIII fragment containing the truncated *fnr* gene from plasmid pGS198 (Spiro and Guest, 1988). Strain GJ2530 (Δ*gltBD* Δ*fnr*) was transformed with pHYD909 and Cm^R colonies were selected for. One such Cm^R colony (GJ2530 / pHYD909) was chosen for further work. This strain carries

chromosomal deletions in *gltBD* and *fnr* genes and harbors an *fnr* mutant gene encoding N-terminally deleted (monomeric) FNR on the plasmid.

3.2.4 Construction of GJ4652:

In the strain GJ2529 and its derivatives viz., GJ2530 and GJ2530 / pHYD909, the Δ*gltBD* is linked to Tn*10* (encoding Tet^R). In order to construct double- or triple- mutant combinations involving Δ*gltBD*, it was decided to have a strain which is Δ*gltBD* but Tet^S. Such a strain would give Tet^R as an additional marker for manipulation of the strain using P1 transductions. To construct such an antibiotic-markerless Δ*gltBD* strain, the following approach was taken, wherein Δ*gltBD* locus was transduced into MC4100, employing *argG* and *nlp*::Tn*10* linked to it, subsequently crossing out the *argG* and *nlp* mutations (Fig. 3.1).

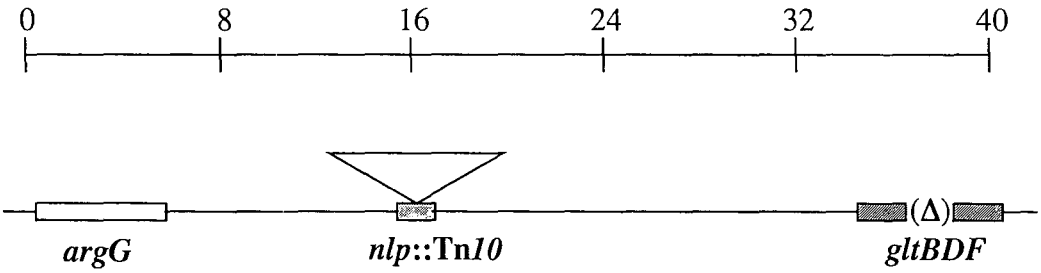


Fig. 3.1 Physical map of the region around *gltBD* (71.5 – 72.5 min). Below the kilobase scale is depicted the physical map of the region near *gltBD*. ▽ represents the Tn*10* insertion in the *nlp* gene in CAG120172, while Δ represents the deletion of the *gltBDF* operon in PA340.

Step 1: *nlp*::Tn*10* located at 70.9 min on the *E. coli* chromosome in the strain CAG12072 was transferred into CSH57 (*argG*) by selecting for Tet^R colonies through P1 transduction. The resultant Tet^R colonies were screened for *argG* by testing the colonies for arginine auxotrophy. One such *argG* Tet^R colony was used for subsequent work.

Step 2: A P1 lysate was grown on the above strain and used to transduce PA340 (Δ*gltBDF500*) by selecting for the inheritance of Tn*10* and *argG* mutations, and screening for the colonies which still retain the Δ*gltBD* locus. This was accomplished by testing the Tet^R colonies for Arg auxotrophy and for GltBD phenotype on W-Salts containing <1 mM NH_4^+. The Tet^R colonies which failed to grow on both the media were considered to have *argG* and Δ*gltBDF* alleles.

Step 3: The three markers namely, *nlp*::Tn*10*, Δ*gltBDF500* and *argG* of the above strain were transduced into MC4100 subsequently through a P1 transduction, and selection for Tet^R was done. The resulting Tet^R colonies were screened for the co-inheritance of *argG* and Δ*gltBDF* loci. One colony which had all the three mutations was selected for the next step.

Step 4: The *argG* mutation was crossed out from the above strain by transducing with P1 lysate prepared on MG1655 (WT strain) and selecting for Arg^+ colonies on MA plates. The loss of *nlp*::Tn*10* among these prototrophs was scored by screening for Tet^S. Such Arg^+ Tet^S colonies were finally screened for the presence of Δ*gltBDF500* allele on W-Salts medium, and the colonies which were unable to grow on such medium were considered to contain Δ*gltBDF500* locus.

Step 5: In the final step, the presence of Δ*gltBDF500* was confirmed as described above in Section 3.2.2 by transducing $GltBD^-$ colonies to *gdhA*::Kan and screening the resultant transductants for Asp auxotrophs. One strain, whose *gdhA*::Kan derivatives were Asp auxotrophic, was designated GJ4652.

3.2.5 Insertional mutagenesis with λp*lac*Mu55:

λp*lac*Mu55 is a plaque-forming derivative of phage λ that carries the two ends of phage Mu (Mu *c* and Mu *S*) and inserts randomly into genes by using the transposition machinery of phage Mu. Each λp*lac*Mu55 phage is a specialized transducing bacteriophage that carries the *c* end of Mu, containing the Mu *C*(ts)62, *ner* (cII) and *A* (transposase) genes, and the terminal sequences from the Mu *S* end (β end). These sequences contain the Mu attachment sites, and their orientation allows the λ genome to be inserted into other chromosomes, resulting in a λ prophage flanked by the Mu *c* and *S* sequences. This phage carries promoterless *lacZY* genes adjacent to the Mu *S* end. Insertion of λp*lac*Mu55 into a gene, in addition to completely inactivating the gene, when inserted in the proper orientation, creates an operon fusion in which *lacZ* and *lacY* are expressed from the promoter of the target gene.

λp*lac*Mu55 phage has previously been shown to integrate with little specificity by the phage Mu transposition system. The Mu *B* function is provided by a helper phage, λpMu507. The absence of the transposase gene and of the neighboring killing function(s) from Mu in the former accounts for the stable, temperature-resistant phenotype of λp*lac*Mu55 lysogens.

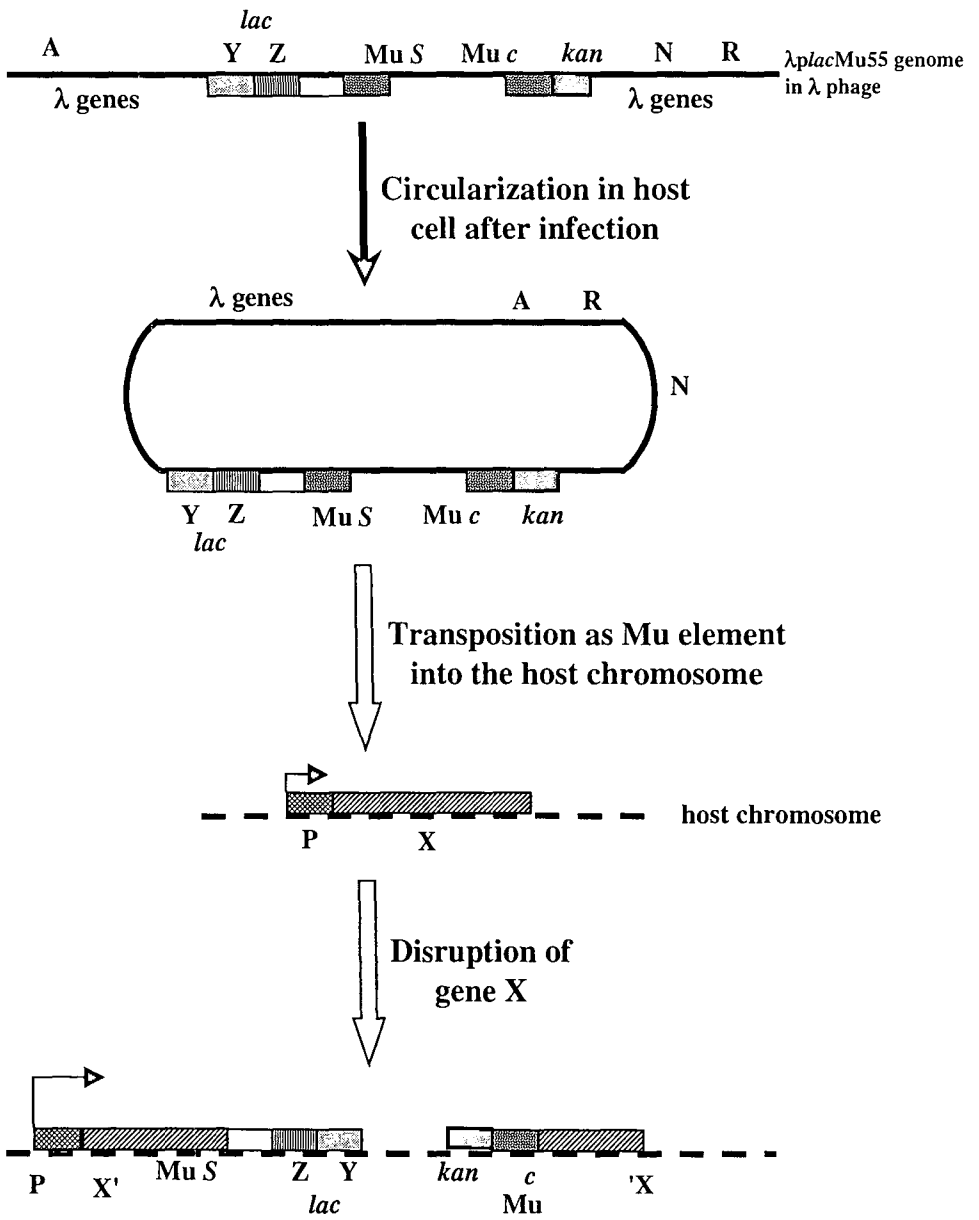


Fig. 3.2 Insertion mutagenesis and formation of promoter-*lac* operon fusion with λp*lac*Mu55. Genes A, N, and R are located on λp*lac*Mu55 while P and X represent the promoter for the gene X in the host chromosome.

The advantages of using λp*lac*Mu55 are that (i) strains containing promoter-*lac* transcriptional (operon) fusions can be isolated in a single step, (ii) the fusions are genetically stable, (iii) they can be conveniently manipulated, (iv) null (loss-of-function) insertions can be isolated, and (v) the presence of the Kan^R gene permits cells containing insertions of these phages to be selected independent of their Lac phenotype (Fig. 3.2).

The Kan^R marker gives a molecular handle to map the mutation both genetically and physically. In the present study, λp*lac*Mu55 was used to generate null mutants (and associated promoter-*lac* fusions) which exhibit NaCl-sensitive phenotype.

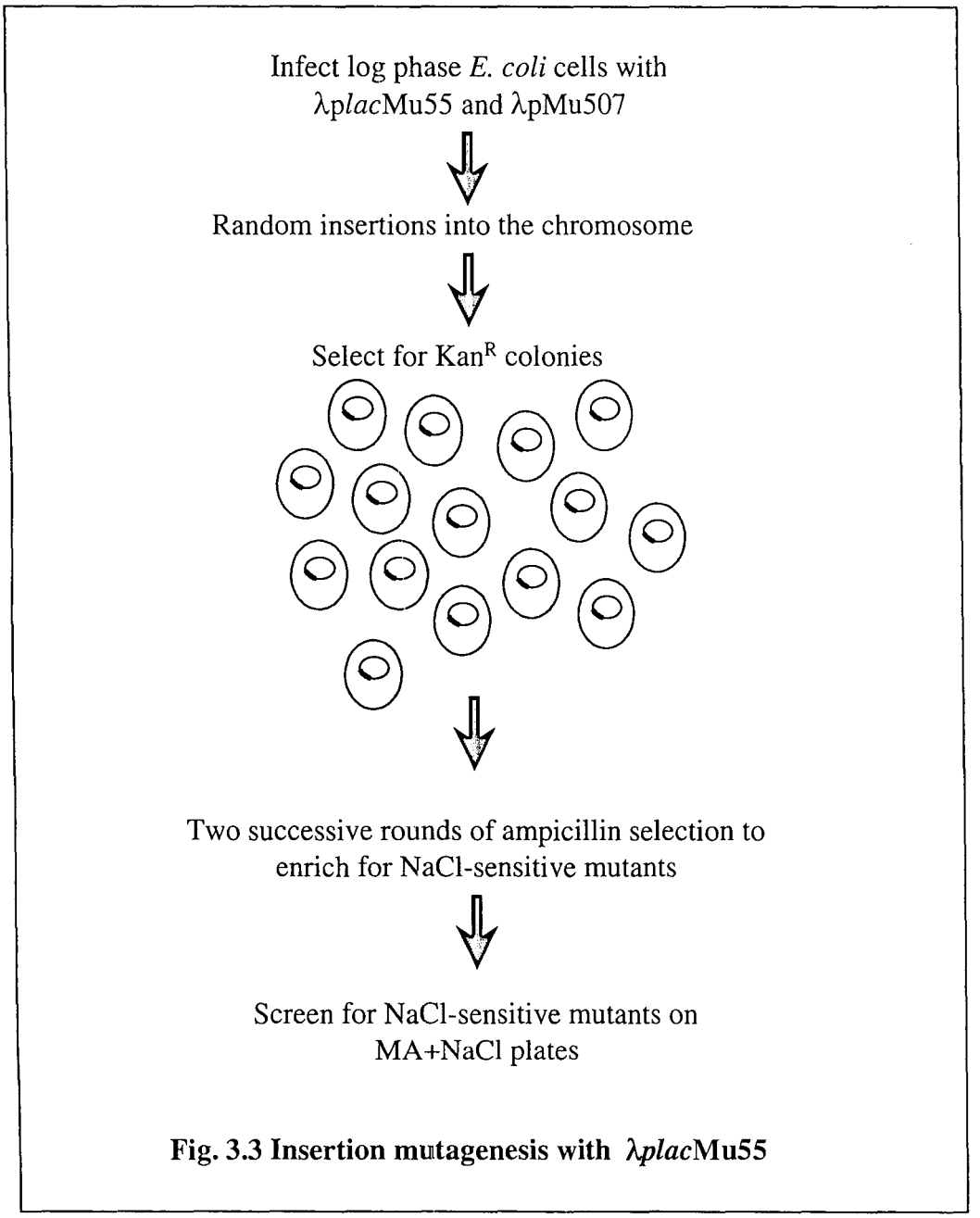


Fig. 3.3 Insertion mutagenesis with λ*lac*Mu55

Insertional mutagenesis of *E. coli* using λp*lac*Mu55 was performed in two independent experiments as described in (Section 2.9.3 of Chapter 2). Enrichment for NaCl-sensitive cells in the mutagenized population was then achieved by the procedure of ampicillin selection (Section 2.14 of Chapter 2). The Kan^R colonies so obtained were tested for NaCl-sensitivity (Fig. 3.3).

3.2.6 Tests of NaCl-sensitivity:

The purified Kan^R colonies were streaked on the surface of MA plates containing either 0.6-0.7 M NaCl with 1mM Bet or 0.4-0.5 M NaCl without Bet at 37°C. Thirty-eight NaCl-sensitive colonies were identified by screening 250 colonies after the two successive rounds of ampicillin enrichment. From these, 20 colonies were picked for further analysis and were designated GJ2533 through GJ2552.

3.2.7 Demonstration that NaCl-sensitivity phenotype is 100% cotransducible with the kanamycin resistance marker:

The NaCl-sensitivity phenotype in the above mutants might be caused by the Kan^R insertion mutation or alternatively by a second point mutation that could either be linked or unlinked to the Kan^R marker. To test these possibilities, linkage between Kan^R and NaCl-sensitivity phenotypes was determined by P1 transduction for each mutant. For this purpose, P1 lysates were prepared on the 20 NaCl-sensitive strains. These lysates were used to transduce the parental strain (GJ2530 / pHYD909) to Kan^R. The resultant Kan^R colonies (4 from each) were then tested for the NaCl-sensitivity phenotype, as described in Materials and Methods. Based on the results obtained (Table 3.1), the strains could be classified as follows:

Class I: 4/4 NaCl-sensitive (e.g. GJ2540, GJ2547). This result suggests that the original NaCl-sensitive strain carried only one *lac*-Kan insertion and that was responsible for the observed NaCl-sensitive phenotype.

Class II: 0/4 NaCl-sensitive (e.g. GJ2538, GJ2551). The above result suggests in these two $NaCl^S$ strains, the NaCl-sensitivity was not due to the *lac*-Kan insertion, but may be due to an unlinked point mutation somewhere in the chromosome. Since the phenotype is not linked to the Kan^R marker, these two mutants were not examined further.

Class III: Some but not all Kan^R colonies were NaCl-sensitive (e.g. GJ2533, GJ2534, GJ2535, GJ2536, GJ2537, GJ2539, GJ2541, GJ2542, GJ2543, GJ2544, GJ2545, GJ2546, GJ2548, GJ2549, GJ2550, GJ2552). This result suggests that there could be two possibilities that would explain the observed NaCl-sensitivity:

(i) there are more than one Kan^R insertion in these strains and only one Kan^R is responsible for the NaCl-sensitivity, while the other has no role to play in the observed phenotype,

(ii) The observed phenotype is not due to the Kan^R insertion but due to a linked point mutation which was co-segregating with the Kan^R marker at a certain frequency.

Table 3.1 Co-transduction of NaCl-sensitivity phenotype with Kan^R marker

Strain derivatives into recipient strain GJ2530 / pHYD909	No. of $NaCl^S$ colonies / Total Kan^R colonies tested[a]
GJ2533	3 / 4
GJ2534	2[b] / 4
GJ2535	3 / 4
GJ2536	3 / 4
GJ2537	2 / 4
GJ2538	0 / 4
GJ2539	1 / 4
GJ2540	4 / 4
GJ2541	3 / 4
GJ2542	3 / 4
GJ2543	2 / 4
GJ2544	3[b] / 4
GJ2545	3 / 4
GJ2546	1 / 4
GJ2547	4 / 4
GJ2548	2 / 4
GJ2549	2 / 4
GJ2550	2 / 4
GJ2551	0 / 4
GJ2552	3 / 4

[a] Four colonies from each transduction into GJ2530 / pHYD909 were streaked on MA supplemented with 0.6 – 0.7 M NaCl and 1 mM Bet for testing NaCl-sensitivity, as described in the text.
[b] partially NaCl-sensitive

To distinguish between the above two possibilities, P1 lysates were prepared on these NaCl-sensitive colonies obtained in the second round and the Kan^R marker was transduced into GJ2530 / pHYD909 afresh. The resultant Kan^R colonies (8 from each) were tested for NaCl-sensitive phenotype, as described earlier. In all the strains, except GJ2534 and GJ2544 (where all Kan^R derivatives were partially $NaCl^S$), all the eight Kan^R derivatives tested were NaCl-sensitive, suggesting that the Kan^R marker in these NaCl-sensitive strains was linked 100% to the NaCl-sensitivity phenotype and that the Kan^R insertion is in fact, responsible for the observed NaCl-sensitivity phenotype. The result also rules out that there is a linked mutation to Kan^R which was responsible for NaCl-sensitivity. If this was indeed the case, then one would have expected only a proportion of Kan^R transductants to be NaCl-sensitive (depending on co-transduction frequency) but not 100% linkage between NaCl-sensitivity and Kan^R transposon. This result thus, supports the former possibility that there were more than one Kan^R insertion in the original NaCl-sensitive isolate and only one of the Kan^R insertions was responsible for the observed NaCl-sensitivity. These confirmed NaCl-sensitive strains in the GJ2531 background were designated as GJ3533 through GJ3553, correspondingly (but leaving out GJ3538 and GJ3551).

3.2.8 Molecular characterization of NaCl-sensitive mutants:

3.2.8.1 Strategy:

In order to physically map the Kan^R mutation and identify the gene disrupted in each of these NaCl-sensitive mutants, the strategy of inverse PCR was employed followed by DNA sequencing.

Inverse PCR is a technique employed to amplify the unknown region flanking the site of transposon insertion with the aid of a pair of primers designed from the known sequence at one end of the transposon element. Inverse PCR involves digesting the genomic DNA with a 4-base recognition restriction enzyme, followed by intramolecular ligation achieved by performing the ligation reaction at high dilution. These ligated molecules serve as the template for the PCR reaction performed with a pair of divergently-oriented primers designed from one end of the transposon. The amplicon obtained after inverse PCR reactions would be sequenced with one of the primers to identify the junction sequence at the site of the transposon insertion, and hence, the identity of the gene disrupted in each case.

λp*lac*Mu55 transposon used in the present study would generate the Mu *c* and Mu *S* regions at either end once the transposon gets integrated into the *E. coli* genome (Fig. 3.4).

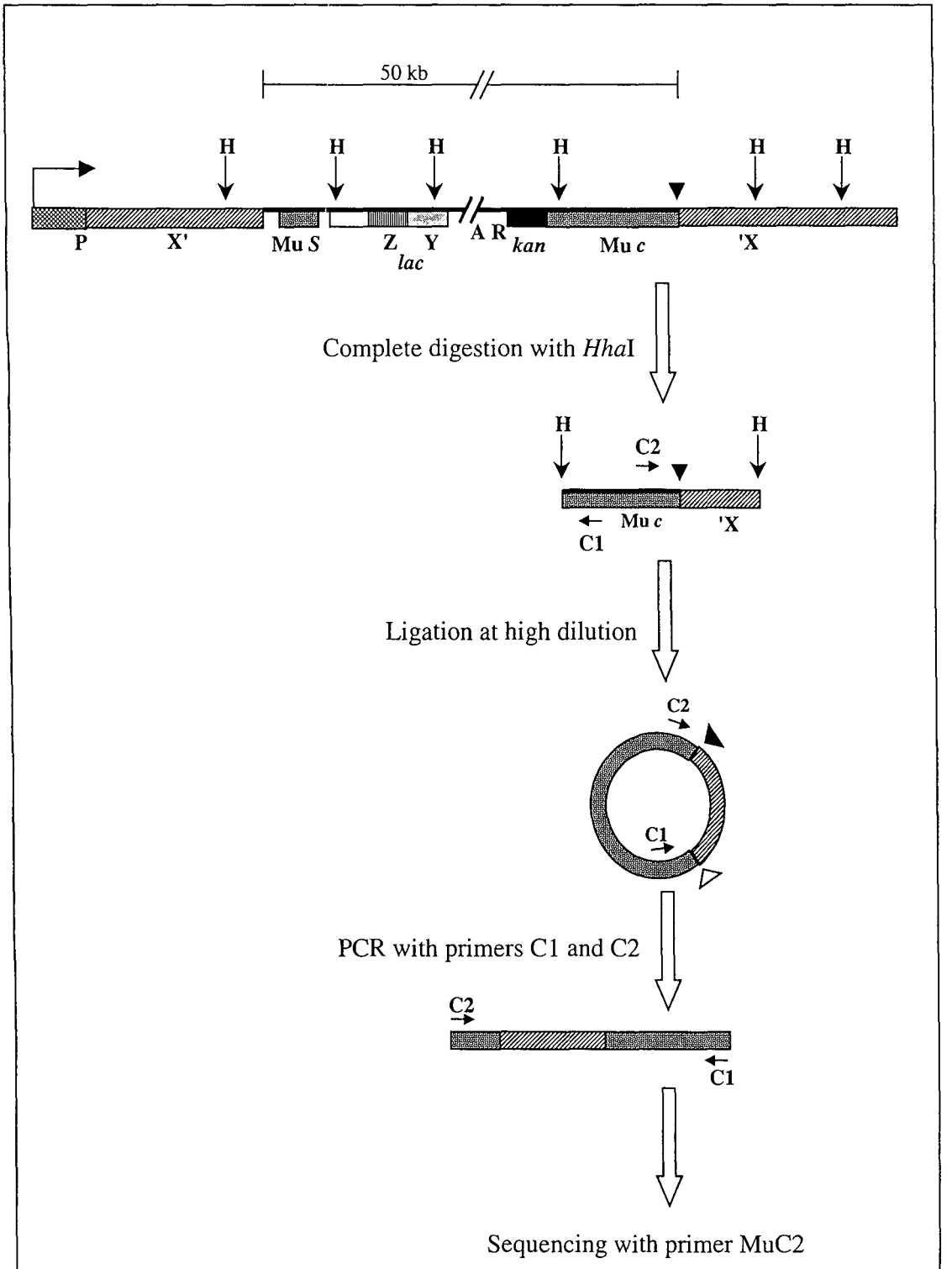


Fig. 3.4 Molecular characterization of mutants by inverse-PCR and sequencing. *Hha*I sites (H), the junction of λ genome and *E. coli* chromosome (filled arrow head), and the new junction created after ligation (open arrow head) are indicated. ▨ represents the sequences of the host gene disrupted by the transposon insertion while ▬ represents the Mu *c* sequences of the transposon

The critical components for inverse PCR are primer design and choice of the appropriate restriction enzymes for digestion of genomic DNA. Here, it was decided to design primers for the Mu *c* end since PCR employing primers from the Mu *S* end might be difficult due to the propensity for formation of inverted repeats in that region (Roy *et al.*, 1995). One primer (MuC2) was designed 70 bp inside the Mu *c* end and reading outwards from the transposon. The second primer (MuC1) was designed 300 bp inside the Mu *c* end and reading into the transposon. The two 4 bp-recognition enzyme, *Hha*I, was chosen for digesting the genomic DNA since this enzyme does not cut between the two regions from which primers were designed. Thus, when genomic DNA prepared from a Kan^R strain was restricted with *Hha*I, the expectation was that it would cut 300 bp inside the Mu *c* end of the transposon and would have frequent cuts (on average, one site per 256 bp) in the chromosomal DNA of *E. coli*.

Intramolecular ligation of such a digested DNA would result in a new junction being generated between the *Hha*I site in the Mu *c* end of the transposon and the *Hha*I site present outside the transposon on the *E. coli* chromosome. This permutation would result in a circular molecule of DNA, which would now consist of Mu *c* end sequences and the unknown *E. coli* genomic DNA sequences corresponding to the disrupted gene. This circularly permutated DNA molecule would serve as the template for amplifying the unknown *E. coli* sequences employing the divergently oriented MuC1 and MuC2 primers. The end PCR product would be a linear DNA molecule consisting of the Mu *c* end and the adjacent unknown *E. coli* chromosomal DNA. Sequencing such an amplicon with the MuC2 primer would yield the identity of the *E. coli* chromosomal region and thus, the gene disrupted by this transposon.

3.2.8.2 Isolation of chromosomal DNA, restriction digestion and inverse PCR:

To map the insertions of λp*lac*Mu55 precisely, chromosomal DNA from each of the NaCl-sensitive mutant strains was isolated according to the procedure mentioned in Section 2.17 of Chapter 2. The genomic DNA was then digested to completion with *Hha*I. The digested samples were then glycogen-precipitated overnight and then 100-200 ng of digested DNA was subjected to intramolecular ligation in a 50μl reaction volume at 16°C for 16 h. 5-10 μl of the ligated sample was used as template for the inverse PCR reaction using MuC1 and MuC2 primers, according to the conditions mentioned in

Section 2.25 of Chapter 2. The PCR products were then electrophoresed on 1% agarose gels and the amplicons were gel-eluted.

In the majority of the strains, PCR products ranging from 300-500 bp were obtained, which gave a ladder-like appearance. The lowermost band corresponding to the lowest molecular weight was more intense than the higher molecular weight bands. The different-sized bands reflect the different-sized products resulting from different ligation products from the same mutant. Depending on the position of the adjacent *Hha*I sites and extent of digestion, the size of the inverse PCR amplicon differs for the same mutant. In some cases, there were only one or two bands. In any case, the lowermost band was cut for gel elution. The gel-eluted, purified PCR products were sequenced with the MuC2 primer. The DNA sequence was then subjected to BLAST search (Altschul *et al.*, 1997) which returned homologous sequences from the *E. coli* database. The first 50-70 bp of the sequence was from the Muc end since the primer was so designed, followed by the *E. coli* sequence.

It was ensured that in each case, the junction sequence between the transposon end and the chromosomal sequences was read. This was to rule out spurious sequencing results arising from misprimed sequencing reactions. Only those DNA sequences that gave the junction sequence were expected to be authentic. Primer MuC1 was not used for sequencing for the following reasons: Firstly, since it was designed 300 bp inside the Mu *c* end and was reading into the Mu transposon, the first few hundred bp would be from the Mu *c* end and then the chromosomal sequences would be read. Secondly, if during the intramolecular ligation step, any other *E. coli* chromosomal DNA fragment gets inserted between the Mu *c* end and the *Hha*I site from the MuC2 primer end, it would result in a permutated junction sequence which has nothing to do with the gene disrupted by the Kan^R insertion. Thus, sequencing with MuC1 primer would lead to *E. coli* sequences unrelated to the junction sequences from the MuC2 primer end and would lead to erroneous results. Thus, it was decided to use the MuC2 primer for sequencing the inverse PCR amplicon.

Sixteen of the 18 NaCl-sensitive mutant strains were subjected to inverse PCR and sequencing. The automated DNA sequencing yielded DNA sequences that were sufficient for BLAST analysis at the NCBI site for fifteen mutants. For one strain, GJ3545, the inverse PCR followed by sequencing yielded only 6 bp sequence which was not sufficient for meaningful BLAST analysis. The results of the BLAST analyses, which identifies the

gene disrupted in each strain, are tabulated in Tables 3.2 and 3.3. Seven different loci conferring NaCl-sensitivity in *E. coli* were obtained.

Table 3.2 Molecular characterization of NaCl-sensitive mutants following inverse PCR

Strain	Gene disrupted	Position on the *E. coli* chr	Observed iPCR product size (bp)	Expected iPCR product size (bp)[a]
GJ3533	*thiI*	9.5	~350	356
GJ3534	*proV*	60.4	~350	343
GJ3535	*thiI*	9.5	only genetic data available	
GJ3536	*argP (iciA)*	65.9	~350	344
GJ3537	*lysC*	91.2	~700	670
GJ3539	*thiI*	9.5	~500	470
GJ3540	*argP (iciA)*	65.9	~400	417
GJ3541	*apaG*	1.1	~450	465
GJ3542	*speC*	66.9	~550	556
GJ3543	*thiI*	9.5	~350	349
GJ3544	*proU*	60.4	only genetic data available	
GJ3545[b]	*glnE*	68.9	~350	ND
GJ3546	*thiI*	9.5	~350	349
GJ3547	*glnE*	68.9	~350	361
GJ3549	*glnE*	68.9	~400	426
GJ3550	*thiI*	9.5	~500	475
GJ3552	*thiI*	9.5	~650	653
GJ3553	*thiI*	9.5	~350	358

[a] Calculated after sequencing the junction sequences of the iPCR amplicon and correcting to the immediate next *Hha*I site.

[b] Sequencing revealed *Hha*I site 6 bp after the junction between the Mu *c* end and *E. coli* chromosome. Subsequent transductional mapping showed that this mutant harbored the insertion in *glnE*. ND, not determined.

3.2.9 Genetic confirmation of sequencing (physical) data:

To rule out the possibility that the sequences obtained are results of spurious misprimed reaction products, a genetic method of confirming the physical data was taken up here by employing the collection of *E. coli* strains (CAG collection) which have Tn*10* insertions encoding Tet^R at various known locations in the *E. coli* chromosome; in some cases, other strains from this laboratory's collection with defined positions of Tet^R or Cm^R

Table 3.3 Physical (molecular) characterization of NaCl-sensitive mutants following sequencing of inverse PCR products.

Mutant strain	Gene disrupted	Position on the *E. coli* chromosome (min)	Insertion site (nucleotide number)	Flanking sequence	Orientation[a] A	B
GJ3533	*thiI*	9.5	440855	CCGGTAAGGATTTTT	Lac^-	CW
GJ3534	*proV*	60.4	2803133	TCCGTGAGGTGCGC	Lac^+	CW
GJ3535	*thiI*	9.5	only genetic data available		Lac^-	CW
GJ3536	*argP* (*iciA*)	65.9	3057871	GCCGTCTCACAGCGC	Lac^+	CW
GJ3537	*lysC*	91.2	4229973	TTGCTGGAGCCGACA	Lac^-	CCW
GJ3539	*thiI*	9.5	440826	CACAGATTGGCTTTTG	Lac^-	CW
GJ3540	*argP* (*iciA*)	65.9	3057753	CCAGGAGCAGACAAC	Lac^+	CW
GJ3541	*apaG*	1.1	51248	CGGCGAGTCGGAATA	Lac^-	CCW
GJ3542	*speC*	66.9	3105073	ATCCGCATCGGTTTC	Lac^-	CCW
GJ3543	*thiI*	9.5	441685	TCGCCGGTGACCAGC	Lac^-	CW
GJ3544	*proU*	60.4	only genetic data available		Lac^+	CW
GJ3545	*glnE*	68.9	only genetic data available		Lac^-	CCW
GJ3546	*thiI*	9.5	441685	TCGCCGGTGACCAGC	Lac^-	CW
GJ3547	*glnE*	68.9	3194673	GCGGCGGACGCGAAC	Lac^+	CCW
GJ3549	*glnE*	68.9	3194693	ACCAGATATTGGGTAA	Lac^-	CCW
GJ3550	*thiI*	9.5	441476	CCGCGTGCGTTTTGTC	Lac^+	CW
GJ3552	*thiI*	9.5	441822	CCGACGGTGAAAGCA	Lac^+	CW
GJ3553	*thiI*	9.5	440857	TCCCGGTAAGGATTTTT	Lac^-	CW

[a] A = Orientation of the *lacZ* gene of λ*plac*Mu55 in each of the mutants w.r.t. transcription of the disrupted gene; B: orientation of the gene w.r.t. the chromosome, CW = clockwise, CCW = counter clockwise. (ND, not determined).

insertions were used. Transductional mapping experiments were performed with these strains as donors. Making use of phage P1 lysates grown on the donor strains, transductions were performed selecting for the marker present in the donor strain (Tet^R or Cm^R) and screening for Kan^S colonies (that is, for loss of the Mu *lac*-Kan insertion marker in the recipient strain by linkage). All the 19 mutants were used as recipients in transductional mapping experiments employing a cohort of donor strains that were chosen based on the sequence data obtained from the inverse PCR experiments.

The results, given in Table 3.4, show that the genetic linkage values were in agreement with the sequencing (physical) data since in each of these mutants when used as the recipient, the incoming Tet^R or Cm^R crossed out the Kan^R transposon marker at an expected frequency.

Once the sequencing results were confirmed by genetic methods, the various alleles of the disrupted genes were designated as follows:

(i) *proV610*::Kan (GJ3534) and *proU611*::Kan (GJ3544).

(ii) *apaG11*::Kan (GJ3541).

(iii) *argP202*::Kan (GJ3536) and *argP203*::Kan (GJ3540).

(iv) *glnE463*::Kan (GJ3545), *glnE464*::Kan (GJ3547) and *glnE465*::Kan (GJ3549).

(v) *lysC82*::Kan (GJ3537).

(vi) *speC100*::Kan (GJ3542).

(vii) *thiI51*::Kan (GJ3533), *thiI52*::Kan (GJ3535), *thiI53*::Kan (GJ3539), *thiI54*::Kan (GJ3543 and GJ3546), *thiI55*::Kan (GJ3550), *thiI56*::Kan (GJ3552) and *thiI57*::Kan (GJ3553).

3.2.10 Effect of the *fnr* status on NaCl-sensitivity and *lacZ* expression of various null insertion mutations:

Since the NaCl-sensitive mutants were obtained in a Δ*gltBD* Δ*fnr* background carrying mutant (monomeric) *fnr* on a plasmid (GJ2530 / pHYD909), it was decided to test the effect of fnr^+ on the NaCl-sensitivity and *lacZ* expression phenotypes of these mutants. The Kan^R insertion representing each of the mutant loci was transduced into GJ2529 (fnr^+ Δ*gltBD zha-6*::Tn*10*) by P1 transduction and the resultant colonies were designated as follows:

GJ2560: Δ*gltBD zha-6*::Tn*10 proV610*::Kan

GJ2561: Δ*gltBD zha-6*::Tn*10 thiI51*::Kan

Table 3.4 Genetic confirmation of physical (sequencing) data obtained for various NaCl-sensitive mutants.

S. No.	Strain	Gene	Min	Donor strain	Donor Tet^R / Cm^R marker (map position in min)	No. of Kan^S / Total colonies screened	Observed linkage (%)	Expected linkage (%)
1.	GJ4533	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	21 / 51	41	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	16 / 50	32	51
2.	GJ4534	*proV*^{a}	60.4	GJ46	*zga-900*::Tn*10* (~60)	4 / 6	67	ND
3.	GJ4535	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	23 / 50	46	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	20 / 52	38	51
4.	GJ4536	*argP* (*iciA*)	65.9	CSH117	*mutY*::Tn*10* (66.8)	1 / 16	6	17
				RI179	Δ*dsbC*::Cm (65.4)	9 / 27	33	42
				JM2071	*galP*::Tn*10* (66.5)	9 / 47	19	34
5.	GJ4537	*lysC*	91.2	CSH123	*thiA*::Tet (90.3)	2 / 38	5	17
				QC1556	Δ*soxRS*::Cm (92.2)	4 / 44	9	13
6.	GJ4539	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	26 / 50	52	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	11 / 51	22	51

Table 3.4 (continued).

S. No.	Strain	Gene	Min	Donor strain	Donor Tet^R / Cm^R marker (map position in min)	No. of Kan^S / Total colonies screened	Observed linkage (%)	Expected linkage (%)
7.	GJ4540	*argP* (*iciA*)	65.9	CSH117	*mutY*::Tn*10* (66.8)	4 / 42	10	17
				RI179	Δ*dsbC*::Cm (65.4)	6 / 49	12	42
				JM2071	*galP*::Tn*10* (66.5)	8 / 49	16	34
8.	GJ4541	*apaG*	1.1	CAG12095	*zab-3051*::Tn*10* (1.8)	16 / 73	22	28
				MG1655	*araD*^{b} (1.4)	10 Ara^S / 50 Kan^R	20	61
9.	GJ4542	*speC*	66.9	CAG18472	*nupG511*::Tn*10* (66.9)	50 / 50	100	100
10.	GJ4543	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	29 / 51	57	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	20 / 49	41	51
11.	GJ4544	*proV*^{a}	60.4	GJ46	*zfi-900*::Tn*10* (~60)	6 / 6	100	ND
12.	GJ4545	*glnE*	68.9	CAG18475	*metC162*::Tn*10* (67.9)	7 / 46	15	13
13.	GJ4546	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	13 / 22	59	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	22 / 52	42	51

Table 3.4 (continued).

S. No.	Strain	Gene	Min	Donor strain	Donor Tet^R / Cm^R marker (map position in min)	No. of Kan^S / Total colonies screened	Observed linkage (%)	Expected linkage (%)
14.	GJ4547	*glnE*	68.9	CAG18475	*metC162*::Tn*10* (67.9)	8 / 75	11	13
15.	GJ4549	*glnE*	68.9	CAG18475	*metC162*::Tn*10* (67.9)	8 / 50	16	13
16.	GJ4550	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	25 / 50	50	73
17.	GJ4552	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	22 / 54	41	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	24 / 51	47	51
18.	GJ4553	*thiI*	9.5	CAG12148	*tsx-247*::Tn*10* (9.3)	11 / 35	31	73
				CAG12017	*zba-3054*::Tn*10* (9.9)	15 / 48	31	51
				GJ1921	*lon-104*::IS*186*::Tn*10*dCm (9.9)	20 / 80	25	51

[a] Since the *lac*Kan fusion was shown to be NaCl-inducible and sequencing of the iPCR product results showed the insertion to be in *proV*, only a few transductants were checked for Tet^R Kan^S (white colonies) or Tet^R Kan^R (red colonies) on MacConkey + 0.2 M NaCl plates.

[b] The Kan^R insertion was moved into MG1655 (*araD*$^+$) and scored for Kan^R to *araD*$^-$ ratio on MA + CAA + 0.2% L-arabinose (MC4100 is *araD*$^-$ and thus is L-arabinose-sensitive and Ara^-). ND = not determined

GJ2562: Δ*gltBD zha-6*::Tn*10 thiI55*::Kan

GJ2563: Δ*gltBD zha-6*::Tn*10 thiI56*::Kan

GJ2564: Δ*gltBD zha-6*::Tn*10 argP202*::Kan

GJ2565: Δ*gltBD zha-6*::Tn*10 argP203*::Kan

GJ2566: Δ*gltBD zha-6*::Tn*10 glnE463*::Kan

GJ2567: Δ*gltBD zha-6*::Tn*10 glnE464*::Kan

GJ2568: Δ*gltBD zha-6*::Tn*10 apaG11*::Kan

GJ2569: Δ*gltBD zha-6*::Tn*10 speC100*::Kan

GJ2570: Δ*gltBD zha-6*::Tn*10 lysC82*::Kan

When these colonies were tested for their ability to grow on high NaCl-containing medium, they behaved similar to the ones carrying monomeric FNR (GJ2530 / pHYD909 background), suggesting that the N-terminal deletion in FNR had no effect on the NaCl-sensitivity phenotype conferred by these mutations. Subsequently, identical results were also obtained when the Kan^R insertions were introduced into the markerless Δ*gltBD* strain, GJ4652 (which is also fnr^+).

The mutant *fnr* gene did not also affect *lacZ* expression in the above *lac*-Kan fusion strains (*apaG11, argP202, glnE464, proV610, speC100, thiI55* and *thiI56*),both in the presence and absence of NaCl.

3.2.11 Classification of NaCl-sensitive mutants:

In order to study the molecular mechanisms of NaCl-sensitivity in the various mutants, they were tested for their tolerance or sensitivity to the other categories of dissolved solutes (that is, the impermeable nonionic solutes and the freely permeable solutes). This section describes the experiments performed to classify the NaCl-sensitive mutants into different categories based on their differential sensitivities to other dissolved solutes in the medium. Also in this chapter, results are presented about the requirement of the *gltBD* locus for exhibiting the NaCl-sensitivity phenotype.

3.2.11.1 Effect of other dissolved solutes on growth of various NaCl-sensitive mutants:

NaCl is the most commonly used solute to impose experimental water stress, and it is an ionic, impermeable solute. In an attempt to classify the NaCl-sensitive mutants, the following additional solutes were tested for their effects on growth for which the

various mutants characterized above were streaked on the surface of MA plates containing sufficiently high concentrations of the solutes:

(a) Ionic impermeable: KCl, K_2SO_4, $(NH_4)_2SO_4$, NH_4Cl;

(b) Nonionic impermeable: sucrose, glucose;

(c) Nonionic freely permeable: glycerol and ethylene glycol.

A. Effect of other ionic impermeable solutes on NaCl-sensitive mutants:

In comparison with the WT strain, all the NaCl-sensitive mutants were also sensitive to other dissolved ionic impermeable solutes, like KCl, K_2SO_4, NH_4Cl and $(NH_4)_2SO_4$ at the concentrations indicated below streaked on MA plates supplemented with 1 mM Bet.

(i) KCl: 0.7 – 0.8 M

(ii) K_2SO_4: 0.6 – 0.7 M

(iii) $(NH_4)_2SO_4$: 0.6 – 0.7 M

(iv) NH_4Cl: 0.7 – 0.8 M

The fact that all the $NaCl^S$ mutants were also sensitive to other ionic impermeable solutes indicates that these mutants are not ionic-stress-sensitive mutants. If the mutants were ionic-stress-sensitive, then the expectation was that they would be sensitive to a particular chemical ion species viz. Na^+-sensitive, or K^+-sensitive or Cl^--sensitive, etc.

B. Effect of nonionic impermeable solutes:

The NaCl-sensitive mutants were tested for their ability to grow on MA Bet plates supplemented with either sucrose (0.7 – 0.9 M) or glucose (0.9 – 1.1 M). All the mutants were sensitive to sucrose and glucose in the medium. These results indicate that the NaCl-sensitive mutants are sensitive to the dissolved ionic or nonionic "impermeable" solute in the medium (Table 3.5).

C. Effect of nonionic freely permeable solutes:

In order to distinguish between osmotic effects from non-osmotic ones, these NaCl-sensitive mutants were next tested for their tolerance or sensitivity to freely permeable nonionic dissolved solutes, like glycerol and ethylene glycol. Glycerol is a nonionic solute that is freely permeable across the plasma membrane. Results from earlier work in the lab (Gowrishankar, 1985) and elsewhere have suggested that glycerol does

Table 3.5 Classification of NaCl-sensitive mutants[a]

Strain no.	Mutation	MA NaCl Bet	MA Sucrose Bet	MA Glucose Bet	MA Glycerol	MA EG	*gltBD* requirement[b]
MC4100	WT	+++	+++	+++	+++	+++	
GJ2529	*gltBD*	++±	++±	++±	++±	++±	
GJ4537	*lysC*	+	+	+	+++	+++	N
GJ2570	*lysC gltBD*	–	–	–	+++	+++	
GJ4545	*glnE*	+++	+++	+++	+++	+++	Y
GJ2566	*glnE gltBD*	–	–	–	+++	+++	
GJ4534	*proU*	+++	+++	+++	+++	+++	Y
GJ2560	*proU gltBD*	++	++	++	+++	+++	
GJ4541	*apaG*	++	++	++	+++	+++	N
GJ2568	*apaG gltBD*	±	±	±	++±	++±	
GJ4536	*argP*	+++	+++	+++	+++	+++	Y
GJ2564	*argP gltBD*	–	–	–	++±	++±	
GJ4542	*speC*	+	+	+	++	++±	N
GJ2569	*speC gltBD*	±	±	±	+	++	
GJ4533	*thiI*	–	–	–	+++	+++	N
GJ2561	*thiI gltBD*	–	–	–	++±	++±	

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl, 0.9-1 M glucose or 0.8-0.9 M sucrose, or MA supplemented with 1.8-2.0 M glycerol or 1.8-2.0 M ethylene glycol, and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 6-point scale (in increasing order): — (no growth), ±, +, ++, ++± and +++ (full growth).

[b] Y means that the mutation confers osmosensitivity in the presence of the *gltBD* mutation, while N means that the mutation confers osmosensitivity even in the absence of the *gltBD* mutation.

not exert any osmotic effect on the cells. Freely permeable nonionic solutes are not expected to impose either osmotic stress or ionic stress.

Previous studies from the lab had also suggested the existence of a novel component of biological water-stress, designated as anhydrotic stress, which is elicited by both impermeable and permeable dissolved solutes. A *speC* strain which was sensitive to all the dissolved solutes in the medium, including glycerol, was called an anhydrosensitive strain (UmaPrasad and Gowrishankar, 1999). Since the *speC* mutant had been classified as being anhydrosensitive based on its sensitivity to only one freely permeable solute, one criticism could be that it is a glycerol (solute)-specific effect, and that it does not represent a general mechanism of growth inhibition.

To address this problem, other freely permeable non-ionic solutes were sought which could be used to impose water-stress. The following criteria were used to select a solute for water-stress experiments: (a) very high concentrations of the solute should be achieved in the medium; (b) the solute should be non-toxic at the concentration used; and (c) the solute should not induce transcription of the classically known osmoresponsive *proU*::*lacZ* fusion. After screening various solutes, ethylene glycol (EG) was selected for further work since it supported the growth of MC4100 up to 2.1 M and it did not induce the *proU*::*lacZ* fusion (therefore, freely permeable). Thus, in the present study, apart from glycerol, EG was used to check the effect of freely permeable nonionic solutes on growth of the NaCl-sensitive mutants. Accordingly, all the mutants were tested on MA plates containing either glycerol (1.5 – 1.7 M) or EG (1.8 – 2 M).

The results showed that *E. coli* strains disrupted in *apaG, lysC, thiI, glnE gltBD, proU gltBD* or *argP gltBD* were tolerant to glycerol and EG in the medium. The *speC* strain alone was sensitive to glycerol (Table 3.5), but even this mutant was not as sensitive to EG as it was to glycerol (in isosmolar concentration).

If the two solutes were inhibiting the *speC* strain through their effect on water-activity of the medium, the expectation was that there should be no difference in the sensitivity pattern of the mutant in these two media. The differential sensitivity of *speC* to glycerol over EG was suggestive of a chemical-specific (inhibitory) effect of the former, rather than a more general water-stress effect. Thus, the earlier classification of *speC* mutant being anhydrosensitive has to be re-evaluated in the light of the present findings, and the evidence in support of a third component of water stress (anhydrotic stress) is itself therefore, at best equivocal.

In the majority of cases, the *gltBD* mutation accentuated the NaCl-sensitivity phenotype. In *argP, proU* and *glnE* strains, mutation in *gltBD* was required to confer sensitivity to NaCl or sucrose, but it did not affect the tolerance of the respective strains to glycerol or EG i.e., *gltBD* derivatives of these mutants were not glycerol-sensitive or EG-sensitive. With *speC* however, the *gltBD* mutation accentuated sensitivity not only to NaCl, glucose and sucrose, but also to glycerol and EG. The basis for this behavior of the *speC* mutant is at present not clear.

3.2.11.2 Requirement of *gltBD* for water-stress sensitivity phenotype:

As mentioned in Section 3.2.1 above, the present screen for genes affecting NaCl-sensitivity was accomplished in a *gltBD* mutant background because the mutation sensitizes the strain to any minor perturbations which might affect the tolerance of *E. coli* to water-stress. This would enable one to pick up even those genes that have a 'weak' $NaCl^S$ phenotype (in addition to the ones which exhibit a 'strong' $NaCl^S$ phenotype). This screen was also expected to result in the identification of genes which interact with *gltBD* in conferring NaCl-sensitivity. To distinguish the genes whose disruption by itself would result in NaCl-sensitivity from those which require *gltBD* to show the NaCl-sensitivity phenotype, the Kan^R null insertions of the various genes were transduced into the WT strain, MC4100 by using the P1 lysates prepared on GJ3533 through GJ3553 and selecting for Kan^R. The Kan^R transductants thus obtained were designated as GJ4533 through GJ4553.

The MC4100 derivatives were then examined for their tolerance / sensitivity to various categories of dissolved solutes. For genes where more than one insertion had been obtained, one or two representative strain(s) were used for the classification. Based on the results obtained (Table 3.5), the mutants were classified into two categories:

Class I: These mutants grew on par with MC4100 on MA medium supplemented with various dissolved solutes like NaCl, sucrose and glucose, e.g., GJ4534 and GJ4544 (*proV*::Kan), GJ4536 and GJ4540 (*argP*::Kan), and GJ4545 and GJ4547 (*glnE*::Kan). This result argues that a mutation in any of these genes was by itself not conferring the growth sensitivity but that it acts synergistically or additively with *gltBD* in conferring the phenotype. It was earlier reported that mutations in *proU* by themselves do not contribute to osmosensitivity but that they do so when combined with mutation in *proP*, which

encodes the second proline and glycine betaine uptake system. In the present study also, *proU* conferred partial osmosensitivity only in combination with Δ*gltBD* mutation.

Class II: The second class of Kan^R mutants was sensitive to growth inhibition imposed by NaCl present in the medium even in a *gltBD*$^+$ background, e.g., GJ4553 (*thiI*), GJ4537 (*lysC*), GJ4541 (*apaG*), and GJ4542 (*speC*), indicating that mutations in these genes confer growth sensitivity irrespective of the *gltBD* locus. When the corresponding Δ*gltBD* derivatives of these mutants were tested, the growth sensitivity phenotype was accentuated.

3.3 Discussion:

Eighteen NaCl-sensitive mutants were isolated following λp*lac*Mu55-mediated random transpositions and Amp-enrichment in a Δ*gltBD* strain. It was established that the NaCl-sensitivity phenotype was linked 100% to the *lac*-Kan insertion. Inverse PCR strategy was standardized and successfully employed to molecularly characterize 16 of the NaCl-sensitive mutants (Section 3.2.8.2). The junction sequences were used to determine the gene disrupted in each case (Table 3.2). The physical data were confirmed by genetic methods (transductional mapping approach; Section 3.2.9). The present screen identified new loci which were hitherto not known to be involved in NaCl-sensitivity viz., *apaG, argP, glnE, lysC,* and *thiI*. The screen also yielded mutants harboring null insertions in *proU* and *speC*, genes that have previously been implicated in the NaCl-sensitivity phenotype. Isolation of *proU* and *speC* mutations conferring NaCl-sensitivity in the present screen lends credence to the strategy employed.

The present study identified genes which confer NaCl-sensitivity only in the *gltBD*$^-$ background (*proU, argP* and *glnE*) and others which conferred the phenotype in both *gltBD*$^-$ and *gltBD*$^+$ backgrounds (*lysC, apaG, speC* and *thiI*; Section 3.2.11). These results suggest an important role for *gltBD* in water-stress adaptational pathway.

All the NaCl-sensitive mutants were classified based on their differential sensitivity to other dissolved solutes. Any dissolved solute at a sufficiently high concentration in a medium is expected to reduce the water activity of that medium [a_w is effectively the concentration (or mole fraction) of water in a solution]. It is widely believed that there are two components of water-stress viz., ionic stress and osmotic stress. Ionic (salinity) stress is the water-stress imposed by dissolved impermeable ionic

solutes whereas osmotic (turgor) stress is imposed by dissolved ionic or non-ionic solutes which are impermeable across the plasma membrane. According to the widely accepted classification, freely permeable non-ionic solutes are not expected to impose either ionic stress or osmotic stress.

The absence of any ionic stress-sensitive mutant in this collection of NaCl-sensitive mutants was not surprising since the ionic-stress component of water-stress is not very prominent in *E. coli* when compared to that of osmotic stress (Section 3.2.11). There are only a few phenomena in *E. coli* that are elicited by NaCl but not by other nonionic solutes viz., transcriptional induction of *nhaA* (Karpel *et al.*, 1991) and *kdp* (Gowrishankar, 1985) operons. It was reported that *nhaA* and / or *nhaB* mutants are Na^+-sensitive at high (basic) pH while *kdp* mutants are sensitive to low [K^+].

In contrast, the majority of phenomena elicited by ionic solutes are also elicited to the same extent by equiosmolar concentrations of impermeable nonionic solutes, like sucrose or glucose. According to the classical definition, any phenomenon elicited to equal extent by both ionic and nonionic dissolved solutes which are impermeable across the biological membrane is considered as belonging to the osmotic stress category. But, implied in the definition is the fact that these phenomena are not elicited when nonionic freely permeable dissolved solutes are used (to distinguish between osmotic effects from non-osmotic ones).

Null insertions in six different loci (*apaG, argP, glnE, lysC, speC* and *thiI*) conferred sensitivity to both ionic impermeable solutes [NaCl, KCl, K_2SO_4, $(NH_4)_2SO_4$ and NH_4Cl) and nonionic impermeable solutes (sucrose and glucose) but not to nonionic freely permeable solutes (glycerol and EG; Section 3.2.11). Thus, these results are consistent with the hypothesis that the genes may be involved in osmoadaptational pathways in *E. coli*. The *speC* mutant was additionally glycerol-sensitive; nevertheless, the earlier classification of *speC* as an anhydrosensitive mutant did not find support with the new findings reported in this study.

Chapter 4

Null insertions conferring osmosensitivity : *proU, apaG, glnE, lysC, thiI, and speC*

4.1 Introduction:

The isolation and molecular characterization of NaCl-sensitive mutants was described in Chapter 3. Mutations in *apaG, lysC, speC* and *thiI* were shown to confer osmosensitivity in a *gltBD*-independent fashion, while mutations in *proU, argP* and *glnE* conferred osmosensitivity in the presence of the *gltBD* mutation. The present chapter describes the characterization of the roles of the various genes (other than *argP*, which is described in Chapter 5) in water-stress adaptation. The chapter is divided into various sections, each section dealing with one locus. Each section has its own introduction describing the literature in brief, followed by detailed phenotypic characterization of the mutant(s) and finally, a discussion of how the gene may be involved in conferring water-stress sensitivity in *E. coli*.

4.2 Characterization of *proU* mutants:

4.2.1 Introduction:

A detailed introduction about the *proU* locus has already been presented in Section 1.1.6.3 of Chapter 1. In brief, the proline and glycine betaine (Bet) transport system encoded by *proU* locus consists of three structural genes, viz., *proV, proW* and *proX*, whose products encode a periplasmic-binding protein-dependent ABC-transporter system (Dattananda and Gowrishankar, 1989; Stirling *et al.*, 1989). These three proteins constitute an efficient translocation system for the substrates across the cytoplasmic membrane (Higgins, 1992; Ames *et al.*, 1990).

Strains harboring mutations in *proU* were isolated during the screen for NaCl-sensitive mutants in *E. coli* (as described in Chapter 3). Two mutants, GJ3534 and GJ3544 were shown to have *lac*-Kan^R insertions in the *proU* locus (see Sections 3.2.8 and 3.2.9 in Chapter 3). GJ3534 (*proV610*) had the Kan^R insertion in the 1.2 kb long *proV* gene 298 nucleotides after the ATG translation start site, in the orientation such that the *lac* genes are transcribed from the *proU* promoters. Since *proV* is the first gene of the *proVWX* (*proU*) operon, the Kan^R insertion was expected to show a polar effect on the downstream *proW* and *proX* genes as well. The insertion in GJ3544 (*proU611*) has not been molecularly characterized, but has been genetically shown to lie in the *proU* locus.

4.2.2 Results

***proU* mutations confer osmosensitivity in *proP* background:**

As described in Section 3.2.11.2 of Chapter 3, the *proU* single mutants (GJ4534 or GJ4544) were no different from MC4100 (WT) for growth on media of high osmolarity, but when combined with the *gltBD* mutation, they were inhibited for growth on such media i.e., the *proU gltBD* mutant was osmosensitive when compared to *gltBD* or MC4100 strains (Table 3.5). It was earlier shown that even when the *proU* locus is disrupted, the second Bet and Pro uptake system viz., *proP* is capable of accumulating enough 'compatible' solutes for osmoadaptation (Gowrishankar, 1986). When both *proP* and *proU* loci are disrupted, the strain becomes osmosensitive.

To test whether the *proV*::Kan insertion isolated in the present screen confers osmosensitivity when combined with the *proP* mutation, a *proV*::Kan *proP* double mutant was constructed in the following fashion. GJ134 (*proP222*) and GJ157 (*proP221 proU*::*lac*) strains, which are uracil auxotrophs due to the Δ(*pyr-76*::Tn*10*) deletion, were made uracil prototrophs with a P1 lysate prepared on MG1655 (WT). Uracil prototrophs of GJ134 and GJ157 were designated GJ4835 and GJ4836, respectively. GJ4836 (*proP221 proU224*::*lacZ*) was used as the osmosensitive control strain.

proP222 (GJ4835) and *proP221 proU*::*lacZ* (GJ4836) strains were subsequently used as recipients to transduce the *proV610*::Kan mutation, using the P1 lysate prepared on GJ3534. The resultant *proV610*::Kan transductants were christened GJ4837 and GJ4838, respectively. *proP222 proV610*::Kan (GJ4837) and *proP221 proV610*::Kan (GJ4838) strains were sensitive to dissolved impermeable solutes like NaCl, KCl, K_2SO_4, sucrose and glucose, clearly demonstrating that the Kan^R insertion in *proV* isolated in the present screen conferred osmosensitivity in a *proP* background.

4.2.3 Discussion:

The results obtained with *proU* mutants in the present study were consistent with the earlier reports i.e., that the *proU* locus is involved in the osmoadaptation of *E. coli*. One can explain the isolation of *proU gltBD* mutant in a screen for NaCl-sensitive mutants if one assumes that *gltBD* and *proU* act independently and additively in conferring NaCl-sensitivity. It is worth mentioning here that obtaining two mutants during the whole genome mutagenesis screen disrupted in a locus (*proU*), which was

previously implicated in osmoadaptation serves to validate the rationale of the experimental approach adopted for the screen.

4.3 Characterization of *apaG* mutant:

4.3.1 Introduction

apaG is the second gene of a three-gene operon (*ksgA, apaG* and *apaH*). The first gene, *ksgA* codes for a methyl transferase of 16S rRNA, mutations in which result in resistance to the antibiotic, kasugamycin. *apaG* is predicted to code for a protein of 13.8 kDa of unknown function while *apaH* codes for the Ap_4A hydrolase involved in the degradation of the dinucleotide AppppA (5',5'''-P^1,P^4-diadenosine tetraphosphate) and other related dinucleotide polyphosphate compounds. ApaH cleaves Ap_4A symmetrically to yield two molecules of ADP, and is strongly stimulated by Co^{2+} and Mn^{2+} (Guranowski *et al.*, 1983; Plateau *et al.*, 1985). In the *ksgA apaGH* operon, two polycistronic promoters controlling the expression of *apaH* (viz., P_K and P_{GH}) are located upstream of *ksgA* and *apaG* respectively (Fig 4.1). The promoter P_{GH} present in *ksgA* gene upstream of *apaG* can account for the expression of the two distal genes, *apaG* and *apaH*. Null insertions in *ksgA* and *apaG* were earlier demonstrated to be polar over *apaH* (Leveque *et al.*, 1990).

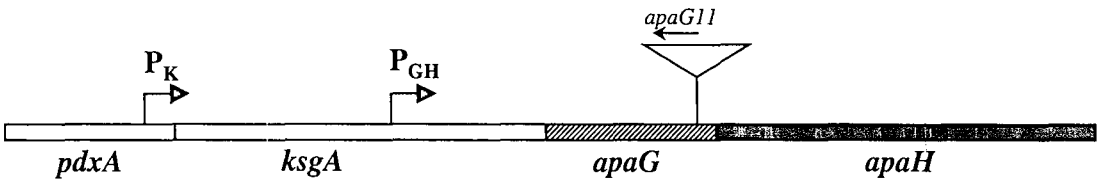


Fig 4.1 Operonic arrangement of the *ksgA, apaGH* genes. The positions of the internal promoters P_K and P_{GH} are indicated. The inverted triangle represents the insertion site in the *apaG* gene in GJ3541 (*apaG11*::Kan). The arrow represents the orientation of transcription of the *lacZ* gene in λp*lac*Mu55.

Zamecnik and co-workers discovered the *in vitro* enzymatic synthesis of Ap_4A by serendipity while studying the aminoacyl tRNA synthetase reactions (Randerath *et al.*, 1966; Zamecnik *et al.*, 1966). Ap_4N and Ap_3N (where N = A, G, C or U) are synthesized in Zn^{2+}-stimulated side reactions catalyzed by certain tRNA synthetases from prokaryotes and eukaryotes (Blanquet *et al.*, 1983; Plateau and Blanquet, 1982).

These adenylated nucleotides are synthesized *in vitro* and presumably *in vivo*, by a side reaction of the amino acyl-tRNA synthetases that can adenylate (from the amino acid adenylate) the nucleotides ATP, ADP, GTP, GDP and ppGpp to form AppppA, ApppA,

AppppG, ApppG and ApppGpp, respectively (Fig. 4.2). Their levels inside the cell are modulated by the action of Ap_4N hydrolase enzyme which cleaves these molecules and recycles the nucleotides.

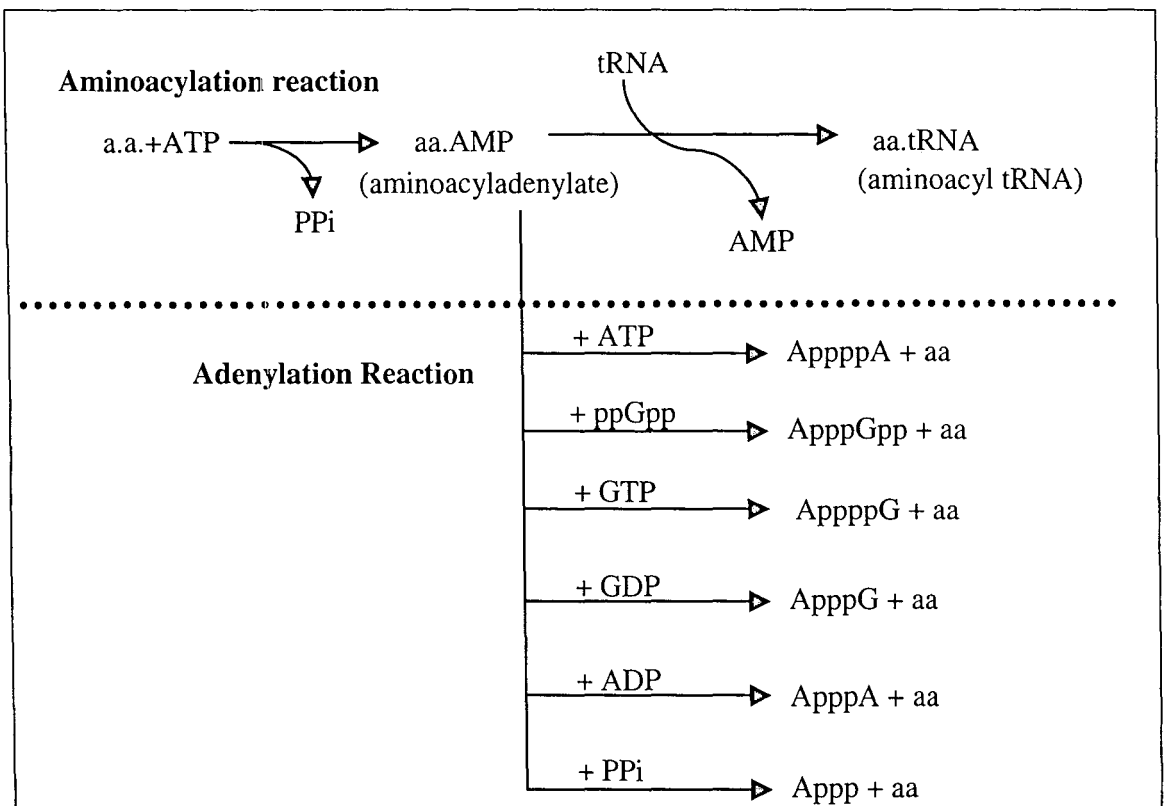


Fig. 4.2 Synthesis of AppppA and other adenylated nucleotides by aminoacyl tRNA synthetase. (Adapted from Bochner *et al.*, 1984)

Postulated functions of dinucleotide polyphosphates:

The precise role of Ap_4A and related molecules in bacterial physiology remains uncertain although several functions have been postulated by different groups.

1. Induction of DNA replication:

It was proposed that Ap_4A is involved in triggering DNA replication, based on an initial finding in permeabilized quiescent mammalian cells that it induced thymidine incorporation and replication bubbles (Grummt, 1978), which was supported by reports that the DNA polymerase alpha complex contains an Ap_4A binding subunit (Grummt *et al.*, 1979; Rapaport *et al.*, 1981) and that Ap_4A could serve as a primer for DNA synthesis (Zamecnik *et al.*, 1982). However, the levels of Ap_4A and related dinucleotides do not change during *E. coli* cell cycle (Plateau *et al.*, 1987a), and although overexpression of

the Ap_4A hydrolase lowered Ap_4A levels by about 10-fold, there was little or no effect on growth rate (Mechulam *et al.*, 1985).

In a related context, it was hypothesized that Ap_4A or related molecules may be involved in DNA repair in eukaryotes (Yoshihara and Tanaka, 1981).

2. Signaling in stress responses:

Based on the work from several labs, including that of Bruce Ames, it was proposed that Ap_4N molecules act as signals of cellular stress ('alarmones') that participate in triggering cellular adaptational responses. *S. enterica* and *E. coli* cells subjected to heat shock or various oxidative stresses were shown to have increased (by upto several 100-fold) levels of Ap_4A and similar nucleotides (Lee *et al.*, 1983a,b; Bochner *et al.*, 1984; Coste *et al.*, 1987). However, detailed observations of the kinetics of dinucleotide production and heat shock or oxidative stress protein production proved that dinucleotides are neither necessary nor sufficient for triggering the cellular responses (VanBogelen *et al.*, 1987; Farr and Kogoma, 1991; Kitzler *et al.*, 1992). Very high levels of dinucleotides caused by disruption of the *E. coli apaH* gene did not induce the stress protein responses (Leveque *et al.*, 1990; Farr *et al.*, 1989), and overproduction of the enzyme had no effect on the heat shock or H_2O_2 stress responses (Plateau *et al.*, 1987b).

By photocrosslinking experiments, Ap_4A has been shown to bind the various proteins specifically; for e.g., DnaK, GroEL and ClpB, which were implicated to have an important function in heat shock and oxidative stress response (Bochner *et al.*, 1986; Johnstone and Farr, 1991; Fuge and Farr, 1993). It was suggested that by binding to these proteins, Ap_4N molecules modulate their function.

3. Control of time of cell division in *E. coli*:

Nishimura and colleagues isolated *E. coli* mutants (designated *cfcA* and *cfcB*) which could accomplish cell division despite inhibition of DNA replication by a temperature-sensitive mutation in *dnaB* (Nishimura *et al.*, 1997; Nishimura, 1998). *cfcA* turned out to be the *glyS* α gene, which codes for a subunit of glycyl tRNA synthetase, and *cfcB* proved to be an IS2 insertion in the *apaH* gene. Both mutants were shown to have elevated Ap4A levels. Subsequently, Nishimura also found that overexpression of *apaH* results in the production of larger cells, implying that normal levels of Ap_4A are required for the normal timing of cell division (Nishimura, 1998). It will require further studies to determine whether Ap_4A is involved in normal control of division timing, rather than merely interfering when present at high levels.

4.3.2 Results:

As described earlier, an insertion in *apaG* gene resulted in osmosensitivity phenotype (Chapter 3). GJ3541 (*apaG11*::Kan) has the insertion 358 nucleotides downstream of the predicted ATG start site in the 0.4 kb long *apaG* gene, and the orientation is such that *lac* is inverse to the direction of *apaGH* transcription (Fig. 4.1). When this mutation was combined with the *gltBD* mutation, the osmosensitivity phenotype was exacerbated, suggesting that they may act additively and independent of each other in conferring the NaCl-sensitive phenotype.

4.3.2.1 Osmosensitivity of *ksgA*, *apaG* and *apaH* mutants:

Since it was earlier shown that mutations in *ksgA* and *apaG* were polar over *apaH*, it was decided to check the osmosensitivity phenotype of null mutations in *ksgA* and *apaH* genes. For this purpose, *ksgA*::Kan, *apaG*::Kan and *apaH*::Kan mutations from strains PAL12103K, PAL12103G and PAL12103A2, respectively (kindly provided by

Table 4.1 Growth of *ksgA*, *apaG* and *apaH* mutants at high osmolarity[a]

Strain	Genotype	MA Bet		
		NaCl (0.6-0.7 M)	Glucose (0.9-1.0 M)	Sucrose (0.8-0.9 M)
MC4100	WT	+++	+++	+++
GJ2529	*gltBD*	++±	++±	++±
GJ4593	*apaG*::Kan	+	+	+
GJ4594	*apaH*::Kan	±	±	±
GJ4595	*ksgA*::Kan	++±	++±	++±
GJ4596	*gltBD apaG*::Kan	±	±	±
GJ4597	*gltBD apaH*::Kan	–	–	–
GJ4598	*gltBD ksgA*::Kan	++	++	++
GJ2568	*gltBDF apaG11*::Kan	+	+	+

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, glucose or sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 6-point scale (in increasing order): — (no growth), ±, +, ++, ++± and +++ (full growth).

Prof. Sylvain Blanquet; Leveque *et al.*, 1990) were transferred to MC4100 and GJ2529 (*gltBD*) through P1-mediated transduction. The resultant Kan^R colonies were tested for their ability to grow on high osmolarity media. The results are summarized in Table 4.1.

The three null insertions differed in their sensitivity to the dissolved solutes, *apaH*::Kan being the most sensitive followed by *apaG*::Kan, while *ksgA*::Kan was the least sensitive. The same order of differential sensitivity was also seen in the *gltBD* background. These differential sensitivities may correlate with the proposed residual activity of the *apaH* gene product in these strains, since the *ksgA* mutant has been shown to have residual Ap_4N hydrolase activity expressed from the second promoter and even in the *apaG* mutant, the Ap_4N hydrolase activity was more than the negligible activity seen in *apaH* mutant, presumably because of leaky expression of *apaH* gene (Leveque *et al.*, 1990).

4.3.2.2 Complementation studies:

A. Cloning *apaGH*$^+$ locus from *E. coli* library:

From the above results it was clear that a strain with the least ApaH enzymatic activity was the most sensitive to the imposed water stress and vice versa. It also demonstrated that *apaH* was necessary for osmotolerance phenotype but did not rule out the importance of *apaG* in osmoadaptation. The latter could be answered by cloning the corresponding genes individually and examining their effect on osmotolerance in the *apaG* and *apaH* null mutants. To check whether *apaH* sequences alone, without the full length *apaG*, can confer osmotolerance in an *apaG*::Kan mutant (in which both *apaG* and *apaH* expression is abolished), the following cloning strategy was adopted.

Kohara *et al.* (1987) have described the construction of an ordered library of the *E. coli* genome in λ phage, and phage λ-105 of this collection carries the *ksgA apaGH* locus. λ-105 DNA was digested with *Bam*HI-*Eco*RI and a 3.5 kb fragment which was expected to contain the above operon was cloned into *Bam*HI-*Eco*RI-digested pCL1921 (low-copy number $Spec^R$ plasmid, 5-6 copies per cell) to generate a recombinant plasmid, pHYD917 (Fig. 4.3).

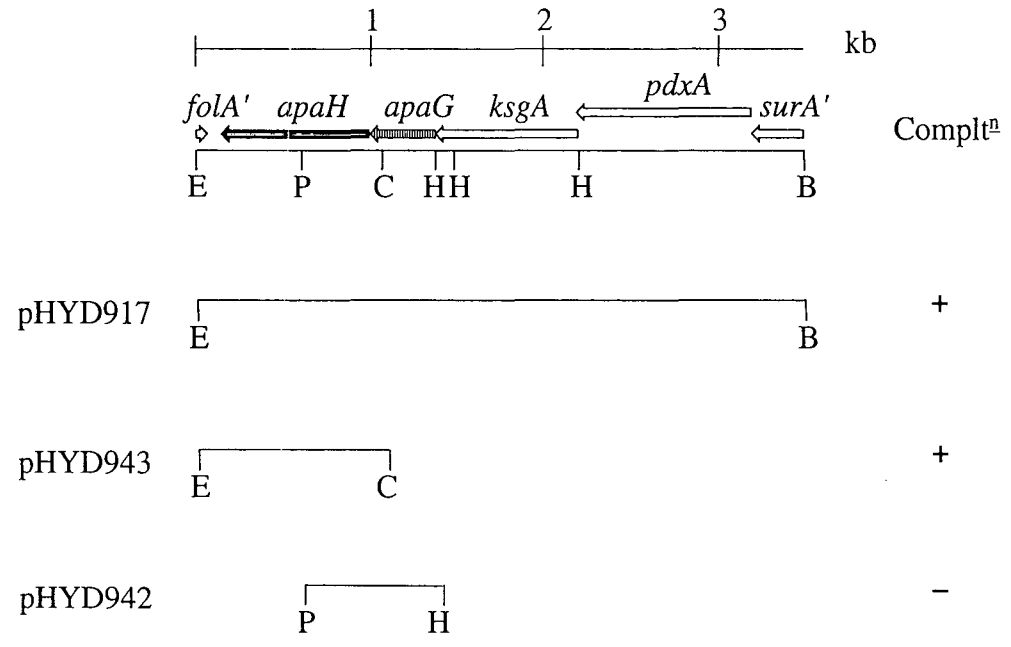


Fig. 4.3 Cloning of *apaGH*$^{+}$ and complementation analysis.
Below the kilobase scale is depicted the physical and gene map of 3.5 kb *Eco*RI-*Bam*HI fragment from Kohara phage λ-105 for the enzymes *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Hpa*I (H) and *Pst*I (P). The position of various genes and their transcriptional organization (arrows) are marked. Each line aligned beneath the physical map represents the extent of chromosomal DNA cloned into pCL1920, whose numerical pHYD designation is given alongside. Also indicated in the column marked Complt^{n} at the right is the ability of the plasmid to complement (+) or not to complement (–) the *apaG* and *apaH* mutants for osmosensitivity.

Plasmids pHYD917 and pCL1921 (as vector control) were introduced into *apaG* (GJ4541), *apaG gltBD* (GJ4661), *apaH*::Kan (GJ4594) and *gltBD apaH* (GJ4597) (along with *gltBD* and MC4100 as control strains) and the resulting Spec^{R} transformants were streaked on high osmolarity plates. The results showed that the plasmid pHYD917 was able to complement the mutant strains for osmosensitivity phenotype. This result (Table 4.2) demonstrated that sequences corresponding to *ksgA*, *apaG* and *apaH* were sufficient to complement the *apaG* and *apaH* mutants for osmosensitivity phenotype.

Table 4.2 Complementation of *apaG and apaH* mutations by *apaGH*$^+$ at high osmolarity[a]

Strain (genotype)	MA Bet Spec					
	NaCl (0.6-0.7M)		Glucose (0.9-1.0M)		Sucrose (0.8-0.9M)	
	pCL1921 (Vector)	pHYD917 (*apaGH*$^+$)	pCL1921 (Vector)	pHYD917 (*apaGH*$^+$)	pCL1921 (Vector)	pHYD917 (*apaGH*$^+$)
MC4100 (WT)	+++	+++	+++	+++	+++	+++
GJ2529 (*gltBD*)	++±	++±	++±	++±	++±	++±
GJ4541 (*apaG*)	+	+++	+	+++	+	+++
GJ4661 (*gltBD apaG*)	±	++±	±	++±	±	++±
GJ4594 (*apaH*)	±	+++	±	+++	±	+++
GJ4597 (*gltBD apaH*)	–	++±	–	++±	–	++±

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, glucose or sucrose, and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): — (no growth), ±, +, ++±, and +++ (full growth).

B. Subcloning of *apaG* and *apaH* and complementation tests:

In order to clone the *apaH* gene alone, the plasmid with 3.5 kb *Bam*HI-*Eco*RI *ksgA apaGH* insert was digested with *Cla*I and *Eco*RI. Since the *Cla*I site in the insert region is sensitive to restriction due to *dam*-methylase activity (which methylates GATC sequences), plasmid pHYD917 was introduced into a *dam*$^-$ strain and plasmid DNA isolated from that strain was subsequently used to digest and release the 1060 bp *Cla*I-*Eco*RI fragment expected to carry the *apaH* gene. The fragment was first cloned at the *Cla*I-*Eco*RI sites of pBS(KS) to obtain the plasmid pHYD941. pHYD941 was then cut with *Bam*HI and *Sal*I to release the 1060 bp *apaH* insert which was then cloned into the *Bam*HI-*Sal*I site of pCL1920 (low copy number Spec^R plasmid, 5 to 6 copies per cell) to generate the plasmid pHYD943.

To clone *apaG*, plasmid pHYD917 was cut with *Hpa*I-*Pst*I and the resulting 775 bp *apaG* insert was cloned into pBluescript [pBS(KS)] at the *Eco*RV-*Pst*I site to generate the plasmid pHYD940. pHYD940 was subsequently digested with *Bam*HI-*Sal*I to release the 775 bp *apaG* insert, which was cloned into pCL1920 at the *Bam*HI-*Sal*I site to obtain plasmid pHYD942.

Table 4.3 Complementation of *apaG and apaH* mutations by *apaH*$^+$ at high osmolarity[a]

Strain (genotype)	Plasmid (genotype)	MA Bet Spec		
		NaCl (0.6-0.7 M)	Glucose (0.9-1.0 M)	Sucrose (0.8-0.9 M)
GJ4596 (*gltBD apaG*)	pCL1920 (vector)	±	±	±
	pHYD942 (*apaG*$^+$)	±	±	±
	pHYD943 (*apaH*$^+$)	+++	+++	+++
GJ4597 (*gltBD apaH*)	pCL1920 (vector)	–	–	–
	pHYD942 (*apaG*$^+$)	±	±	±
	pHYD943 (*apaH*$^+$)	+++	+++	+++

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, glucose or sucrose, and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): — (no growth), ± and +++ (full growth).

Plasmids pHYD942 (*apaG*$^+$) and pHYD943 (*apaH*$^+$) along with pCL1920 (as vector control) were introduced into GJ4596 (*gltBD apaG*::Kan) and GJ4597 (*gltBD apaH*::Kan) and the Spec^R transformants were tested on high osmolarity media. The results (Table 4.3) showed that both the *apaG* and the *apaH* insertion mutants were complemented by *apaH*$^+$ but not by *apaG*$^+$. (It may be noted that the insertion mutation in *apaG* abolishes both *apaG* and *apaH* function, whereas the *apaH* insertion mutant is *apaG*$^+$.) The fact that the plasmid pHYD943 was able to complement both *apaG*::Kan and *apaH*::Kan mutants suggests that ApaH function is necessary and sufficient for the cell to survive under low a_w stress conditions, and that the osmosensitivity phenotype of the *apaG* mutant is due to its polar effect on the *apaH* gene.

4.3.2.3 Growth curves of *apaG* and *apaH* mutants:

In order to quantify the osmosensitivity phenotype in terms of growth rate (culture doubling times), stationary phase cultures of WT and *apaH* mutants were subcultured 1:100 in MA liquid medium supplemented with either 0.8 M NaCl or 1.2 M glucose. Growth was monitored with the aid of a Klett-Summerson photoelectric colorimeter. The comparison of growth curves for MC4100 (WT) and GJ4828 (*apaH*) showed that *apaH* was severely inhibited for growth in high osmolarity media (Fig. 4.4).

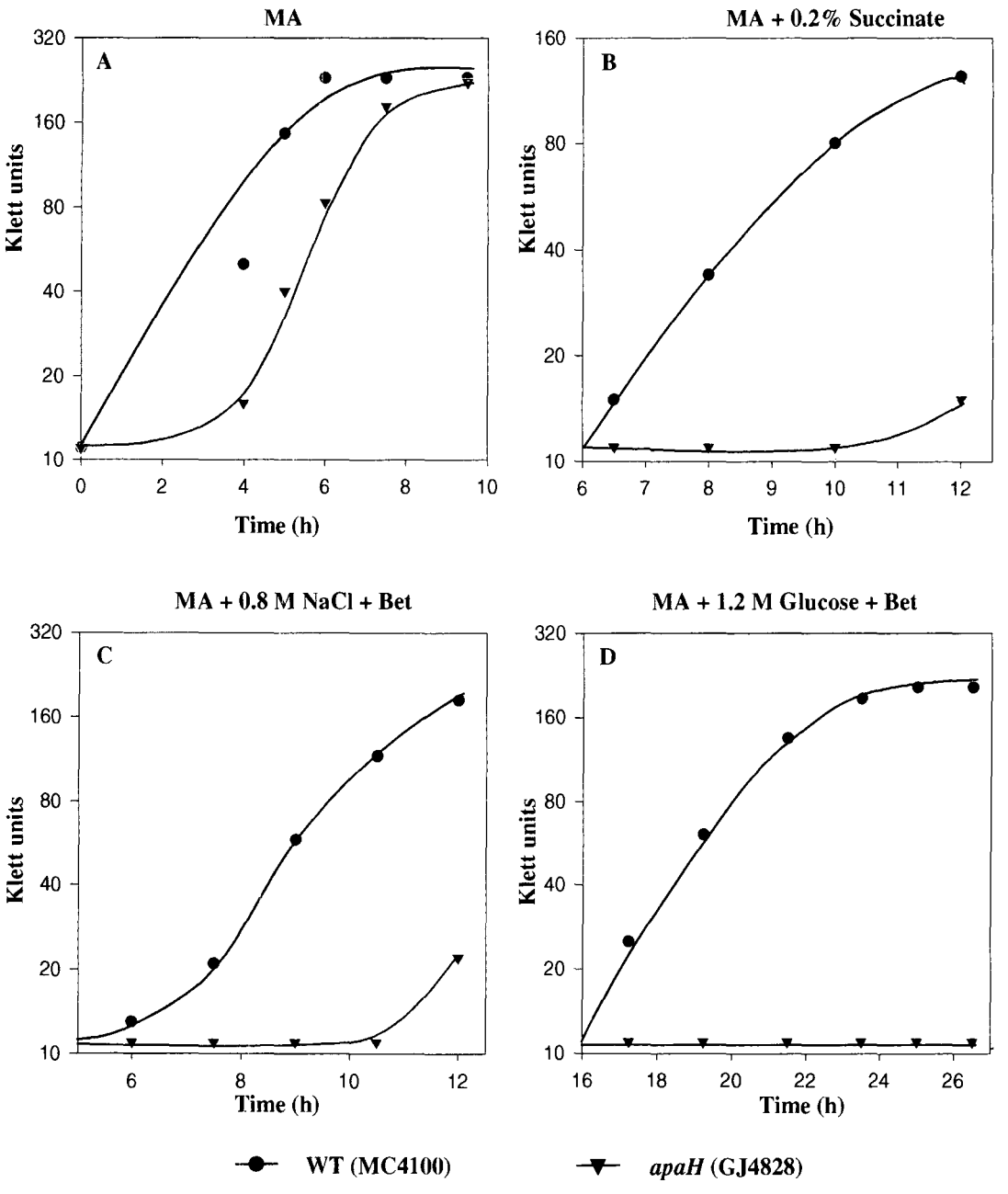


Fig. 4.4 Growth of *apaH* strain in different growth media.
Cultures of the different strains were inoculated into MA medium, MA with 0.2% sodium succinate instead of glucose as the sole carbon source, MA medium supplemented with 0.8 M NaCl and 1 mM glycine betaine, and MA medium supplemented with 1.2 M glucose and 1 mM glycine betaine, and optical density (Klett units) for each was monitored as a function of time of incubation in a shaker water-bath at 37°C.

Table 4.4 Doubling times (min) of WT and *apaH* strain in various media

Strain	MA	MA + Succinate	MA + Bet	
			NaCl (0.8 M)	Glucose (1.2 M)
WT	54	96	96	90
apaH	78	>600	>600	>600

To test the possibility that the *apaG* and *apaH* mutants were slow-growing even in the absence of water stress, WT and the two mutant strains were subcultured in MA medium and their growth was monitored. The results (Table 4.4 and Fig. 4.4) suggest that the *apaH* mutant is indeed slower growing compared to MC4100. The apparent osmosensitivity of the *apaH* mutant may hence, simply be a manifestation of the slow-growing nature of the mutants and may not be a true defect in water stress adaptation. Similar results were observed when the *apaG* mutant was tested, with the difference that it grew slightly better than *apaH* on both non-stressed and osmotically stressed media.

The slow-growth phenotype of *apaH* was in disagreement with earlier published work (Leveque *et al.*, 1990), in which the authors had shown that there was no difference in colony size or morphology for the *ksgA*, *apaG* and *apaH* mutants on solid medium, or in growth curves in liquid cultures for strains (both in $recA^+$ and $recA^-$ backgrounds), when they were grown in MOPS medium supplemented with glucose. All the three strains had exhibited similar growth properties at 30°C, 37°C and 42°C.

4.3.2.4 Criteria for defining that a mutant is osmosensitive:

In view of the above result (that the *apaG* and *apaH* mutants may not be truly osmosensitive but only non-specifically sick), the following criteria were devised in defining a strain as osmosensitive, and used in tests with the remaining mutants.

1. The mutant must grow slower than the WT on MA + NaCl or MA + sucrose or MA + glucose (osmotically stressed) medium.
2. Ideally, the mutant must grow as well as the WT on MA media without osmotic stress (like MA with 0.2% glucose or succinate or acetate as the sole carbon source). Succinate and acetate are poor carbon sources and the WT strain itself grows slower in such a medium. A mutation specifically affecting the NaCl-tolerance of a strain is not expected to affect its ability to grow in such a medium.

3. Even if the mutant was non-specifically more sick than WT, one could still argue that it is osmosensitive, provided it grows faster than one or more control non-specific sick mutants (such as *recA, recB* and *ruvABC*) on media without osmotic stress and slower than them on osmotically-stressed medium. The latter genes are involved in DNA recombinational repair functions, and mutations in them would make the strains slow-growing (sick) but not affect their specific osmotolerance phenotype.

To construct the control strains, the *recA, recB* and *ruvABC* alleles were introduced into WT and *gltBD* strains. The *recA56* (linked to *srl*::Tn*10*) and *recB*::Tn*10* alleles were introduced by transduction after selecting for Tet^R colonies, into the Tet^S Δ*gltBD* strain GJ4652 whose construction has been described in Section 3.2.4.

A P1 lysate grown on GJ216 (*recA56* linked 75% to *srl*::Tn*10*) was used to transduce MC4100 and GJ4652. The resultant Tet^R colonies were screened for the inheritance of the *recA56* allele by testing for UV-sensitivity as described in Section 2.15.1 of Chapter 2. One UV-sensitive colony from each transduction, carrying the *recA56* allele was selected for further work and designated GJ4584 (*recA*) and GJ4843 (*gltBD recA*).

The *recB268*::Tn*10* allele was transferred to MC4100 and GJ4652 by employing the P1 lysate prepared on JJC315. The resultant Tet^R colonies were designated GJ4839 (*recB*) and GJ4840 (*gltBD recB*). The P1 lysate made on JJC754 (Δ*ruvABC*::Cm) was used to transduce MC4100 and GJ4652. The Cm^R colonies harboring the *ruvABC* mutation were designated GJ4841 (*ruvABC*) and GJ4842 (*gltBD ruvABC*).

When the various non-specific mutants and *apaG* and *apaH* mutants were tested for osmosensitivity by both liquid culture method and on solid media, it was observed that *apaH* was more sick than any of the mutants on MA medium with and without osmotic stress. In the present case, the rank order of growth of the strains on the two sets of media was not different (Table 4.5). This result conclusively established that *apaH* cannot meet the criteria described above to define an osmosensitive mutant. As regards the *apaG* strain, it grew better than *apaH, recB* and *ruvABC* strains but was slower than *recA* on non-stressed plates. Even on high osmolarity plate, *apaG* grew better than non-specific sick mutants viz., *recB* and *ruvABC*.

Table 4.5 Rank order of growth of *apaG* and *apaH* mutants vis-à-vis the 'nonspecific sick' mutants.

Non-stressed (MA with 0.2% glucose or succinate or acetate as sole carbon source):

WT > *gltBD* > *recA* > *apaG* ≥ *recB* > *ruvABC* > *apaH*

Osmotically stressed (MA + 1 mM Bet with 0.6-0.7 M NaCl or 0.8-0.9 M Sucrose or 0.9-1.0 M Glucose):

WT > *gltBD* ≥ *recA* ≃ *apaG* > *ruvABC* > *recB* ≃ *apaH*

The above pattern was also observed when *gltBD* derivatives of *apaG* and *apaH* were analyzed for their growth phenotype on various media. Thus, neither the *apaG* and *apaH* single mutants nor their *gltBD* derivatives met the criteria set for defining osmosensitive mutants and they were not further characterized.

4.3.3 Application of the new criteria for testing the other NaCl-sensitive mutants:

For the other NaCl-sensitive mutants obtained in the present study, the null hypothesis was taken to be that they were also slow-growing on non-stressed media and were consequently, severely inhibited on osmotically-stressed media. To test this hypothesis, their growth rates were compared as described above.

4.3.3.1 Comparison of growth on solid media:

The various mutants viz., *lysC, speC, thiI, argP gltBD* and *glnE gltBD* along with control strains *recA, ruvABC, gltBD recA* and *gltBD ruvABC* were tested on MA, MA supplemented with either 0.2% acetate or succinate as sole carbon source, MA Bet supplemented with either 0.6-0.7 M NaCl or 0.8-0.9 M glucose. The results, summarized as growth rank orders in Table 4.6 show that:

1. On MA and MA with either succinate or acetate, *lysC, speC* and *thiI* strains grew on par with WT (MC4100), while *recA* and *ruvABC* strains were slower than WT.
2. On osmotically stressed plates, *recA* and *ruvABC* strains were slower than WT, but grew much better than *lysC, speC* and *thiI* mutants i.e., the rank order of growth of the strains on the two sets of media was different.
3. On media not imposing osmotic stress, *argP gltBD* and *glnE gltBD* mutants grew on par with *gltBD* control strain, whereas *gltBD recA* and *gltBD ruvABC* were slow-growing.

4. On the other hand, the *argP gltBD* and *glnE gltBD* mutants were severely inhibited for growth compared to *gltBD recA* and *gltBD ruvABC*, which in turn were slightly slower than *gltBD* strain on high osmolarity plates i.e., once again, the rank orders of growth of the NaCl-sensitive and non-specifically sick strains on the two sets of media were different.

Table 4.6 Rank order of growth of various mutants.

Non-stressed (MA with 0.2% glucose or succinate or acetate as sole carbon source):

WT = *thiI57* = *lysC82* = *speC100* > *recA* ≥ *ruvABC*

gltBD = *gltBD argP202* = *gltBD glnE463* ≥ *gltBD recA* ≥ *gltBD ruvABC*

Osmotically stressed (MA + 1 mM Bet with 0.6-0.7 M NaCl or 0.8-0.9 M Sucrose or 0.9-1.0 M Glucose):

WT > *recA* ≥ *ruvABC* >> *lysC82* = *speC100* > *thiI57*

gltBD > *gltBD recA* ≥ *gltBD ruvABC* > *gltBD glnE463* > *gltBD argP202*

4.3.3.2 Comparison of growth in liquid culture:

The growth rate of the above strains was monitored in MA, MA with 0.2% succinate (non-stressed conditions), and MA Bet supplemented with 0.8 M NaCl or 1.2 M glucose (osmotically stressed conditions) liquid cultures, as described above. The results (summarized in Fig. 4.5 and Table 4.7) mirrored those obtained on solid media.

Table 4.7 Doubling times (min) of the various NaCl-sensitive mutants

Strain	MA	MA + Succinate	MA + Bet	
			NaCl (0.8 M)	Glucose (1.2 M)
WT[a]	54	96	96	90
recA	ND	102	126	102
lysC	50	90	>450	>450
speC	51	96	>450	>450
thiI	50	90	>600	>600
gltBD	54	102	108	120
gltBD recA	ND	102	228	138
gltBD ruvABC	ND	114	150	126
gltBD argP	54	108	>600	>600
gltBD glnE	57	115	>600	>600

[a] These values are the same as in Table 4.4.

Since the mutations in *lysC, speC, thiI, argP* and *glnE* (the latter two in *gltBD* background) were shown to be genuinely osmosensitive, they were characterized further.

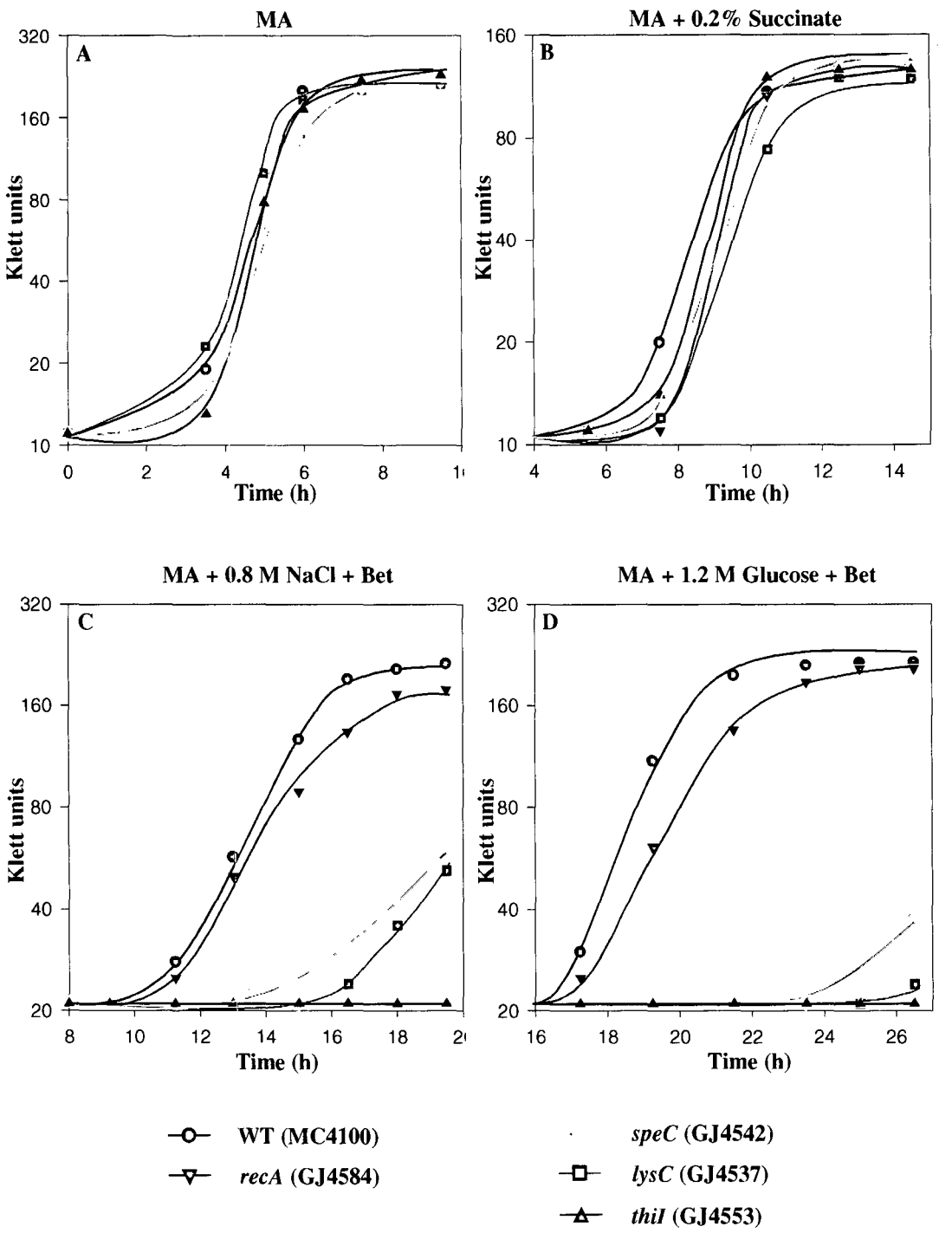


Fig. 4.5 Growth of osmosensitive mutants and control strains in different growth media (Panels A to D).

Cultures of the different strains were inoculated into MA medium, MA with 0.2% sodium succinate instead of glucose as the sole carbon source, MA medium supplemented with 0.8 M NaCl and 1 mM Bet, and MA medium supplemented with 1.2 M glucose and 1 mM Bet, and optical density (Klett units) for each was monitored as a function of time of incubation in a shaker water-bath at 37°C.

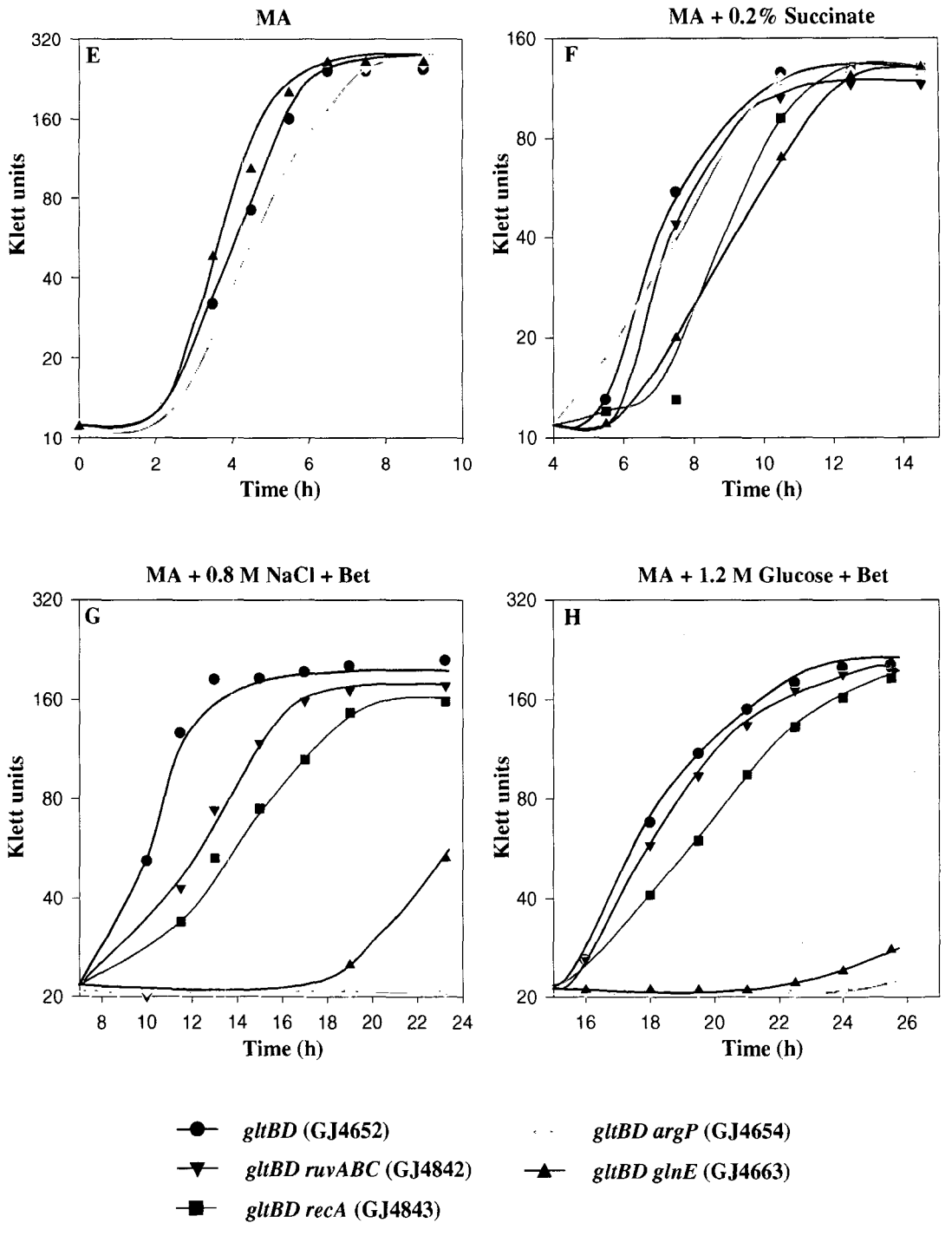


Fig. 4.5 Growth of osmosensitive mutants and control strains in different growth media (contd). (Panels E to H).
Legends are the same as for panels A-D.

4.4 Characterization of the *glnE* mutants:

4.4.1 Introduction:

A detailed introduction to *glnE* has been presented in Chapter 1. In brief, *glnE* is involved in nitrogen assimilation in *E. coli* and encodes the adenyltransferase enzyme which catalyzes the adenylation and deadenylation of glutamine synthetase, thus controlling the latter's enzymatic activity.

4.4.2 Results:

Three NaCl-sensitive mutants were isolated, which had null insertions in the *glnE* gene. Molecular characterization of GJ3547 (*glnE464*::Kan) and GJ3549 (*glnE465*::Kan) indicated that they harbor the Kan^R insertions in the 2.9 kb long *glnE* gene, 1009 and 2588 nucleotides downstream, respectively of the predicted ATG start site. Their orientations are such that the *lac* genes can be transcribed from the *glnE* in the former but not so in the latter. The exact sequence details of the Kan^R insertion in GJ3545 (*glnE463*::Kan) could not be ascertained since repeated efforts to sequence the flanking region sufficient for BLAST analysis from the amplicon obtained after inverse PCR had failed because there was an *Hha*I site (which was the enzyme used to prepare the inverse PCR template) just six nucleotides after the Kan^R insertion. But, as described in Section 3.2.9, it was proved by genetic methods that GJ3549 has the Kan^R insertion in the *glnE* gene by employing the P1 lysate of CAG18475 (*metC162*::Tn*10*), which gave a Tet to Kan linkage value very similar to that obtained for GJ3545 and GJ3547.

4.4.2.1 *glnE gltBD* mutant has compromised NH_4^+-assimilation phenotype:

NH_4^+-assimilation and Glu biosynthesis are intimately connected, and a defect in NH_4^+-assimilation would be reflected in a strain's inability to synthesize Glu *in vivo*. In a *gltBD* mutant, one of the primary ways of Glu biosynthesis (GOGAT, encoded by the genes *gltBD*) is absent, while the remaining glutamate dehydrogenase (GDH) pathway is efficient in NH_4^+-assimilation only in media with $\geq$ 1mM NH_4^+.

The degree of severity in NH_4^+-assimilation in the *gltBD glnE* mutant was tested on W-salts supplemented with varying $(NH_4)_2SO_4$ concentrations, and compared with that of other control strains. The results are summarized in Table 4.8, and show that the *glnE gltBD* strain is crippled for NH_4^+ assimilation (exhibiting poor growth even at 2 mM whereas the *gltBD* strain is able to grow at 1 mM). The *glnE* single mutant was no

different from the WT. (The *argP gltBD* double mutant was even more crippled for NH_4^+ assimilation than the *glnE gltBD* mutant. The significance of this result is discussed in Chapter 5.)

Table 4.8 Growth of the *glnE gltBD* mutant on low-[NH_4^+] medium[a]

Strain	Genotype	W-salts + NH_4^+ (mM)				
		0	0.3	0.6	1.0	2.0
MC4100	WT	++	+++	+++	+++	+++
GJ4652	*gltBD*	—	±	+	++	++
GJ4545	*glnE463*	++	+++	+++	+++	+++
GJ4663	*glnE463 gltBD*	—	—	±	+	++
GJ4654	*argP202 gltBD*	—	—	—	—	+

[a] The indicated strains were streaked on W-salts plates supplemented with varying concentrations of $(NH_4)_2SO_4$ and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): — (no growth), ±, +, ++, and +++ (full growth).

4.4.2.2 Alleviation by Asp of osmosensitivity in *glnE gltBD* mutant:

When the effect of exogenous Asp (1 mM) on high-osmolarity-medium growth was tested, a dramatic increase in tolerance of the *glnE gltBD* mutant was observed, whereas the control strains viz., MC4100, *gltBD* and *thiI* were only marginally growth-promoted under the same conditions. There was >0.1 M NaCl (or equiosmolar glucose and sucrose) increase in the upper threshold of tolerance for the *glnE gltBD* strain in the presence of Asp. At the same time, Asp promoted the growth of MC4100, *gltBD* and other strains by <0.1 M NaCl.

4.4.2.3 Effect of multicopy *spoT*$^+$ on NH_4^+ assimilation and osmotolerance in *glnE gltBD* mutants:

As described in Section 1.2.2.4 of Chapter 1, work published earlier from the lab (Saroja and Gowrishankar, 1996) suggested that a *gltBD fnr* mutant is compromised for both osmotolerance and growth on low NH_4^+, and that multicopy *spoT*$^+$ is able to

suppress both phenotypes. The suppression of growth sensitivity was shown to be through bypassing of the need for GOGAT in low NH_4^+ growth medium. Since *glnE gltBD* was also crippled for growth at low NH_4^+ and was osmosensitive, the effect of multicopy *spoT*$^+$ was tested. Plasmids pBR329 (vector) and pHYD809 (vector with 7.8 kb insert spanning *spoT* region) were introduced into *glnE gltBD, fnr gltBD, thiI gltBD, argP gltBD* and *gltBD* strains and the resultant Tet^R Cm^R transformants were tested for their ability to grow on high osmolarity plates and on W-salts medium supplemented with low NH_4^+. The results showed that multicopy *spoT*$^+$ did not suppress either the osmosensitivity or the NH_4^+ assimilation defect in *argP gltBD* and *glnE gltBD* mutant strains, but the plasmid promoted the growth of the *gltBD* single mutants, *fnr gltBD* and *thiI gltBD* strains.

4.4.2.4 Cloning of *glnE*$^+$ locus from Kohara library:

The phage λ-508 clone from the ordered library of Kohara *et al.* (1987) covers the *glnE* locus. λ-508 was clonally purified and amplified. λ-508 phage DNA, when restricted with *Bam*HI-*Kpn*I enzymes, generated a 5.2 kb fragment which was expected to contain the *glnE* gene. This fragment was cloned into *Bam*HI-*Kpn*I cut pCL1920 vector. The resultant Spec^R recombinant plasmid was designated pHYD916 (Fig. 4.6). In order to check whether pHYD916 would complement the *glnE* mutant, GJ4613 (*glnE463*::Kan *gltBD recA*), GJ4614 (*glnE464*::Kan *gltBD recA*) and GJ4652 (*gltBD* control) strains were transformed with the plasmid (or with pCL1920 as vector control). The resultant Spec^R colonies were tested for their ability to grow on high osmolarity plates. The results (Table 4.9) show that the 5.2 kb *glnE*$^+$ insert in pHYD916 was able to complement the *glnE gltBD* mutants for growth on high NaCl plate, as also on media supplemented with high glucose and sucrose, demonstrating that the cloned *glnE*$^+$ gene was capable of complementing the *glnE* mutants for osmosensitivity.

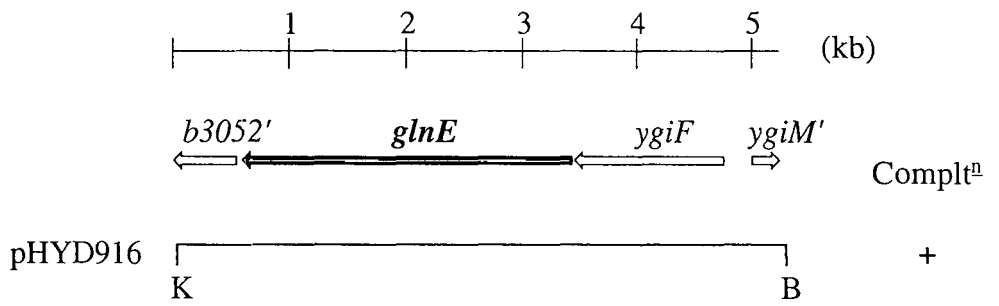


Fig. 4.6 Cloning of *glnE*$^+$ and complementation analysis.
Below the kilobase scale is depicted the physical and gene map of 5.2 kb *Kpn*I (K) -*Bam*HI (B) fragment from Kohara phage λ-508. Other descriptions are as in Fig. 4.3.

Table 4.9 Complementation of *glnE*::Kan mutations by *glnE*$^+$ at high osmolarity[a]

Strain (genotype)	MA Bet Spec NaCl (0.6-0.7 M)	
	pCL1920 (vector)	pHYD916 (*glnE*$^+$)
GJ4652 (*gltBD*)	+++	+++
GJ4613 (*glnE463 gltBD recA*)	–	+++
GJ4614 (*glnE464 gltBD recA*)	–	+++

[a] The indicated strains were streaked on MA + 1 mM Bet Spec plates supplemented with 0.6-0.7 M NaCl and incubated for 42-48 h at 37°C. No growth (–) and full growth (+++). Similar observations were made when NaCl was replaced with 0.9-1.0 M glucose or 0.8-0.9 M sucrose.

4.4.3 Discussion:

Null mutations in the *glnE* gene, encoding adenyltransferase, in a *gltBD* background have been shown for the first time in the present study to confer osmosensitivity. The *glnE* gene product is involved in the control of catalytic activity of glutamine synthetase (GS) through adenylation and deadenylation, which represents a negative feedback mechanism. A high Gln to 2-ketoglutarate ratio favors adenylation, a modification which results in an inactive GS while a low ratio favors deadenylation, which makes GS catalytically active.

4.4.3.1 Impaired NH_4^+ assimilation in the *glnE gltBD* mutant as a cause of osmosensitivity phenotype:

The osmosensitivity phenotype of the *glnE gltBD* mutant was reversed by the addition of 1 mM Asp (Section 4.4.2.2). Since Asp had only a minor growth-promoting activity for WT, *gltBD* and other NaCl-sensitive mutant strains but a major effect in promoting the growth of *glnE gltBD*, it can be argued that Asp specifically alleviates the osmosensitivity phenotype. Asp addition is known to increase the internal Glu pools since it acts as an amino group donor for rapid conversion of 2-ketoglutarate to Glu in a simple transamination reaction.

The growth promotion of *glnE gltBD* by Asp on media of low a_w pointed to the probable impairment in the double mutant of Glu synthesis and ammonia assimilation (Section 4.4.2.1), because both these are intimately connected in *E. coli*. A *gltBD* single mutant can synthesize Glu only through glutamate dehydrogenase (GDH)-catalyzed reaction, which might be just sufficient for it to survive under conditions of osmotic stress (when the cell's need for Glu is elevated). Hence, it can be argued that the *glnE gltBD* mutants are osmosensitive because the *glnE* mutation further compromises ammonia assimilation and Glu biosynthesis.

4.4.3.2 Mechanisms by which Glu synthesis may be reduced in *glnE* mutant:

As discussed earlier, in *E. coli* there are primarily two pathways of Glu biosythesis viz., GS-GOGAT and GDH pathways. GS-GOGAT, taken together, catalyze the conversion of NH_3 and 2-ketoglutarate to Glu, and GDH does likewise. In a GOGAT-deficient (Δ*gltBD*) strain, Glu is synthesized only through the GDH pathway. In a *glnE* strain, due to the absence of the modifying GlnE enzyme, the GS enzyme is always unmodified and therefore, might be catalytically active constitutively, resulting in the continuous conversion of Glu to Gln. Because of this, in a *gltBD glnE* double mutant, there is an uncontrolled shunting / channeling of Glu to Gln in a manner which is insensitive to the Gln to 2-ketoglutarate ratio, resulting in impaired NH_4^+ assimilation, and subsequently leading to low intracellular Glu pools and osmosensitivity.

The inability of GlnE (ATase) to adenylate GS might result in constitutively high production of GS. This is based on the fact that adenylated GS inhibits the transcription of *glnA* and deadenylated GS fails to repress its own transcription (autoregulation) (Foor

et al., 1975). This will therefore lead to a situation wherein the intracellular Gln to Glu ratio would be very high since in a *glnE* mutant, the deadenylated GS cannot autorepress its own transcription.

In *S. enterica*, *glnE* mutants were shown to excrete Glu and further drain their Glu pool into Gln when they are shifted from N-limiting to NH_4^+-excess conditions (Kustu *et al.*, 1984). This loss of Glu (intracellular) pools is accompanied by the loss of K^+ ions from the cell, which means that the intracellular concentrations of two important solutes (K^+ and Glu) involved in the osmoadaptation of *S. enterica* are decreased in a *glnE*-defective strain (Yan *et al.*, 1996). A similar situation might exist in *E. coli*, which gets compounded in the *gltBD* background, resulting in an osmosensitivity phenotype.

The synthetic osmosensitivity of the *gltBD glnE* strain may be analogous to the *Mycobacterium tuberculosis* system wherein *glnE* was shown to be essential for viability in certain culture conditions (Parish and Stoker, 2000). It was shown that GlnE is required to maintain intracellular balance of Glu/Gln and that in the absence of this regulation, GlnA activity would deplete the intracellular pool of Glu, resulting in cell growth inhibition. In *Streptomyces coelicolor, glnE* mutants were shown to have a lowered ratio of Glu to Gln (Fink *et al.*, 1999).

While looking for suppressors of NaCl-sensitivity in a *gltBD glnE* strain using the localized P1 mutagenesis approach (described in detail in Section 6.3.2.2. of Chapter 6), it was observed that a majority of the suppressors had become $NaCl^T$ by crossing out Δ*gltBD* with the Cm^R linked to $gltBD^+$ region, while one had become $glnE^+$ by the crossing out of *glnE*::Kan. These results suggested that:

(i) that the *glnE* gene is indeed involved in $NaCl^T$ since in a whole-genome screen looking for $NaCl^T$ suppressors in a *gltBD glnE* strain, one suppressor was obtained which had become $glnE^+$; and

(ii) there is a strong interaction (synergism) between *gltBD* and *glnE* in conferring $NaCl^S$ to the strain since vast majority of the suppressors have now become $gltBD^+$, reinforcing the notion that Glu pools and thus, *gltBD* play an important role in the osmoadaptation of *E. coli*.

4.5 Characterization of *lysC* mutants:

4.5.1 Introduction:

In all *Enterobacteriaceae* members, aspartokinases (AK) catalyze the first step in the common biosynthetic pathway from Asp to Lys and diaminopimelate, Met, Thr, and Ile (Cohen *et al.*, 1969). *E. coli* has three AK isozymes (Fig. 4.7):

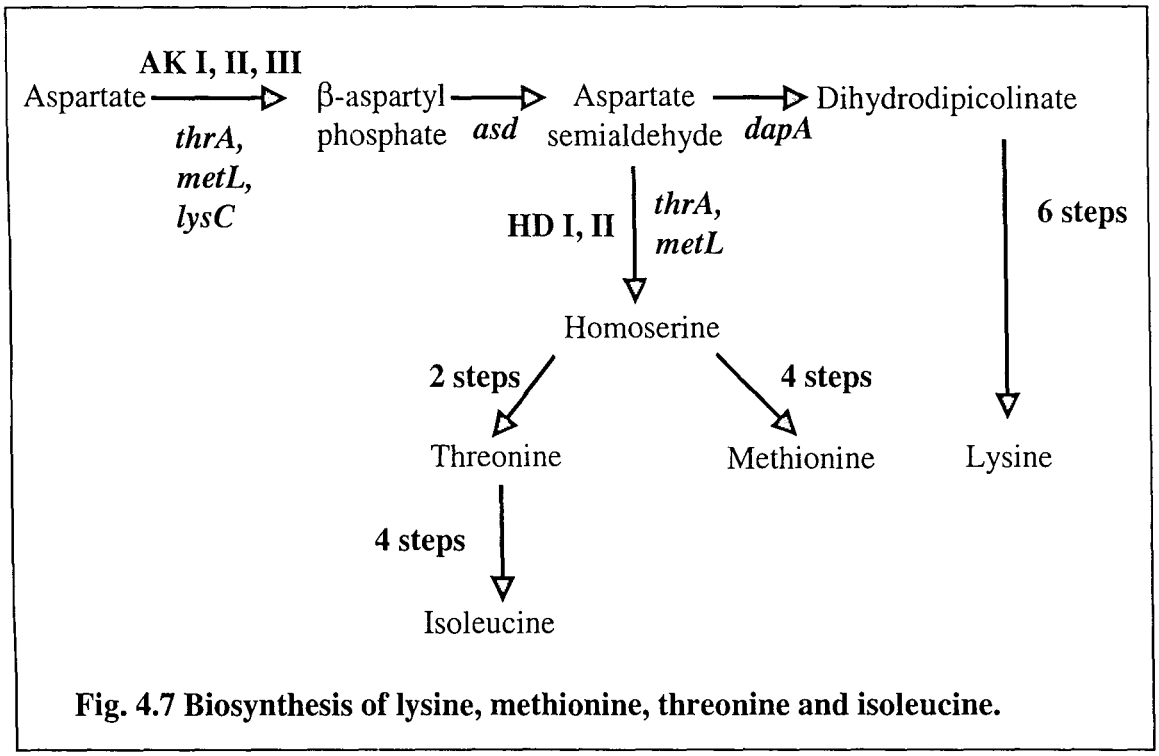


Fig. 4.7 Biosynthesis of lysine, methionine, threonine and isoleucine.

(a) AK-I–HD-I (Aspartokinase I – homoserine dehydrogenase I), encoded by *thrA* is a bifunctional protein having both AK and HD activities. Its enzymatic activity is inhibited allosterically by Thr and its synthesis is repressed by Thr plus Ile;

(b) AK-II–HD-II (Aspartokinase II – homoserine dehydrogenase II), which is also a bifunctional protein having both AK and HD activities, and is encoded by *metL*. It is not inhibited by any ligand, including Met, but its synthesis is repressed by Met;

(c) AK-III (Aspartokinase III) is encoded by *lysC* (located at 91.2 min on the *E. coli* chromosome) and has only the AK activity. Its synthesis is repressed by Lys and its activity is also inhibited by Lys. Feedback inhibition by Lys is non-competitive with respect to Asp (Stadtman *et al.*, 1961). Lys inhibition is co-operative (Cohen and Saint-Girons, 1987); 0.2 mM Lys, a concentration comparable to that of the intracellular Lys pool, inhibits ~50% of the enzyme activity (Truffa-Bachi and Cohen, 1966). The purified enzyme has an M_r of 100,000 and is comprised of two identical subunits. The subunit

polypeptide length is 449 amino acids. Based on its sequence similarities with other AKs, AK-III is believed to consist of two domains, an N-terminal domain with catalytic activity and a C-terminal domain through which Lys acts to exert its inhibitory effect.

The specific activity of AK-III varies in response to intracellular Lys concentration. Approximately a 10-fold repression is observed when Lys is added to the growth medium (Stadtman *et al.*, 1961), whereas a 15-fold derepression is seen during Lys-limited growth in a chemostat (Cohen and Saint-Giron, 1987). Although Lys is a specific effector, Arg also plays a role in AK-III regulation. When both amino acids are present simultaneously, Arg prevents Lys from maximally repressing AK-III synthesis (Cassan *et al.*, 1975).

4.5.2 Results:

One *lysC* mutant was isolated during the screen for mutations affecting NaCl-tolerance in *E. coli*. The *lac*-Kan^R transposon was inserted 839 nucleotides after the ATG translation start site of the 1.35 kb-long *lysC* gene, as revealed by sequencing the junction sequences in GJ3537 (*lysC82*::Kan). GJ4660 (*gltBD lysC*) and GJ4537 (*lysC*) were inhibited for growth on plates supplemented with either ionic or nonionic impermeable solutes, indicating that the observed phenomenon was osmotic in nature. *lysC* mutation by itself conferred growth sensitivity on these various media but the phenotype was exacerbated in the presence of *gltBD* mutation.

4.5.2.1 Construction of *thrA* null mutants:

To test whether null insertions in *thrA* (which encodes aspartokinase I) affect the osmotolerance of a strain, *thrA* null mutant derivatives of MC4100 (WT) and GJ4652 (*gltBD*) were constructed using the P1 lysates prepared on CAG18425 (*thrA*::Tn*10*Kan) and CAG18442 (*thrA*::Tn*10*). The insertions in *thrA* gene disrupted the downstream *thrBC* genes of the *thrABC* operon involved in Thr biosynthesis. The resultant Kan^R and Tet^R derivatives of both GJ4652 and MC4100 were screened for Thr auxotrophy to confirm that they harbor mutations in the *thrA* gene, and one colony each was selected for further work and designated as GJ4831 (*thrA*::Kan), GJ4832 (*gltBD thrA*::Kan), GJ4833 (*thrA*::Tet), and GJ4834 (*gltBD thrA*::Tet). These strains were then tested for their ability to grow on high osmolarity medium. The results, summarized in Table 4.10, show that null mutations in *thrA* do not affect the ability of the strain to grow under high osmotic stress conditions.

Table 4.10 Osmosensitivity of various *lysC* and *thrA* mutants[a].

Strain	Genotype	MA Bet[b] NaCl (0.6-0.7 M)	Glucose (0.9-1.0 M)	Sucrose (0.7-0.8 M)
MC4100	WT	+++	+++	+++
GJ4537	*lysC82*::Kan	+	+	+
GJ4652	*gltBD*	++	++	++
GJ4831	*thrA*::Kan	+++	+++	+++
GJ4832	*gltBD thrA*::Kan	++	++	++
GJ4833	*thrA*::Tn*10*	+++	+++	+++
GJ4834	*gltBD thrA*::Tn*10*	++	++	++
GJ4660	*gltBD lysC82*::Kan	–	–	–
GJ4759	*gltBD lysC1001*	–	–	–
GJ4760	*gltBD thrA1101*	++	++	++

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, glucose or sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 4-point scale (in increasing order): — (no growth), +, ++, and +++ (full growth).
[b] Thr was supplemented at 40 µg/ml concentration when *thrA* alleles were tested.

4.5.2.2 Testing the different alleles of *lysC* and *thrA* for osmosensitivity phenotype:

To check whether other alleles of *lysC* and *thrA* would behave like the null alleles tested above, *lysC1001* and *thrA1101* (both from Gif106M1) were each individually transferred to GJ4652 (*gltBD*) strain in the following manner.

A. Construction of GJ4759 and GJ4760:

Gif106M1 (*lysC1001 thrA1101 metL1000*) strain was transduced to Tet^R with P1 lysates prepared on CSH123 (*thiA*::Tn*10* located near *lysC*) or CAG18462 (*mdoB*::Tn*10* located near *thrA*) and the resultant colonies were screened for *lysC1001* and *thrA1101* by testing the colonies for $(Lys + Met)^S$ for *thrA1101* and $(Thr + Met)^S$ for *lysC1001*. [A *lysC* mutant would be $(Thr + Met)^S$ because it is starved for Lys under these conditions.

Similarly, a *thrA* mutant would be (Lys + Met)^S due to Thr starvation.] One *thiA*::Tn*10* transductant which is *lysC1001 thrA1101 metL1000* was designated as GJ4757 (Tet^R linked to *lysC1001*) and an *mdoB::Tn10* transductant which was *mdoB*::Tn*10 thrA1101 lysC1001 metL1000* was christened GJ4758 (Tet^R linked to *thrA1101*). GJ4652 (*gltBD*) was transduced to Tet^R separately using the P1 lysates prepared on the above two strains. The Tet^R colonies resulting from GJ4757 lysate were screened for *lysC* by streaking them on MA supplemented with Thr and Met, and one colony that did not grow on such a plate was selected for further analysis and designated GJ4759 (*lysC1001 thiA*::Tn*10* Δ*gltBD*). The Tet^R colonies of GJ4652 obtained employing GJ4758 lysate were screened for (Lys + Met)^S and one such colony was designated GJ4760 (*thrA1101 mdoB*::Tn*10* Δ*gltBD*).

B. Testing *lysC1001 gltBD* (GJ4759) and *thrA1101 gltBD* (GJ4760) for osmosensitivity:

The above two strains were streaked on MA NaCl (0.6-0.7 M) Bet, MA Sucrose (0.7-0.8 M) Bet, and MA Glucose (0.8-0.9 M) Bet, and the results indicated that GJ4759 was sensitive to the dissolved solutes in the medium but GJ4760 was not sensitive. This result (Table 4.10) shows that *gltBD lysC1001* (GJ4759) is osmosensitive while *gltBD thrA1101* is not, consistent with the results obtained earlier with the null mutants (Section 4.5.2.1).

4.5.2.3 Effect of various amino acids on osmosensitivity phenotype of *lysC* mutant:

Since the AK-I, II and III isozymes are involved in the biosynthesis of Met, Thr and Lys, it could be argued that the mutation in *lysC* results in decreased pools of the end product amino acids leading to osmosensitivity but having no perceptible effect on MA media. To test this hypothesis, Lys, Thr and Met were supplemented at 40 μg/ml to MA plates without and with 0.6 to 0.7 M NaCl Bet, 0.8 to 0.9 M sucrose or 0.9 to 1.0 M glucose. The results are summarized in Table 4.11. The salient features of these data are:

(i) Exogenous Lys did not affect the osmosensitivity of either GJ4537 (*lysC*) or GJ4660 (*gltBD lysC*);

(ii) Exogenous Met or Thr, when added individually, exacerbated the osmosensitivity of the *lysC* mutant GJ4537;

(iii) There was no effect on the growth of the *lysC* mutant when either Lys + Thr or Lys + Met was added to the high osmolarity media;

(iv) Lys + Met + Thr, when added to the high osmolarity plates, promoted the growth of the *lysC* mutant marginally;

(v) Diaminopimelate supplementation had no effect on the osmosensitivity phenotype of *lysC*.

Table 4.11 Effect of various amino acids on the osmosensitivity phenotype of the *lysC* mutant[a]

Amino acid addition	MC4100 (WT)		GJ4537 (*lysC*::Kan)	
	MA	MA + NaCl	MA	MA + NaCl
Nil	+++	+++	+++	+
Lys	+++	+++	+++	++
Met	+++	+++	+++	±
Thr	+++	+++	++	–
Met + Thr	+++	+++	–	–
Lys + Thr	+++	+++	+++	++
Lys + Met	+++	+++	+++	+
Lys + Met + Thr	+++	+++	+++	+++

[a] The indicated strains were streaked on non-stressed (MA) and osmotically-stressed media (MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl). All the amino acids were added at 40 μg/ml concentration, and the plates were incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): — (no growth), ±, +, ++ and +++ (full growth).

4.5.2.4 Phenotypic effects of combining different NaCl-sensitive mutations:

To examine the interactions, if any, between *lysC* and *glnE*, a *lysC glnE gltBD* triple mutant was constructed as follows. The strain GJ4663 (*glnE gltBD*) was transduced to Cm^R employing the P1 lysate prepared on the strain GJ4736, in which *lysC*::Kan was linked ~90% to Cm^R. The resultant Cm^R colonies were screened for Met + Thr sensitivity. One such sensitive colony was selected for further work and designated GJ4737. On media of high osmolarity, the triple mutant strain (GJ4737) was more severely crippled for growth than either the *lysC* or *glnE gltBD* mutants. This result showed that *lysC* and *glnE* have an additive effect on the osmosensitivity phenotype, suggesting that they act through independent pathways.

4.5.3 Discussion:

Possible explanations as to why *lysC* mutants are osmosensitive:

A null insertion in *lysC* was obtained which conferred osmosensitive phenotype in *E. coli*. Subsequently, a previously characterized *lysC1001* allele was also shown to confer osmosensitivity. Mutations in *thrA* did not confer osmosensitivity, suggesting that it is the LysC function which is, for some reason, essential for cell survival under low a_w conditions.

A *lysC* mutant grown in the presence of Met has only ThrA (AK-I) functional, while in the presence of Thr, it has only MetL (AK-II) functionally active. Osmosensitivity was exacerbated in both these situations. This result indicates that at least two AK isoenzymes (one of which should necessarily be LysC) need to be functionally active for a strain to survive under hyperosmolarity conditions. This suggests that some of the intermediates of the pathway or the end products themselves may be important for the strain to survive under low a_w stress.

4.5.3.1 Inhibition of ThrA or MetL under water stress conditions:

Among the three AKs, it is AK-III (*lysC* encoded) which is predominantly responsible for the conversion of Asp to β-aspartyl phosphate, while the other two play a minor role. If one were to hypothesize that either ThrA or MetL activity (or both) is severely crippled under low a_w conditions, then the continued function of LysC becomes essential for cell survival. The partial alleviation of the osmosensitivity phenotype in the presence of these three amino acids suggests that the strain under these conditions might be starved for the end product amino acids or their derivatives.

Alternatively, in a *lysC* mutant some of the intermediates of the biosynthetic pathway may be limiting on a high osmolarity medium (but sufficient enough for growth on non-stressed media) resulting in osmosensitivity phenotype.

4.5.3.2 Novel function for AK-III?

If one assumes that AK-III has an additional novel function (apart from its catalytic phosphorylation of Asp for the synthesis of three amino acids), which is essential for *E. coli* to survive under low a_w stress, then one can explain the osmosensitivity phenotype of a *lysC* strain. The fact that the WT strain was not affected by the addition of exogenous Lys + Met + Thr on either osmotically stressed or non-

stressed media suggests that it might be a Lys-insensitive function of LysC (distinct from its Lys-sensitive AK-III activity) which is important for the cell to survive under water-stress conditions. LysC may play a role in osmoregulation either by catalyzing the phosphorylation of an unidentified substrate or through some specific protein-protein interaction. This kind of interaction between AK-III and other protein(s) is not unusual as there is a report of such interaction between AK and Enzyme I^{Ntr} in *Bradyrhizobium japonicum* (King and O'Brian, 2001), and it was suggested that *lysC* has a role in addition to amino acid biosynthesis in this organism. Enzyme I^{Ntr} encoded by *ptsP* is suggested to play a role in oligopeptide transport while the other paralogs are involved in coordinating nitrogen and carbon metabolism. Phosphorylation of Enzyme I^{Ntr} is inhibited in the presence of AK, and unphosphorylated Enzyme I^{Ntr} is involved in the transport of oligopeptides. A connection, if any, between Enzyme I^{Ntr} and AK in N metabolism has yet to be elucidated.

4.6 Characterization of *thiI* mutants:

4.6.1 Introduction

The *thiI* gene product is essential for thiamin (Vitamin B_1) biosynthesis and thiolation of uracil at position 8 in tRNAs. The biosynthesis of thiamin and 4-thiouridine (s^4U) in tRNA have been linked by the observation that some mutants lacking s^4U in their tRNAs were also auxotrophic for thiamin (Ryals *et al.*, 1982). Two groups independently showed that the cloned *thiI* gene could complement both s^4U and thiamin-deficient mutants in *E. coli* and *S. enterica* (Mueller, 1998; Webb *et al.*, 1997).

Base modification in tRNA is found in all organisms and represents the fine-tuning of the many functions of tRNA in protein translation (Bjork, 1995; Madore *et al.*, 1999). Thionucleosides in particular have been found to influence tRNA aminoacylation (Madore *et al.*, 1999; Sylvers *et al.*, 1993), codon-anticodon specificity (Yokoyama *et al.*, 1985) and reading frame maintenance (Urbonavicius *et al.*, 2001), as well as the binding of the tRNA to the ribosome (Ashraf *et al.*, 1999).

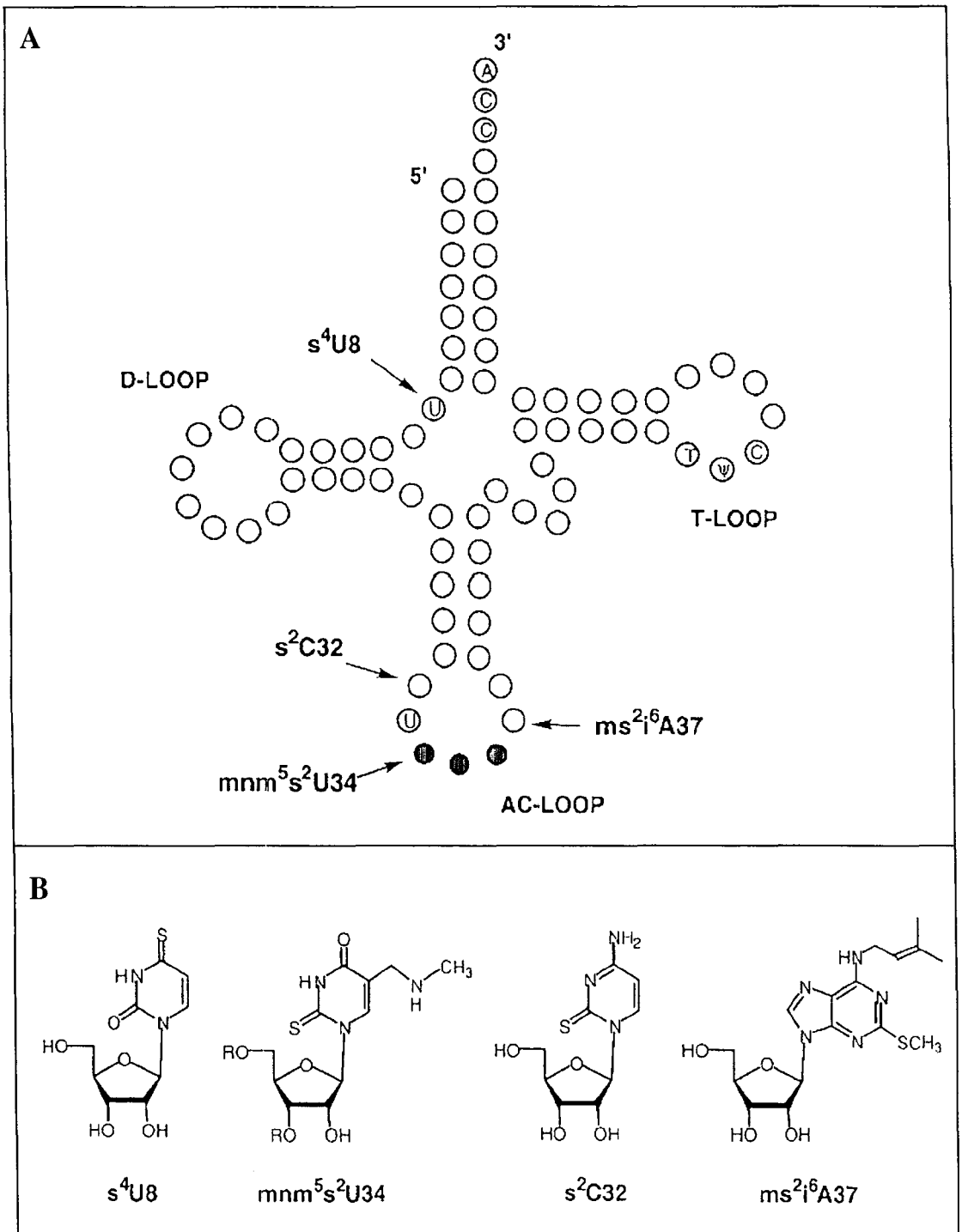


Fig. 4.8. Structures of the four naturally occurring thionucleosides and their locations in *E. coli* tRNA. Invariant nucleosides are shown in the tRNA secondary structure, and anticodon bases are represented by solid circles. s^4U8, 4-thiouridine 8; mnm^5s^2U34, 5-methylaminomethyl-2-thiouridine 34; s^2C32, 2-thiocytidine 32; ms^2i^6A37, 6-*N*-dimethylallyl-2-methylthioadenosine 37. (Adapted from Lauhon, 2002).

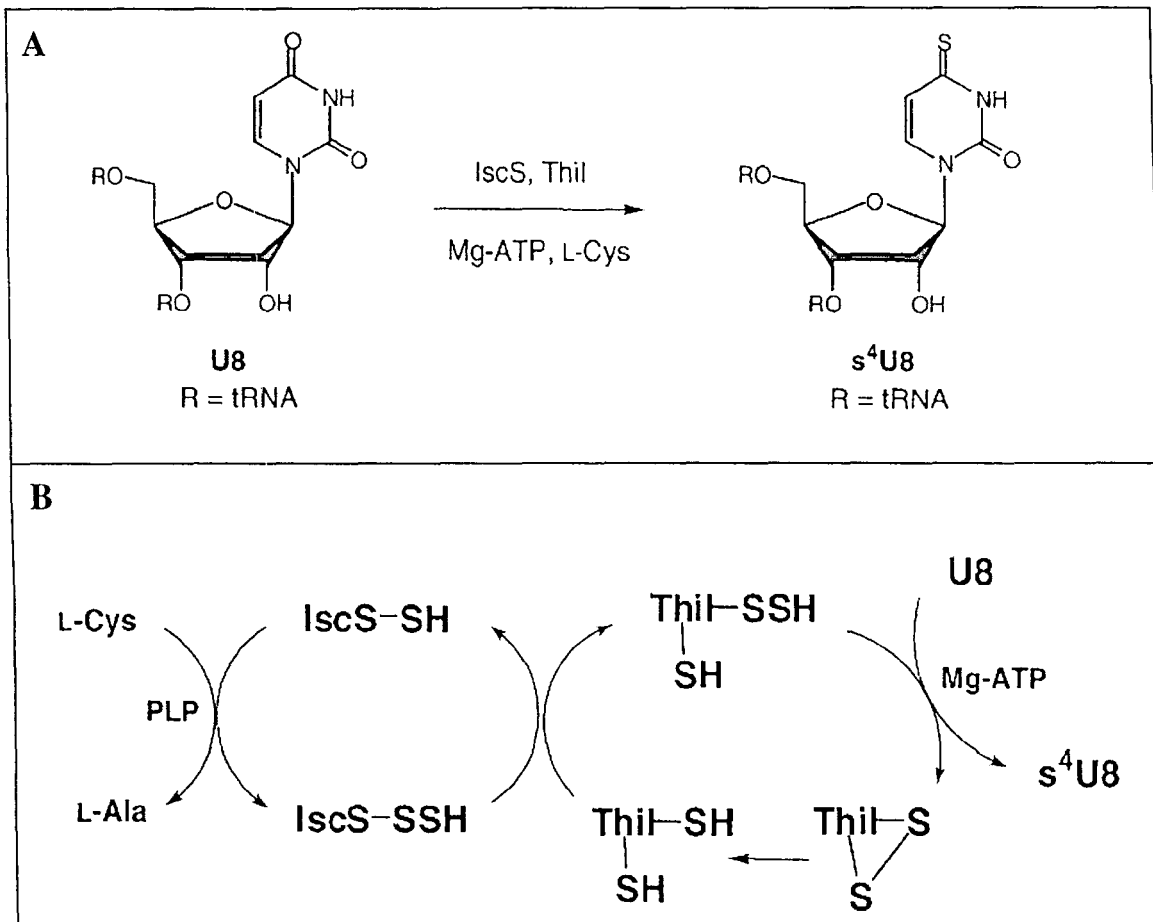


Fig. 4.9. Biosynthesis of 4-thiouridine in *E. coli*. (A) Factors necessary for conversion of uridine (U8) to 4-thiouridine (s^4U8) in *E. coli*. (B) Currently proposed mechanism for sulfur transfer from IscS to ThiI during 4-thiouridine biosynthesis. ThiI also utilizes Mg-ATP for activation of the uridine O-4 and requires free thiol for reduction of a disulfide that is formed internally during turnover. IscS-SH, unmodified IscS protein; IscS-SSH, IscS persulfide; PLP, pyridoxal-L-phosphate. (Adapted from Lauhon, 2002).

In *E. coli*, there are four naturally occurring thionucleosides viz., 4-thiouridine (s^4U), 2-thiocytidine (s^2C), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) and 6-*N*-dimethallyl-2-methylthioadenosine ms^2i^6A (Fig. 4.8). s^4U is the predominant thionucleoside formed by post-transcriptional modification of the nearly-invariant uridine at position 8 in tRNAs. The biosynthesis of s^4U has been shown to require both the thiamin pathway enzyme, ThiI (Mueller *et al.*, 1998) and the cysteine desulfurase, IscS (Kambampati and Lauhon, 2000; Fig. 4.9A). The mechanism of sulfur transfer initially involves mobilization of sulfur from Cys by IscS to form a persulfide at Cys328 in the active site of the latter (Flint, 1996; Fig. 4.9B). This persulfide or sulfane sulfur is then transferred to a Cys on ThiI, which in turn transfers it to the tRNA (Kambampati and Lauhon, 2000) by a mechanism that probably involves oxidation of ThiI. This type of mechanism also operates for the synthesis of 2-thiouridine at position 34 in tRNAs

specific for Lys, Glu and Gln, and is catalyzed by IscS and MnmA (formerly *trmU,* which encodes a tRNA methyltransferase; Kambampati and Lauhon, 2003). *miaA* (which encodes tRNA Δ^2-isopentenylpyrophosphate transferase) and *miaB* (encoding thiotransferase) are involved in the synthesis of ms^2i^6A at position 37.

Some tRNA modifications are thought to act as biochemical sensors within the cell for environmental stress. For example, 4-thiouridine has been proposed to act as a sensor for near-UV radiation (Thomas and Favre, 1975), leading to intramolecular crosslinks in tRNA that result in poor aminoacylation (Blondell and Favre, 1988) and hence, inducing the stringent response (Kramer *et al.*, 1988). It was suggested that s^4U, found in 65% of tRNA species, undergoes a photoactivated 2 + 2 cycloaddition with cytidine at position 13 when the tRNA is exposed to near-UV light of a wavelength similar to the 334 nm absorbance maximum of s^4U (Favre *et al.*, 1969; Bergstrom *et al.*, 1971; Favre *et al.*, 1971; Leonard *et al.*, 1971). These cross-linked tRNAs have been shown to be poor substrates for aminoacylation. The accumulation of nonaminoacylated tRNA serves to trigger the stringent response via the *relA* gene product, which is involved in the synthesis of ppGpp. The alarmone, ppGpp, then acts to inhibit stable RNA synthesis while inducing amino acid biosynthesis (Stephens, 1975). This response is known as the near-UV (NUV) phenotype, which is a cellular adaptational mechanism in *E. coli* to survive UV exposure. The presence of 6-N-dimethylallyl-2-methylthioadenosine (ms^2i^6A) has been shown to require soluble iron and is absent in tRNA from bacteria isolated from lethally infected animals, in which iron is scarce (Griffiths *et al.*, 1978). It has recently been shown that MiaB, which is implicated in sulfur insertion of ms^2i^6A (Esberg, 1999) is an Fe-S protein (Pierrel *et al.*, 2002).

E. coli contains three genes *iscS, csdA* (Mihara *et al.*, 1997) and *csdB* (Mihara *et al.*, 1999) that code for cysteine desulfurases. Each gene product has been shown to catalyze cysteine desulfurase activity with varying efficiency *in vitro* (Mihara *et al.*, 2000). It has recently been shown that IscS is the major cysteine-desulfurase required for biosynthesis of all thionucleosides in *E. coli* (Lauhon, 2002), whereas *csdA* and *csdB* mutants showed no variation in thionucleoside levels. It has been established that *iscS* mutants are defective in a wide range of metabolic pathways involving sulfur. These include the biosynthesis of vitamins, such as thiamin and nicotinic acid (NAD), as well as the amino acids Ile, Val and Met (Lauhon and Kambampati, 2000; Schwartz *et al.*, 2000; Skovran and Downs, 2000; Tokumoto and Takahashi, 2001). Many of the metabolic

defects are the result of the key role that *iscS* plays in the formation and function of proteins encoded by the *isc* gene cluster that are necessary for Fe-S cluster biosynthesis (Zheng *et al.*, 1998).

ThiI is also required for the biosythesis of the thiazole moiety of thiamin. *iscS* mutants were shown to be thiamin- and NAD- auxotrophs and deficient in s^4U modification. Based on these results, it was suggested that IscS is a cysteine desulfurase, which mobilizes sulfur for biosynthesis of thiazole moiety. The NAD requirement of *iscS* mutant was explained based on the role of the IscS protein in Fe-S cluster assembly. The quinolinate synthetase (NadA) protein involved in NAD biosynthesis is an Fe-S cluster protein which becomes inactive in the absence of Fe-S cluster assembly in an *iscS* mutant. To explain the thiamin requirement, one could assume that IscS functions as the sulfur donor in the formation of the thiazole ring (which itself contains sulfur). Alternatively, an enzyme in the thiamin biosynthetic pathway (ThiH) has been proposed to contain an Fe-S cluster (Begley *et al.*, 1999) and the activity of this enzyme may be insufficient in the *iscS* mutant. Subsequently, IscS has been shown to have a major role in *in vivo* Fe-S cluster formation in *E. coli*. IscS provides the sulfur that subsequently becomes incorporated during *in vivo* Fe-S cluster synthesis.

With regard to its role in catalyzing s^4U modification in tRNA, it probably has a mixed function: ATP-dependent activation of uridine and sulfur-atom transfer. A catalytically critical 'P-loop' motif, SGGXD(S/T), has been suggested to catalyze the adenylation and subsequent substitution of carbamoyl oxygen of uracil at the expense of ATP (Mueller and Palenchar, 1999).

In addition, ThiI from *E. coli, H. influenzae* and *S. enterica* have a C-terminal extension with sequence similarity to those of rhodanese-like sulfurtransferases (Palenchar *et al*, 2000). Although no function has been assigned to the N-terminal portion of the ThiI protein, a PSI-BLAST search of the N-terminal region of ThiI as the query detected similar sequences in a variety of predicted RNA methylases from Archaea, eukaryotes and bacteria (Aravind and Koonin, 2001). The search detected a similar region in an Archaea-specific family of predicted pseudouridine synthases (PSUSs) from *Methanococcus jannaschii*. The conserved region is shared by enzymes that are predicted to carry out at least three unrelated types of RNA-modification viz., methylation, pseudouridylation and thiouridylation, and named it as the THUMP domain (after thiouridine synthases, methylases and PSUSs).

4.6.2 Results:

As described in Section 3.2.8 of Chapter 3, eight Kan^R insertion mutants were isolated in the screen for NaCl-sensitive mutants. Molecular characterization revealed that they all carried null insertions in the *thiI* gene. Of these eight mutants, at least six are independent insertions, as revealed by sequencing results (Fig. 4.10). The Kan^R transposon was inserted 54, 83, 85, 704 and 1050 nucleotides downstream of the predicted ATG translation start site of *thiI* in GJ3539 (*thiI53*::Kan), GJ3533 (*thiI51*::Kan), GJ3553 (*thi57*::Kan), GJ3550 (*thiI55*::Kan), and GJ3552 (*thiI56*::Kan), respectively. Two strains, GJ3543 and GJ3546 had Kan^R insertions 866 nucleotides downstream of the ATG start site, and both are therefore designated *thiI54*::Kan. All the *thiI*::Kan mutants obtained in this screen behaved identically as far as their osmosensitivity phenotype was concerned.

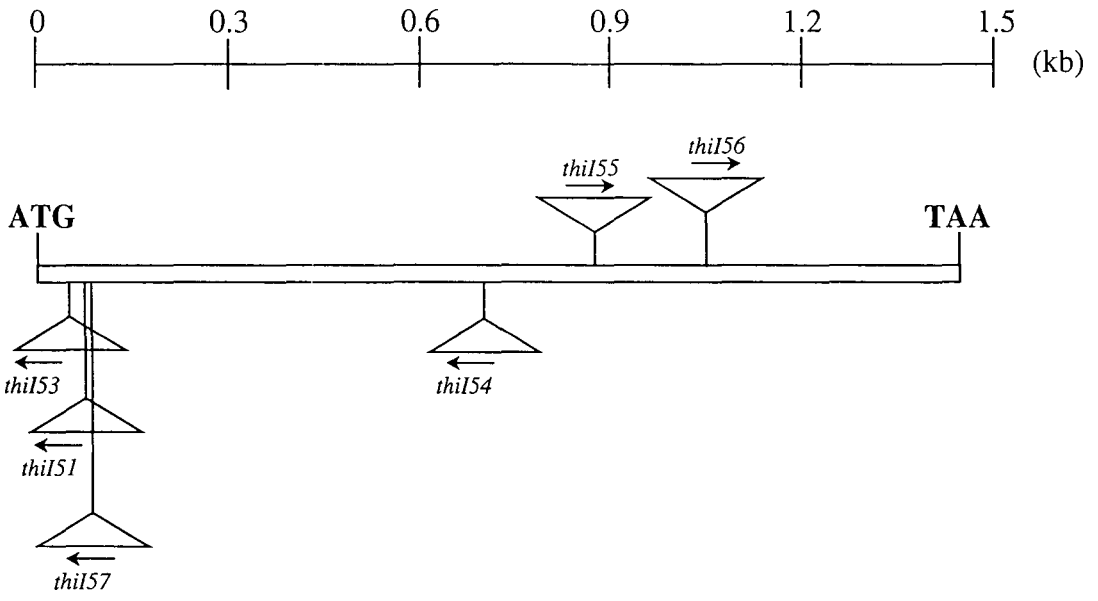


Fig. 4.10 Schematic representation of the λp*lac*Mu55 insertions in the *thiI* gene. The inverted triangles represent the insertion sites in the *thiI* gene in GJ3533 (*thiI51*::Kan), GJ3539 (*thiI53*::Kan), GJ3543 and GJ3546 (*thiI54*::Kan), GJ3550 (*thiI55*::Kan), GJ3552 (*thiI56*::Kan) and GJ3553 (*thiI57*::Kan). The arrows represent the orientation of transcription of the *lacZ* gene in λp*lac*Mu55. Start (ATG) and stop (TAA) sites in the *thiI* coding region are shown.

4.6.2.1 Other phenotypes of *thiI* mutants:

A. Thiamin auxotrophy:

All the eight *thiI* mutants were thiamin auxotrophs consistent with the earlier published results.

B. NUV phenotype:

To test whether *thiI* mutants isolated in this screen have lost the NUV phenotype, *thiI*::Kan mutation was transferred into MG1655 strain which is *relA*$^+$ (the NUV phenotype is more pronounced in a *relA*$^+$ background). MG1655 and its *thiI* derivative (GJ4564) were irradiated with near-UV light, as mentioned in Section 2.15.7, for 40-60 min and then transferred into MA medium. There was a considerable lag when near-UV-irradiated WT (MG1655) cells were freshly subcultured (NUV phenotype) and such a lag was absent in the *thiI* mutant (GJ4564), consistent with earlier reports (Fig. 4.11).

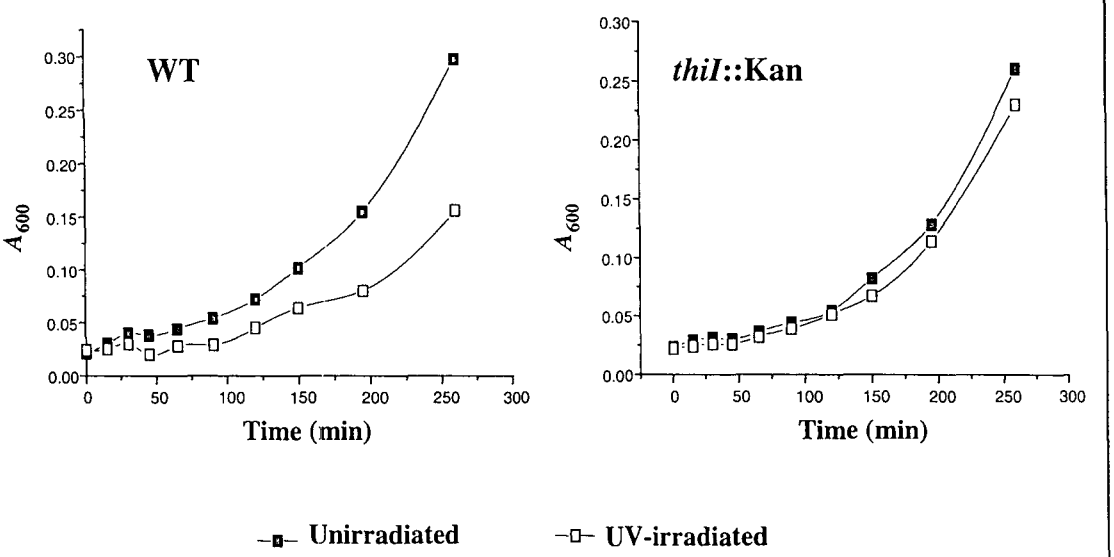


Fig. 4.11 NUV phenotype of the *thiI* mutant.

4.6.2.2 Measuring s^{4}U levels in the *thiI* mutant:

Since s^{4}U is the chromophore implicated in NUV phenotype, it was decided to test for the presence of s^{4}U in the *thiI* mutant (GJ4533). tRNA was isolated according to the procedure described in Section 2.20 of Chapter 2. Absorption spectra of the tRNA samples was measured from 300-400 nm. WT cells (MC4100) had a characteristic broad absorption band with its peak around 334 nm, because of absorption by s^{4}U and this was absent in a *thiI* mutant, suggesting the absence of s^{4}U modification in the latter (Fig 4.12).

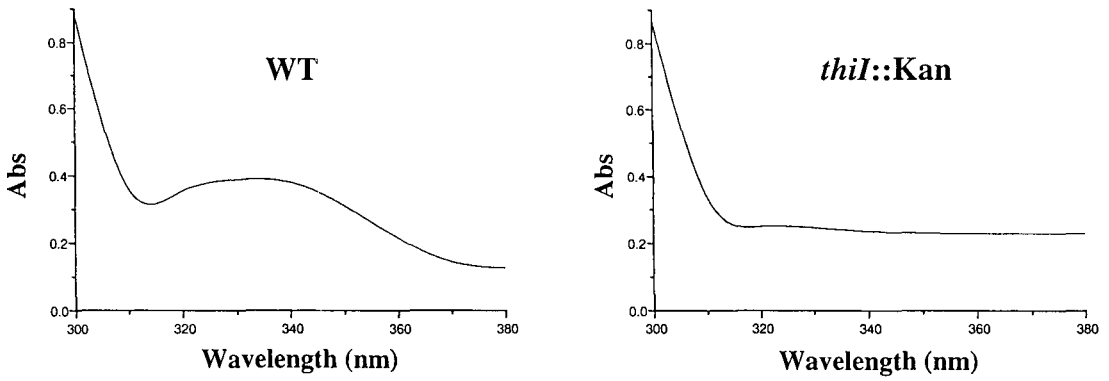


Fig. 4.12 tRNA absorption spectra of *thiI* mutant. For s^4U, λ_{max} = 334 nm

4.6.2.3 Testing previously described *thiI* alleles for osmosensitivity:

Two different *thiI* mutant alleles obtained from other sources were also tested for their association with osmosensitivity. Strain VJS2890 contains the *thiI*::Kan allele, which was transferred to MC4100 (WT) and GJ4652 (Δ*gltBD*) by P1 transduction; the resulting Kan^R colonies, designated as GJ4741 and GJ4742 respectively, were tested for growth on high osmolarity media and thiamin auxotrophy. All the Kan^R colonies were sensitive to the different dissolved solutes and were also thiamin auxotrophs.

HB117 *nuvC* has a point mutation in the *thiI* (previously known as *nuvC*) gene, which was transferred to MC4100 (WT) and GJ4652 (Δ*gltBD*) in the following manner. In the first step, *tsx-247*::Tn*10* which is located near *thiI* was introduced into HB117 *nuvC* and one resultant colony which was still a thiamin auxotroph was selected and designated as GJ4752. A P1 lysate grown on GJ4752 was then used to transduce MC4100 and GJ4652 to Tet^R and the resultant colonies were screened for thiamin auxotrophy. One colony each from the above transductions which was thiamin auxotroph was designated GJ4754 (*thiI*) and GJ4756 (*thiI* Δ*gltBD*). GJ4754 and GJ4756 were tested for their ability to grow on low a_w media. The results show that they behaved similar to GJ2561 (Δ*gltBD thiI*::Kan) and GJ4533 (*thiI*::Kan) that is, they were osmosensitive (Table 4.12).

Table 4.12 Osmosensitivity of various *thiI* mutants[a]

Strain	Genotype	MA + Bet		
		NaCl (0.6-0.7 M)	Glucose (0.9-1.0 M)	Sucrose (0.8-0.9 M)
MC4100	WT	+++	+++	+++
GJ4652	Δ*gltBD*	++	++	++
GJ4533	*thiI*::Kan	±	±	±
GJ4659	*thiI*::Kan *gltBD*	–	–	–
GJ4741	*thiI*::Kan	±	±	±
GJ4742	*gltBD thiI*::Kan	–	–	–
GJ4754	*thiI*	±	±	±
GJ4756	*thiI* Δ*gltBD*	–	–	–

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, glucose or sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 4-point scale (in increasing order): — (no growth), ±, ++ and +++ (full growth).

4.6.2.4 Cloning of WT *thiI*$^+$ from Kohara library and complementation analysis:

A 5.9 kb *Sal*I fragment carrying the *thiI* gene [of phage λ147 of the ordered library of Kohara et al (1987)] was cloned into pCL1920 in both orientations to generate the recombinant plasmids pHYD910 (Orientation I) and pHYD911 (Orientation II) (Fig. 4.13). Plasmid pHYD912 was generated from pHYD911 by *Pst*I digestion followed by recircularization. This results in a 2.8 kb insert which is expected to encode a ThiI gene product deleted for 18 amino acids in the carboxy terminal domain (CTD). This 18 amino acid CTD had previously been reported to be essential for *thiI* function in *S. enterica* (Webb *et al.*, 1997), but another group had reported that it is dispensable for thiamin and NUV phenotypes in *E. coli* (Mueller *et al.*, 1998). All the three plasmids were introduced into *thiI recA* mutants and the resultant transformants were examined for complementation of various phenotypes.

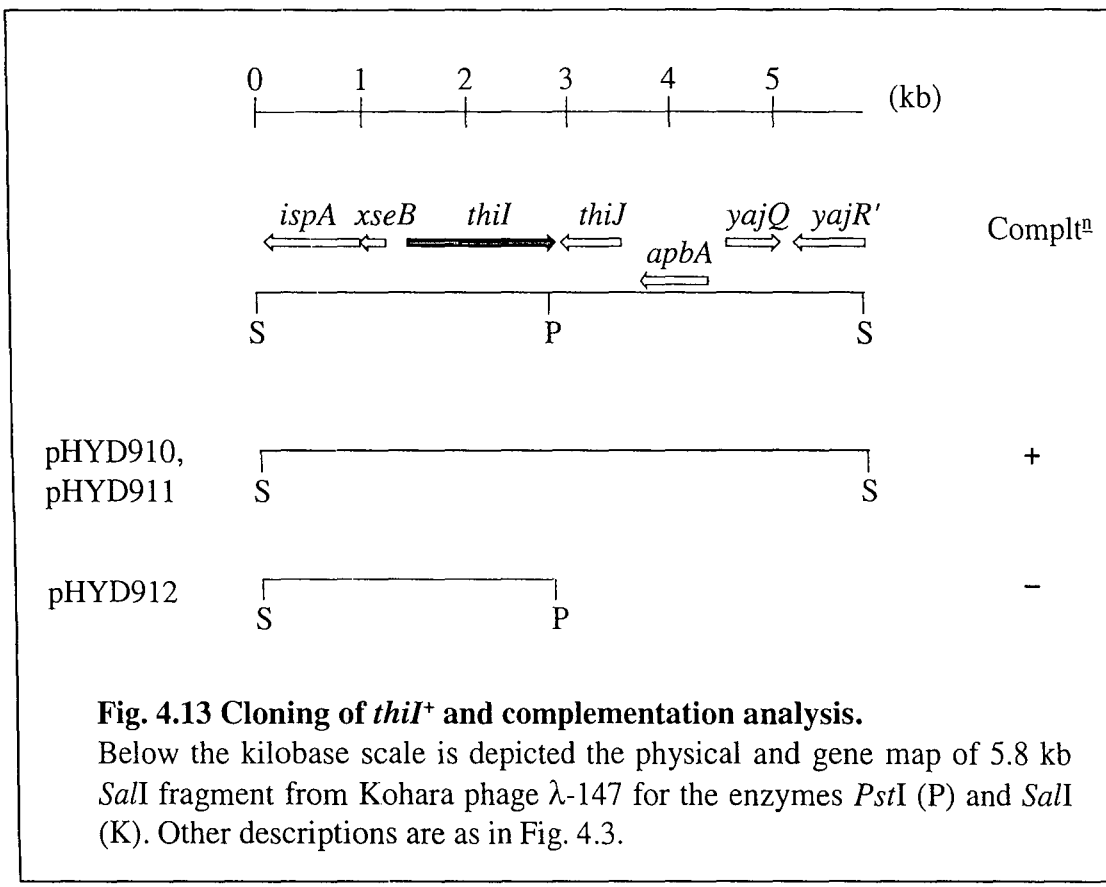


Fig. 4.13 Cloning of *thiI*$^+$ and complementation analysis.
Below the kilobase scale is depicted the physical and gene map of 5.8 kb *Sal*I fragment from Kohara phage λ-147 for the enzymes *Pst*I (P) and *Sal*I (K). Other descriptions are as in Fig. 4.3.

The 5.9 kb insert, in either orientation, was able to complement the thiamin auxotrophy, osmosensitivity and NUV-phenotypes (Table 4.13). The results with pHYD912 established that the 18 amino acid-CTD-deleted ThiI protein is unable to complement the *thiI* mutant for any of its phenotypes.

Table 4.13 Complementation of *thiI*::Kan mutations by *thiI*$^+$ for various phenotypes

Strain (genotype)[a]	Plasmid (genotype)	Thiamin phenotype[b]	MA Bet Spec + NaCl[c]	NUV phenotype[d]
GJ4584 (WT)	pCL1920 (vector)	+	+++	+
GJ4585 (*thiI51*)	pCL1920 (vector)	–	–	–
GJ4585 (*thiI51*)	pHYD910 (*thiI*$^+$)	+	+++	+
GJ4585 (*thiI51*)	pHYD911 (*thiI*$^+$)	+	+++	+
GJ4585 (*thiI51*)	pHYD912 (*thiI'*)	–	–	–

[a] All strains are *recA*$^-$ derivatives.
[b] + and – represent the ability or inability to complement thiamin auxotrophy.
[c] The indicated strains for osmosensitivity were streaked on MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl and incubated for 42-48 h at 37°C. — represents no growth, while +++ represents full growth.
[d] + and – represent ability to complement the NUV phenotype.

4.6.3 Discussion:

Loss-of-function mutations in *thiI* were shown to confer an osmosensitivity phenotype in *E. coli*. These mutants, as expected, also exhibited thiamin auxotrophy and NUV phenotype. Complementation analysis showed that the C-terminal 18 amino acid region of ThiI is essential for its function. Since ThiI in *E. coli* is proposed to contain a C-terminal extension with sequence similarity to rhodanese-like sulfurtransferases (Palenchar *et al.*, 2000), these 18 amino acids may form part of such a sulfurtransferase motif.

Possible roles of *thiI* in conferring water-stress tolerance:

In order to explain the osmosensitivity of the *thiI* mutant, the following hypotheses can be considered.

(a) ThiI acts as an intermediate in the sulfur-transfer reaction during thiazole moiety formation and s^4U modification. ThiI transiently accepts the sulfur from Cys via IscS and then transfers it to the substrate. It has been shown that strains defective in *iscS* have reduced activities for proteins containing [4Fe-4S] clusters (e.g. aconitase B, 6-phosphogluconate dehydratase, glutamate synthase [GOGAT], fumarase A, FNR, NADH-dehydrogenase I and succinate dehydrogenase; Schwartz *et al.*, 2000). It is possible that ThiI plays an accessory role in the formation of [4Fe-4S] clusters in some of the above and also in other hitherto unidentified proteins. Absence of the ThiI protein might drastically decrease their biological activities, one or more of which may specifically be required for survival in low a_w medium, and hence the *thiI* strain is osmosensitive. (Here, ThiI may be acting in the *de novo* synthesis of the Fe-S clusters.)

(b) Apart from the inability of *thiI* strains in *de novo* Fe-S cluster assembly, another additional complication in these strains growing on low a_w media would be to cope with oxidative damage to such clusters. This argument is based on the assumption that oxidative damage may be a consequence of low a_w stress in aerobic conditions. Under such conditions, the labile Fe-S clusters are damaged which, if unrepaired, would be detrimental to the cell. (Here, ThiI may be acting in the repair of Fe-S clusters.)

(c) The third hypothesis envisages tRNAs themselves as having a prominent role in the cellular hyperosmolar adaptational pathway. 65-70% of the total tRNAs are usually thiolated at U and the thiolated tRNAs are thought to be essential for bacterial NUV stress response. A similar response may be needed for the cells to survive in low a_w stress, in the

absence of which the growth of the cells is inhibited. Alternatively, thiolated tRNAs themselves may bind to some cellular factors and regulate gene expression. Thus, in a *thiI* mutant, the absence of thiolated tRNAs may result in the deficiency of gene function(s) needed for water stress adaptation.

Results from a recent study (Urbonavicius *et al.*, 2001) show that several structurally different modified nucleosides share a common function namely, that they all serve to improve reading frame maintenance. At the same time, the vastly different chemical structures and their presence in different tRNAs (in different locations), and their influence on different reactions in which tRNAs participate suggest that the various nucleoside modifications may also have discrete and specific functions. For example, *tgt* (which encodes tRNA guanosine transglycosylase involved in the epoxy-Q modification of G at position 34) and *miaA* (involved in the generation of ms^2i^6A at position 37) mutants of *Shigella flexneri* were shown to have reduced levels of VirF, the virulence-related transcriptional regulator (Durand *et al.*, 2000). It was suggested that the primary result of epoxy-Q and ms^2i^6A undermodification of the tRNA is the poor translation of *virF* mRNA but not of any other mRNA, whose product acts downstream of the action of VirF. In a similar way, it is therefore plausible that in a *thiI* mutant, defective translation of a particular mRNA may lead to the absence of a critical regulatory protein, resulting in NaCl-sensitive phenotype.

In an unrelated study, insertion mutations in the *miaA* gene, involved in tRNA modification, increased the spontaneous mutation frequency (Connolly and Winkler, 1989). A model was proposed according to which tRNA with ms^2i^6A undermodification acts as a physiological switch to increase spontaneous mutation frequency as a cellular response to certain environmental stress conditions that do not involve exposure to mutagens. Near UV-mediated DNA damage induces SOS response which is dependent on s^4U (Caldeira de Araujo and Favre, 1986; Garcia *et al.*, 1986). It is known that osmotic stress also induces DNA damage (Kultz and Chakravarty, 2001). Thus, under low a_w stress it could be hypothesized that DNA damage induces SOS response in a *thiI*$^+$ but not *thiI* mutant strain, resulting in the NaCl-sensitivity of the latter.

4.7 Characterization of the *speC* mutant:

4.7.1 Introduction

A detailed introduction to *speC* has been presented in Chapter 1. In brief, *speC* encodes a 732 amino acid-long ornithine decarboxylase involved in polyamine biosynthesis which converts Orn to Put.

One of the NaCl-sensitive mutants isolated in the initial screen for null mutants defective for growth in high NaCl-containing medium had Kan^R insertion in the *speC* gene. Inverse PCR followed by sequencing revealed that the strain GJ3542 (*speC100*::Kan) had harbored a Kan^R insertion in *speC*, 2160 nucleotides after the ATG translation start site of the 2.2 kb long *speC* gene and the orientation is such that *lac* is inverse to the direction of *speC* transcription.

4.7.2 Results:

4.7.2.1 Testing other *speC* alleles for osmosensitivity phenotype:

As described in Chapter 3, GJ4542 (*speC*) and GJ4662 (Δ*gltBDF speC*) strains carrying the *speC* insertion mutation obtained in this study are sensitive to ionic impermeable solutes like NaCl, KCl, K_2SO_4, and to nonionic impermeable solutes like glucose and sucrose.

GJ1380 [Δ(*speC-glc*)*63*] and GJ1293 (*speC3*) containing previously described *speC* alleles were examined for their growth inhibition on low a_w medium imposed by various dissolved solutes. The results indicate that these mutant alleles of *speC* behave similar to *speC*::Kan and that the resultant strains are growth-inhibited to an equivalent extent as GJ4542 on media of high osmolarity (Table 4.14).

Table 4.14 Growth of different *speC* mutants at high osmolarity[a]

Strain	Genotype	MA Bet		
		NaCl (0.6-0.7 M)	Glucose (0.9-1.0 M)	Sucrose (0.7-0.8 M)
MC4100	WT	+++	+++	+++
GJ4652	*gltBD*	++	++	++
GJ4542	*speC100*::Kan	+	+	+
GJ4662	*speC100*::Kan *gltBD*	±	±	±
GJ1380	Δ(*speC-glc*)63	+	+	+
GJ1293	*speC3*	+	+	+

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl, 0.9-1 M glucose or 0.8-0.9 M sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 4-point scale (in increasing order): ±, +, ++ and +++ (full growth).

4.7.2.2 Conditions that suppress water-stress sensitivity in *speC*:

(a) Effect of Arg, Cit and Orn:

It was earlier reported from the lab (UmaPrasad and Gowrishankar, 1998) that on media of low a_w containing uracil, addition of Arg and Cit suppressed and Orn accentuated the growth sensitivity phenotype of the *speC* mutant. The three compounds were therefore tested for their ability to affect the growth of the *speC*::Kan mutant obtained in the present screen on high osmolarity media without uracil. The results summarized in Table 4.15 show that addition of exogenous Arg and Cit relieved the growth inhibition of *speC* mutants on low a_w media. However, Orn also had a slight growth promoting ability under the present conditions. Arg was the best among these compounds in promoting growth while Orn was the least.

(b) Effect of polyamines:

Given that the intermediates in the Arg biosynthetic pathway ameliorated the osmosensitivity of the *speC* mutant, it was decided also to check the effect of polyamines on its growth phenotype. Put and Spd were added at 1 mM concentration to the media of high osmolarity. The results (Table 4.15) showed that exogenous Put and Spd reversed the growth inhibition of the *speC* mutant.

Table 4.15 Effect of Arg, Cit, Orn, Put, Spd and *argR* mutation on growth of *speC* mutant in high osmolarity medium[a].

Strain	Genotype	MA + Bet + NaCl[b]					
		–	Arg	Cit	Orn	Put	Spd
MC4100	WT	+++	+++	+++	+++	+++	+++
GJ4631	*argR64*	+++	+++	+++	+++	+++	+++
GJ4542	*speC*::Kan	±	+++	+++	++	+++	+++
GJ4560	*speC*::Kan *argR64*	+++	+++	+++	+++	+++	+++

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): ±, ++ and +++ (full growth).
[b] Similar results were obtained when glucose (0.9-1.0 M) or sucrose (0.8-0.9 M) was used to impose water stress.

4.7.2.3 Effect of *argR* mutation on osmosensitivity phenotype of *speC*:

Since Arg and the intermediates in the Arg biosynthetic pathway (Cit and Orn) and polyamines (Put and Spd) derived from Arg, when added exogenously promoted growth of the *speC* mutant on high osmolarity plates, it was decided to examine the effects, if any, of derepression of Arg biosynthetic pathway. *argR* mutations are known to derepress the pathway, resulting in increased pools of Arg and intermediates in the cell.

argR derivatives of *speC* mutants were constructed in the following manner. A P1 lysate prepared on GJ901 (*argR*$^+$ with a *zha-6*::Tn*10* marker linked around 75% to *argR*$^+$) was used to transduce MA1034 (*argR64*) to Tet^R. The Tet^R transductants were screened for *argR* phenotype, by streaking on MA plates containing uracil (40 μg/ml) and Can (65 μg/ml) (see Section 2.15.5 of Chapter 2). As expected, around 25% of the transductants retained the *argR64* mutation. The *argR64* mutation was thus tagged with a linked Tn*10* marker. One such colony was designated GJ4559.

In the next step, a P1 lysate prepared on GJ4559 (*argR* linked to Tn*10*) was used to transduce GJ4542 (*speC*::Kan) to Tet^R. The resulting Tet^R colonies were screened for *argR* phenotype as described in Section 2.15.5 of Chapter 2. One such *argR64* strain was selected for further work and christened GJ4560. The *argR* derivative of *speC* was then

tested for its ability to grow on high osmolarity media. The results (Table 4.15) show that *argR* completely abolished the growth sensitivity of the *speC* mutant as seen by the comparable growth abilities of GJ4560 (*argR speC*) and GJ4631 (*argR*).

4.7.2.4 Complementation studies:

In order to check whether the *speC*$^+$ gene would complement for the growth inhibition observed in the *speC*::Kan strain, plasmid pODC, containing the *speC*$^+$ gene cloned at the *Pst*I site of pBR322 (Boyle *et al.*, 1984), was introduced into GJ4542. The transformants were streaked on various media containing different dissolved solutes. pODC complemented the mutant strains for all the phenotypes examined, demonstrating that it is the *speC* deficiency that resulted in the observed growth inhibition.

4.7.3 Discussion

Mutations in *speC* gene which encodes the enzyme ornithine decarboxylase catalyzing the conversion of Orn to Put, resulted in osmosensitivity. The growth inhibition of the *speC* mutant was relieved by supplementation with Arg, Cit or Orn, as also by introduction of an *argR* mutation. The *argR speC*$^+$ strain was also able to cross-feed *argR*$^+$ *speC* for restoration of water-stress tolerance. Based on the above observations, it can be argued that increase in the cellular Arg pools alleviates the water-stress sensitivity of a *speC* mutant. It is worth mentioning here that in a screen for extragenic suppressors of *speC* for osmosensitivity, several were shown to have *argR* mutations (see Section 6.3.2.4, Chapter 6).

Addition of Put and Spd also alleviated the osmosensitivity of the *speC* mutant. This result was somewhat unexpected (although it was in agreement with an earlier published observation from the lab [UmaPrasad and Gowrishankar, 1998]), because previous reports had suggested that upon osmolarity shock, *E. coli* excretes Put, which has been explained as an attempt by the cells to balance the internal ionic strength resulting from K^+ uptake under such conditions (Munro *et al.*, 1972).

One model which can explain the above observations is that *E. coli* needs increased Put / Spd pools under osmotic stress conditions. It has been shown that K^+ accumulation occurs concomitantly with the excretion of polyamines. One polyamine molecule with two positive charges is extruded for accumulation of two K^+ ions, thereby maintaining electrical neutrality even as the intracellular osmolarity is increased for turgor restoration. Because of the defect in polyamine synthesis in *speC* mutants, K^+

accumulation may be compromised leading to osmosensitivity. Increased Arg pools may be used for the conversion of Arg to polyamines through the alternate *speAB* pathway, thus suppressing the osmosensitivity phenotype of *speC*.

4.8 Effect of multiple copies of WT genes in various NaCl-sensitive mutants:

In order to examine whether the presence of WT genes in multiple copies in non-cognate mutant background would affect the osmotolerance ability, the following experiment was undertaken. Plasmids pHYD924 (pBR329 with *argP*$^+$), pHYD923 (pBR329 with *thiI*$^+$) and pHYD918 (pBR329 with *speC*$^+$) were introduced into WT, *argP gltBD* (GJ4654), *glnE gltBD* (GJ4663), *thiI* (GJ4553), *lysC* (GJ4537) and *speC* (GJ4542) strains. The results showed that the above plasmids:

(i) complemented the respective mutants for osmosensitivity phenotype, but did not increase the tolerance levels more than the WT levels;

(ii) did not complement the strains in a non-cognate mutant background. There was hence no cross-protection of mutants for osmosensitivity phenotype, suggesting that the corresponding genes act in independent pathways in conferring osmoadaptation in *E. coli*.

Chapter 5

Role of *argP* in osmosensitivity and arginine metabolism

5.1 Introduction:

As described in Chapter 3, null mutations in *argP* / *iciA*, which confer osmosensitivity phenotype in a *gltBD* mutant background, were obtained in a screen for NaCl-sensitive mutants following transposon-mutagenesis. A detailed description of previous work by other groups on *argP* / *iciA* has been given in Chapter 1. Briefly, *argP* has previously been described by two different groups to be involved in Arg transport and in the initiation of chromosome replication in *E. coli*.

The present chapter describes the novel phenotypes of loss-of-function (null) and gain-of-function mutations in *argP*. The results demonstrate that ArgP acts as a transcriptional activator of a previously uncharacterized ORF, known as *yggA*, and are consistent with the hypothesis that the ArgP protein, through its action on *yggA*, is involved in Arg metabolism in *E. coli*. Null mutations in *argP* and *yggA* conferred a Can^{SS} (i.e., supersensitivity) phenotype. The *argP* null mutations in combination with *gltBD* mutation were shown to confer osmosensitivity. The data suggest that *argP* is also involved in *gltBD*-independent NH_4^+ assimilation in *E. coli* and that its deficiency in a *gltBD* mutant background confers osmosensitivity through an inability to meet the demand for increased Glu synthesis. Addition of exogenous Lys to $argP^+$ strains made them phenotypically $argP^-$, suggesting that Lys supplementation might, directly or indirectly, lead to inhibition of ArgP function inside the cell. The physiological relevance of the antagonistic effects of two basic amino acids (Arg and Lys) on ArgP function in *yggA* gene regulation, NH_4^+ assimilation, and the osmosensitivity phenotype are also discussed.

Results:

5.2 Phenotypic characterization of *argP*

5.2.1 Characterization of *argP* mutants:

As described in Section 3.2.8 of Chapter 3, two independent insertions were obtained in *argP* which conferred osmosensitivity in *E. coli*. The *argP*::Kan insertion by itself did not confer water-stress sensitivity (strains GJ4536 and GJ4540) but it conferred sensitivity to various dissolved solutes when combined with the *gltBD* mutation (GJ4654 and GJ4655; Table 5.1). However, the difference was that the *argP202 gltBD* strain (GJ4654) was more osmosensitive than the *argP203 gltBD* strain (GJ4655), suggesting

that in the latter, there may be residual background transcription of *argP* from a fortuitous promoter created by Mu *c* end.

Table 5.1 Growth of *argP*::Kan mutants at high osmolarity[a]

Strain	Genotype	MA + Bet		
		NaCl (0.6-0.7 M)	Glucose (0.9-1 M)	Sucrose (0.8-0.9 M)
MC4100	WT	+++	+++	+++
GJ2529	Δ*gltBD*	++±	++±	++±
GJ4536	*argP202*::Kan	+++	+++	+++
GJ4540	*argP203*::Kan	+++	+++	+++
GJ4654	*argP202*::Kan Δ*gltBD*	—	—	—
GJ4655	*argP203*::Kan Δ*gltBD*	±	±	±

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with 0.6-0.7 M NaCl, 0.9-1 M glucose or 0.8-0.9 M sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 4-point scale (in increasing order): — (no growth), ±, ++±, and +++ (full growth).

The sequencing results indicated that GJ4654 carried the Kan^R insertion in *argP* 99 nucleotides downstream of the predicted ATG start site (designated *argP202*::Kan). GJ4655 has the Kan^R insertion 19 nucleotides upstream of the predicted ATG start site, that is, between the predicted promoter sequences and the ATG translation start site for the *argP* gene (designated *argP203*::Kan; Fig. 5.1).

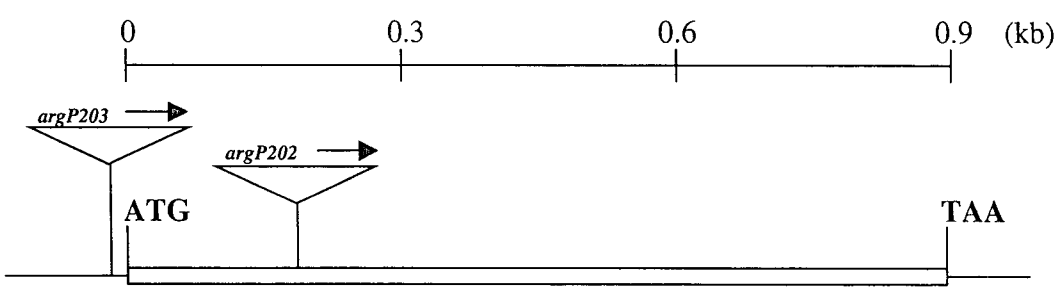


Fig. 5.1 Schematic representation of the λp*lac*Mu55 insertions in the *argP* gene. The inverted triangles represent the insertion sites in the *argP* gene in GJ3536 (*argP202*::Kan) and GJ3540 (*argP203*::Kan). The arrows represent the orientation of transcription of the *lacZ* gene in λp*lac*Mu55. Start (ATG) and stop (TAA) sites in *argP* coding region are shown.

5.2.2 Effect of Bet on osmosensitivity of *argP gltBD* mutant:

The *gltBD argP202* strain (GJ4654) when streaked on MA plates containing NaCl (0.4–0.5 M), glucose (0.6–0.8 M), or sucrose (0.5–0.7 M) without Bet supplementation did not exhibit a marked osmosensitivity when compared with the *argP*$^+$ *gltBD* (GJ4652). However, on MA plates containing 1mM Bet with NaCl (0.6 – 0.7 M), glucose (0.9 – 1.1 M) or sucrose (0.8 – 1.0 M), the *argP gltBD* double mutant grew more poorly than either *gltBD* or *argP* single mutants i.e., *argP gltBD* mutant exhibited severe osmosensitivity in the presence of Bet than when tested without Bet. This result was analogous to the previously described osmosensitivity of the *proU proP* double mutant (that is deficient in both Bet transporters, ProU and ProP), wherein the double mutant was osmosensitive only in the presence of Bet (at higher concentrations of NaCl) but was no different from WT without Bet (Gowrishankar, 1985). This result suggested that *gltBD argP* may behave like the *proU proP* mutant. Since the *proU* and *proP* loci (as described in Section 1.1.6 of Chapter 1) are involved in transport of the compatible solutes Pro and Bet, one possibility considered was that *proU,* or *proP,* or both may fail to be expressed in an *argP gltBD* mutant.

To investigate whether *argP* regulates the *proU*::*lacZ* and *proP*::*lacZ* fusions, the following approach was taken. GJ134 (*proU*::*lacZ* and Ura$^-$ due to the deletion of the *pyr* locus) was transduced to Ura$^+$ by employing the P1 lysate grown on WT strain, MG1655. Ura$^+$ colonies would have crossed out the *pyr* deletion. The *argP*::Kan mutation was introduced into this strain using the P1 lysate prepared on GJ4820 (*argP*::Kan, in which the *lacZ* gene within λp*lac*Mu55 has been inactivated by a Tet insertion), and the resultant strain was designated GJ4873 (*argP proU*::*lac*). The β-galactosidase specific activity of this strain was measured, as described in Chapter 2, in the presence and absence of 0.2 M NaCl Bet. The results are summarized in Table 5.2.

As can be gleaned from the results, the *argP* mutation did not alter the osmoinducibility of the *proU*::*lacZ* expression significantly. Thus, it appears unlikely that *argP* has a direct role in controlling its expression.

Table 5.2 Effect of *argP* mutation on *proU*::*lac* expression[a]

Strain	Genotype	β-gal activity	
		0	0.2 M NaCl Bet
GJ4836	*proU*::*lacZ argP*$^+$	2	370
GJ4873	*proU*::*lacZ argP*	1	355

[a] Isogenic *argP*$^+$ and *argP* strains containing *proU*::*lacZ* fusion were grown in half-strength MA medium without NaCl (0) and 0.2 M NaCl + 1 mM Bet at 37°C to mid-exponential phase (A_{600} ~0.6 – 0.8) for β-galactosidase assays. Enzyme specific activity values are reported in Miller Units (Miller, 1992).

The effect of *argP* mutation on *proP*::*lacZ* expression was studied by transducing the *argP*::Kan (with *lacZ*::Tet) mutations, as done above, into GJ184 (*proP*::*lacZ*). The resultant transductants were streaked on MA Xgal ± NaCl Bet and MacConkey ± NaCl Bet plates. There was no discernible difference in their β-galactosidase expression (as determined by the intensity of the color on the indicator plates) compared to the *argP*$^+$ *proP*::*lacZ* strain (GJ184), suggesting that the *proP*::*lacZ* fusion also was not affected by *argP* mutation. Thus, *argP* may not act through Pro/Bet uptake in conferring osmosensitivity.

5.2.3 *argP gltBD* mutant has compromised NH_4^+-assimilation phenotype:

As described in Chapter 1, NH_4^+-assimilation and Glu biosynthesis are intimately connected, and a defect in NH_4^+-assimilation would be reflected in the biosynthesis of Glu. In a *gltBD* mutant, one of the primary ways of Glu biosynthesis (GOGAT, encoded by the genes *gltBD*) is absent, while the remaining glutamate dehydrogenase (GDH) pathway is efficient in NH_4^+-assimilation only in media with ≥ 1 mM NH_4^+.

In order to investigate the degree of severity in NH_4^+-assimilation in the *gltBD argP* mutant, this strain was tested for its ability to grow on W-salts supplemented with varying $(NH_4)_2SO_4$ concentrations, along with other control strains. The results are summarized in Table 5.3 and show that the *argP gltBD* strain is severely crippled for NH_4^+ assimilation (exhibiting poor growth even at 2 mM). The *argP* single mutant is not

different from the WT, whereas the *gltBD* single mutants are slightly defective (as described earlier and in Chapter 4).

Table 5.3 Growth of the *argP gltBD* mutant on low-ammonia medium[a]

Strain	Genotype	W-salts + NH_4^+ (mM)				
		0	0.3	0.6	1.0	2.0
MC4100	WT	++	+++	+++	+++	+++
GJ2529	*gltBD*	—	±	+	++	++
GJ4652	*gltBD*	—	±	+	++	++
GJ4536	*argP202*	++	+++	+++	+++	+++
GJ4654	*argP202 gltBD*	—	—	—	—	+
GJ4663	*glnE gltBD*	—	—	±	+	++

[a] The indicated strains were streaked on W-salts plates supplemented with varying concentrations of $(NH_4)_2SO_4$ and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): — (no growth), ±, +, ++, and +++ (full growth).

When compared to the *gltBD glnE* mutant (described in Chapter 4), the *gltBD argP* mutant is more severely affected in NH_4^+ assimilation on W-salts medium, which is also reflected on high osmolarity plates wherein *argP gltBD* mutant is more sensitive than *glnE gltBD*.

Consistent with the finding that the *argP gltBD* mutant was compromised for growth on W-salts media with low [NH_4^+] was the observation that the strain also grows poorly on one-third strength MA medium. (Since full strength MA has 15 mM [NH_4^+], the one-third strength medium has 5 mM). The poor growth was alleviated by additional supplementation of 4-6 mM NH_4^+ to the one-third strength medium.

5.2.4 Asp-mediated alleviation of osmosensitivity in *argP gltBD*:

Asp is known to alleviate the NH_4^+-assimilation defect of the *gltBD* mutant by directly feeding into Glu. When Asp effect on high osmolarity plates was tested, the result was a dramatic increase in tolerance specifically of *argP gltBD* mutant whereas MC4100,

gltBD and *thiI* strains were only marginally growth-promoted. The *argP gltBD* mutant which stops growth at MA NaCl Bet (0.5 M) was able to grow at MA NaCl Bet (0.7 M) in the presence of 1mM Asp i.e., there was >0.2 M NaCl (or equiosmolar glucose and sucrose) increase in the growth-ability of the strain. At the same time, Asp promoted the growth of MC4100, *gltBD* and other strains by <0.1 M NaCl.

On the other hand, other small molecular weight substances that were tested, including Arg, Cit, Lys and Orn, which are postulated to be either substrates of LAO-bp mediated transport or intermediates in the Arg / polyamine biosynthetic pathway (Cit, Orn, Put, Spd) promoted the growth of the *gltBD argP* mutant and other strains like MC4100, *gltBD, thiI* etc marginally. This suggested that their modest growth-promoting effect was probably nonspecific since it was observed for other strains tested.

5.2.5 Effect of multicopy *spoT*$^+$ on NH_4^+ assimilation and osmotolerance in *argP gltBD* mutants:

As described in Section 1.2.2.4 of Chapter 1, work published earlier from the lab (Saroja and Gowrishankar, 1996) had suggested that a *gltBD fnr* mutant is compromised for both osmotolerance and growth on low NH_4^+, and that multicopy *spoT*$^+$ is able to suppress both phenotypes. The suppression of growth sensitivity was by bypassing of the need for GOGAT in low NH_4^+ growth medium. Since *argP gltBD* was also crippled for growth at low NH_4^+ and was osmosensitive, the effect of multicopy *spoT*$^+$ was tested. Plasmids pBR329 (vector) and pHYD809 (vector with 7.8 kb insert fragment spanning *spoT* region) were introduced into *argP gltBD* and *gltBD* strains and the resultant Tet^R Cm^R transformants were tested for their ability to grow on high osmolarity plates and on W-salts medium supplemented with low NH_4^+. The results showed that multicopy *spoT*$^+$ did not suppress either the osmosensitivity or the NH_4^+ assimilation defect in *argP gltBD* mutant strain.

5.2.6 Effect of other NaCl-sensitive mutations on osmotolerance of *argP gltBD* strain:

To examine whether other NaCl-sensitive mutations affect the ability of *argP* strains (with and without the *gltBD* mutation) to grow on high osmolarity media, double and triple mutants were constructed in the following manner.

5.2.6.1 Construction of *argP speC* double mutant:

Strain GJ1380 [Δ(*speC-glc*)*63*] was transduced to Kan^R by P1 transduction employing the P1 lysate prepared on *argP202*::Kan strain (GJ4536). The resultant Kan^R strain was designated GJ4571 (*argP speC*).

5.2.6.2 Construction of *argP thiI* double mutant:

The *argP202*::Kan strain was transduced to Tet^R employing P1 lysate grown on GJ4572 (*thiI*::Kan linked 50% to *tsx*::Tn*10*). The resultant Tet^R transductants were screened for thiamine auxotrophy (and therefore, for inheritance of *thiI*::Kan), and one such colony was selected for further work and designated GJ4578 (*argP thiI*).

5.2.6.3 Construction of *argP gltBD lysC* triple mutant:

To construct the triple mutant, a P1 lysate prepared on GJ4736 (*lysC*::Kan linked ~90% to Cm^R) was used to transduce GJ4654 (*argP gltBD*) to Cm^R. The resultant Cm^R colonies were then screened for inheritance of *lysC*::Kan by checking their ability to grow on MA supplemented with Thr and Met at 40 μg/ml concentrations. (A *lysC* mutant strain would be Thr + Met-sensitive due to starvation for Lys, as described in Section 4.5.2.2 of Chapter 4.) One such Thr + Met-sensitive colony was selected for further work and numbered GJ4738 (*argP gltBD lysC*).

5.2.6.4 Construction of *argP gltBD glnE* triple mutant:

To construct the *argP gltBD glnE* triple mutant, a P1 lysate prepared on GJ4890 (*argP202*::Kan linked 30% to the Δ*dsbC*::Cm marker) was used to transduce GJ4663 (*gltBD glnE*) to Cm^R. The resultant Cm^R colonies were then screened for inheritance of *argP*::Kan by examining their growth on MA plates supplemented with 10 μg/ml Can (as described later in Section 5.2.7 of this Chapter, *argP* null mutants are Can-supersensitive). One such Can^{SS} derivative was selected for further work and named as GJ4891 (*argP gltBD glnE*).

5.2.6.5 Testing the osmosensitivity phenotypes of the double and triple mutants:

When the above double and triple mutant strains were tested for their growth on high-osmolarity media, as described in Section 2.15.2 of Chapter 2, the following results were obtained.

(i) *argP speC* double mutant was more growth-inhibited than either single mutant;

(ii) In the case of the *argP thiI* double mutant, additivity with respect to osmosensitivity phenotype could not be established conclusively;

(iii) *argP gltBD lysC* triple mutant was more crippled for growth than *argP, gltBD,* or *lysC* single mutants, as well as the *argP gltBD* or *lysC gltBD* double mutants;

(iv) Similarly, the *argP gltBD glnE* triple mutant was more growth-inhibited than *argP, gltBD* or *glnE* single mutants, as well as the *argP gltBD* or *glnE gltBD* double mutants.

The above results demonstrated that, in general, the *argP* mutation when combined with other $NaCl^S$ mutations identified in the present study confers an additivity of growth inhibition on media of high osmolarity.

5.2.6.6 Testing the NH_4^+ assimilation phenotype of the double and triple mutants:

For testing the additivity, if any, of the *argP* mutation with other mutations for growth on low NH_4^+ media, the above double and triple mutants were analyzed for their growth on W-salts + < 1mM NH_4^+. The *argP gltBD lysC* triple mutant grew somewhat slower than the *argP gltBD* or *lysC gltBD* strains, suggesting that *lysC* might have a minor effect on the NH_4^+-assimilation property. However, the triple mutant *argP gltBD glnE* was significantly more crippled for growth than either double mutant i.e., *argP gltBD* or *glnE gltBD*. This result suggested that *glnE* and *argP* do act additively in conferring a reduced NH_4^+-assimilation phenotype. When the other double mutants viz., *argP speC* or *argP thiI* were tested, they were no different from the single mutants or the WT strain for growth on low-NH_4^+ media.

5.2.7 Can phenotype of the *argP* null mutants:

Since previous work had implicated *argP* to be involved in Arg transport (Celis, 1973, 1990, 1999), the two *argP*::Kan mutants (GJ4654 and GJ4655) were tested for their sensitivity / tolerance to Can (the toxic analog of Arg) by comparing their growth with that of the WT on MA plates with and without Can at 40 μg/ml. The results showed that both mutants were Can-sensitive relative to WT. This was unexpected since, according to Celis' (1999) model, null insertions in a gene involved in Arg uptake are expected to give Can resistance. As discussed in Chapter 3, sequencing results showed that the Kan^R insertions in these two mutants differed in their insertion site, and therefore, had a small difference in their water-stress sensitivity phenotypes. It was therefore decided to check

whether they exhibit differential sensitivity towards Can also. It was also decided to check whether the *gltBD* mutation contributes to the Can phenotype.

Accordingly, GJ4654 (*gltBD argP202*::Kan), GJ4655 (*gltBD argP203*::Kan), GJ4536 (*argP202*::Kan) and GJ4540 (*argP203*::Kan) were streaked on MA plates containing varying concentrations of Can. The results are summarized in Table 5.4.

Table 5.4 Can sensitivity of *argP*::Kan mutants[a]

Strain	Genotype	MA + Can (μg/ml)						
		0	1	2	4	10	20	40
MC4100	WT	+++	+++	+++	+++	+++	+++	+++
GJ2529	Δ*gltBD*	+++	+++	+++	+++	+++	+++	+++
GJ4536	*argP202*::Kan	+++	+++	—	—	—	—	—
GJ4540	*argP203*::Kan	+++	+++	++	++	++	—	—
GJ4654	*argP202*::Kan Δ*gltBD*	+++	+++	—	—	—	—	—
GJ4655	*argP203*::Kan Δ*gltBD*	+++	+++	++	++	++	—	—

[a] The indicated strains were streaked on MA plates supplemented with 0, 1, 2, 4, 10, 20 and 40 μg/ml Can and incubated for 24 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): — (no growth), ++ and +++ (full growth).

Both the *argP*::Kan strains were Can-sensitive in a *gltBD*-independent manner, but they did differ in their sensitivity to Can. *argP202*::Kan (GJ4536 and GJ4654) was sensitive to ≥2 μg / ml of Can while *argP203*::Kan (GJ4540 and GJ4655) was sensitive to ≥20 μg /ml Can concentration. This result may be correlated with the difference in the water-stress sensitivity phenotype, wherein GJ3536 and GJ4654 (both *argP202*::Kan Δ*gltBD*) were more growth-inhibited than GJ3540 or GJ4655 (both *argP203*::Kan Δ*gltBD*), and may be explained on the ground that *argP202* is a total null for *argP* whereas *argP203* may exhibit a leaky expression of the *argP* gene (since the λp*lac*Mu55 insertion is upstream of the translation start site). Since both the *argP* mutants were more sensitive to Can than WT, the phenotype has been named as Canavanine-supersensitivity (Can^{SS}), to distinguish it from Can^{S} (Can-sensitive) of WT and Can^{R} (Can-resistant) of an *argR* mutant. The WT strain fails to grow at 1 mg/ml Can while the *argR* strain grows well even at this concentration.

In strains with the *argP* mutations, there was no difference between *gltBD*$^+$ and *gltBD*$^-$ backgrounds in their sensitivity to Can. This result demonstrates that the *gltBD* locus has no effect on the Can phenotype of *argP* alleles (compare GJ4654 with GJ4536, and GJ4655 with GJ4540), unlike the water-stress sensitivity phenotype wherein it was shown in Section 5.2.1, that *argP* by itself is not osmosensitive but when combined with *gltBD* mutation confers osmosensitivity phenotype. The above results also clearly show that loss-of-function mutations in *argP* confer Can^{SS}.

5.2.8 Effect of Arg, Cit, Orn and Lys on the Can^{SS} phenotype of *argP* mutants:

To examine whether the intermediates in the Arg biosynthetic pathway would affect the Can^{SS} phenotype of *argP* mutants and to test whether the presence of other basic amino acids like Lys and Orn (which are reported to share their transport systems with Arg) would affect the Can^{SS} phenotype, these mutants were streaked on MA glucose Can plates supplemented with various amino acids. It was observed that Arg, Cit, Orn or Lys (each at 40 μg/ml) reverse the Can^{SS} phenotype of *argP* strains.

5.2.9 Effect of *argR* mutation on Can^{SS} phenotype of *argP* mutants:

In the next set of experiments, the effect of *argR* mutation on the Can^{SS} phenotype of *argP* mutants was tested. As described in Section 1.3.1 of Chapter 1, *argR* encodes the ArgR repressor which, in the presence of the co-repressor Arg, is involved in repressing the genes of Arg biosynthetic pathway. *argR* mutations would lead to the derepression of the biosynthetic pathway, thereby increasing the intracellular Arg pools.

5.2.9.1 Construction of *argR* derivatives of *argP* mutants:

A P1 lysate prepared on GJ4559 (*argR64* linked to Tn*10*; described in Section 4.7.2.3) was used to transduce MC4100 to Tet^R. The Tet^R transductants were screened for *argR* phenotype, and one such colony that was selected for further work was designated GJ4631 (*argR64 zha-6*::Tn*10*). The *argP*::Kan mutations were then introduced into GJ4631 by transduction using P1 lysates made on GJ4536 (*argP202*::Kan) or GJ4540 (*argP203*::Kan), and selecting for Kan^R. The resulting *argP*::Kan derivatives of GJ4631 were designated GJ4617 (*argR64 argP202*::Kan) and GJ4618 (*argR64 argP203*::Kan).

5.2.9.2 Testing the *argR argP* mutant for Can^{SS} phenotype (tests of epistasis):

The *argR*$^+$ and *argR*$^-$ derivatives of *argP* mutants were examined for their Can phenotype on MA Can plate, as described above. The results are depicted in Table 5.5. The *argR* mutation completely abolished the Can^{SS} phenotype associated with *argP* mutants.

Table 5.5 Effect of *argR* mutation on the Can^{SS} phenotype of *argP* strain[a]

Strain	Genotype	MA + Can (μg/ml)	
		0	20
MC4100	WT	+	+
GJ4631	*argR64*	+	+
GJ4536	*argP202*::Kan	+	–
GJ4617	*argP202*::Kan *argR64*	+	+
GJ4540	*argP203*::Kan	+	–
GJ4618	*argP203*::Kan *argR64*	+	+

[a] The indicated strains were streaked on MA plates supplemented with Can (0 and 20 μg/ml). The plates were incubated for 24 h at 37°C. – (no growth), + (full growth).

When epistasis tests were carefully performed at higher Can concentrations, it was observed that *argP* decreased the Can tolerance levels of *argR* strains considerably. The rank order of growth (proceeding from most sensitive to most tolerant) of the above strains on MA + uracil + Can (400 μg/ml to 1000 μg/ml) was: *argP202* = *argP203* = *argP202 argR64* < WT << *argP203 argR64* ≤ *argR64*. (As explained in Section 2.15.5, addition of uracil sensitizes strains to Can, presumably by reducing carbamoyl phosphate levels needed for Arg synthesis.) The results show that the *argP202*::Kan null mutation has decreased the tolerance level of the *argR* strain considerably. The *argP203*::Kan mutation has (presumably) also decreased the tolerance limits of the *argR* strain but to a less extent than the *argP202*::Kan. The results were therefore, simply interpreted as suggesting that neither *argP* nor *argR* is epistatic over the other.

5.2.10 Arg cross-feeding ability of *argR* strain harboring *argP* null mutation:

By virtue of the fact that they are derepressed for Arg synthesis, *argR* strains excrete considerable amounts of Arg into the medium which can be visualized as a halo of growth of an Arg auxotroph seeded in the medium. Since *argP* is believed to affect the transport of Arg, it was decided to test whether the *argP* mutation would affect the Arg cross-feeding ability of an *argR* strain. The *argR*, *argP* and *argR argP* strains were spotted with an inoculation loop on a MA plate supplemented with tetrazolium chloride (indicator dye) and mixed with cells of an Arg-auxotrophic (*argE*) strain. The plates were incubated for 48 to 60 h at 37°C (Fig. 5.2). The results indicated that the *argP* mutation moderately decreased the cross-feeding ability of the *argR* strain (compare the halos around the *argR* and *argR argP* strains).

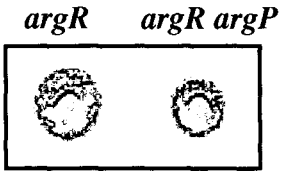


Fig. 5.2 Arg cross-feeding ability of *argR* and *argR argP* strains

5.2.11 Molecular cloning and complementation studies

5.2.11.1 Cloning of *argP*$^+$ gene from Kohara phage λ–471:

In order to initiate molecular genetic studies on *argP*, the *argP*$^+$ gene was cloned as follows: Kohara et al. (1978) have described the construction of an ordered library of the *E. coli* genome in λ phage, and phage λ–471 of this collection carries the *argP* locus. λ471 DNA was digested with *Bam*HI / *Kpn*I, and a 2.9 kb fragment which was expected to contain the *argP* sequence was cloned into *Bam*HI / *Kpn*I-digested pCL1920 (low copy number plasmid, 5 to 6 copies per cell) to generate a recombinant plasmid, pHYD913 (Fig. 5.3). A 1.86 kb *Sal*I fragment, which would be expected to carry the *argP* gene along with its promoter was then subcloned from pHYD913 into the *Sal*I site of pCL1920 in both orientations to generate the recombinant plasmids pHYD914 and pHYD915.

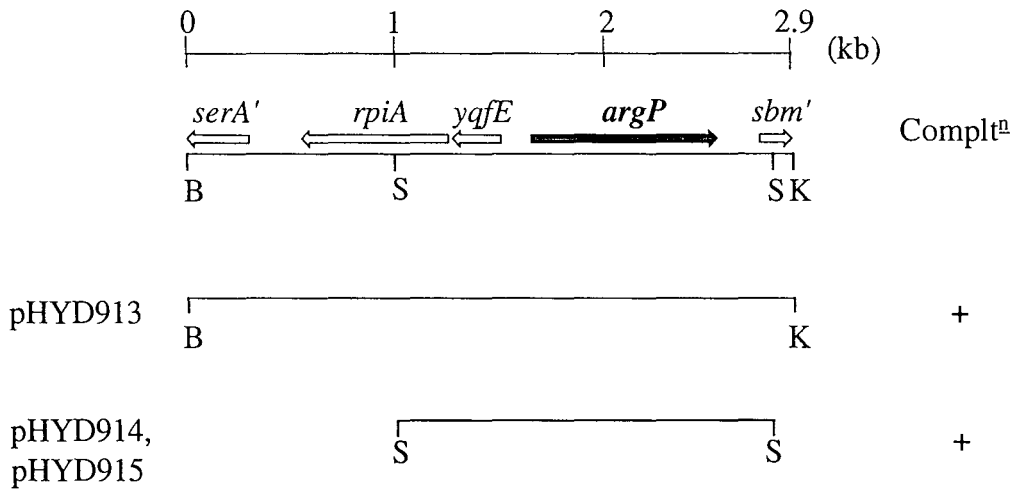


Fig. 5.3 Cloning of *argP*$^{+}$ and complementation analysis.
Below the kilobase scale is depicted the physical and gene map of 2.9 kb *Bam*HI-*Kpn*I fragment from Kohara phage λ-471 for the enzymes *Bam*HI (B), *Kpn*I (K), and *Sal*I (S). The position of various genes and their transcriptional organization (arrows) are marked. Each line aligned beneath the physical map represents the extent of chromosomal DNA cloned into a plasmid, whose numerical pHYD designation is given alongside. Also indicated in the column marked Complt^{n} at right is the ability of the plasmid to complement (+) or not to complement (–) the *argP* mutant for osmosensitivity and Can^{SS} phenotype.

5.2.11.2 Complementation of osmosensitivity and Can^{SS} phenotype by *argP*$^{+}$:

The three plasmids described above were introduced into *argP202*::Kan Δ*gltBD recA* strain (GJ4612). The above strains were streaked on MA + 1 mM Bet supplemented with 0.6-0.7 M NaCl, 0.8-0.9 M sucrose or 0.9-1.0 M glucose.

The results (Table 5.6) demonstrate that all the three plasmids complement the *argP*::Kan *gltBD* mutant for its osmosensitivity phenotypes, and therefore that the *argP202* allele is recessive to *argP*$^{+}$.

Table 5.6 Complementation of *argP*::Kan mutations by *argP*$^+$ at high osmolarity[a]

Recipient strain (chromosomal genotype)	Plasmid (genotype)	MA + 1 mM Bet Spec		
		NaCl (0.6-0.7 M)	Glucose (0.9-1 M)	Sucrose (0.8-0.9 M)
MC4100 (WT)	pCL1920 (vector)	+++	+++	+++
GJ2529 (Δ*gltBD*)	pCL1920 (vector)	++±	++±	++±
GJ4612 (*argP202*::Kan Δ*gltBD*)	pCL1920 (vector)	—	—	—
	pHYD913 (2.9 kb *argP*$^+$)	+++	+++	+++
	pHYD914 (1.8 kb *argP*$^+$)	+++	+++	+++
	pHYD915 (1.8 kb *argP*$^+$)	+++	+++	+++

[a] The indicated strains were streaked as described in the footnote to Table 5.1 with the modification that Spec was added to the plates.

Complementation for Can^{SS} was also tested for plasmid pHYD915 as follows. This plasmid and pCL1920 vector (as control) were introduced into GJ4536 (*argP202*::Kan) and GJ4540 (*argP203*::Kan) strains, and the resulting Spec^R transformants were streaked on MA + Can (20 μg / ml) plate. The results are tabulated in Table 5.7. The results show once again that pHYD915 was able to complement both the *argP*::Kan mutants for Can^{SS} phenotype, and this phenotype was, as expected, *gltBD*-independent (data not shown).

Table 5.7 Complementation of the Can^{SS} phenotype of *argP*::Kan mutants by *argP*$^+$ gene[a]

Recipient strain (genotype)	Plasmid (genotype)	MA + Spec + Can	
		0	20
MC4100 (WT)	pCL1920 (vector)	+	+
GJ4536 (*argP202*::Kan)	pCL1920 (vector)	+	–
GJ4536 (*argP202*::Kan)	pHYD915 (*argP*$^+$)	+	+
GJ4540 (*argP203*::Kan)	pCL1920 (vector)	+	–
GJ4540 (*argP203*::Kan)	pHYD915 (*argP*$^+$)	+	+

[a] The indicated strains were streaked on MA plates supplemented with Spec (for maintaining the plasmid pCL1920 and its derivatives) and Can (0 and 20 μg/ml). The plates were incubated for 24 h at 37°C.
– (no growth), + (full growth).

5.3 Identification and characterization of Can^R *argP* mutants

5.3.1 MNNG mutagenesis of *argP*:

The identification in the present study of null *argP* mutants as Can^{SS} was in apparent conflict with the suggestion of Celis (1999) that ArgP is an activator of Arg uptake and his identification of Can^R mutations in *argP*. In an attempt to resolve this issue, an MNNG mutagenesis approach was taken to isolate plasmid-borne Can^R *argP* alleles. The characterization of Can^R *argP* alleles was expected to contribute to the understanding of the role of *argP* in Arg metabolism. MNNG mutagenesis of MC4100 carrying pHYD915 (*argP*$^+$) was performed according to the procedure already described in Chapter 2 (Section 2.13). Briefly, MC4100 carrying pHYD915 was mutagenized with MNNG, by exposing aliquots of the culture to MNNG for different time periods. Point mutations (A.T → G.C) were expected to arise in such mutagenized cells. Since the target of interest (*argP*) is present on the plasmid, isolation of plasmid DNA from the pool of mutagenized cells and their transfer into the *argP*::Kan null strain would enrich for plasmid-borne mutations. The Spec^R transformants obtained were then screened for their Can phenotype by streaking on MA + Spec + uracil + Can (65 μg/ml) plates. This screen was expected to yield *argP* mutant alleles which confer increased Can resistance / tolerance above that observed for the GJ4536 / pHYD915 control strain. Although the mutagenesis was also expected to generate *argP* alleles associated with Can^{SS}, these were not specifically targeted in the screening procedure.

Seven Can^R colonies were obtained after screening ~800 colonies. In order to confirm that the Can^R phenotype was plasmid-borne, plasmid DNA was isolated from each of the Can^R colonies and re-introduced into GJ4536. All transformants tested were Can^R, indicating that all seven plasmids breed true for the Can^R phenotype. The seven plasmids carrying Can^R *argP* gene were designated pHYD926 through pHYD932.

5.3.2 Recessivity / dominance testing of Can^R *argP* alleles:

To determine whether the Can^R *argP* alleles obtained in the above screen were recessive or dominant to the *argP*$^+$ allele, the plasmid DNAs of pHYD926 to pHYD932 were introduced into MC4100. The resultant Spec^R colonies were tested for Can^R phenotype, along with the MC4100 / pHYD915 control strain. The results (Table 5.8) suggest that six of the seven plasmids viz., pHYD926 to pHYD930 and pHYD932 exhibit

the Can^R phenotype even in the presence of the WT copy of the *argP* gene, whereas in pHYD931, the colonies were Can^S (growing on par with MC4100 / pHYD915 control). Based on these results, it can be concluded that plasmids pHYD926 to pHYD930 and pHYD932 contain *trans*-dominant *argP* (*argP*^{d}) alleles.

Table 5.8 Molecular characterization of MNNG-mutagenized Can^R *argP* alleles[a]

Plasmid	Dominance / Recessivity[b]	G.C → A.T Sequence change[c]	Amino acid change[d]
pHYD926	D	930	S94L
pHYD927	D	971	P108S
pHYD928	D	1079	V144M
pHYD929	D	1299	P217L
pHYD930	D	1529	L294F
pHYD931	R	1532	R295C
pHYD932	D	852	A68V

[a] The indicated Can^R *argP* alleles contained in pCL1920 vector were sequenced using a set of four primers as described in the text.
[b] Dominance (D) or recessivity (R) of Can^R alleles was tested.
[c] Co-ordinates marked relative to the *Sal*I site of 1815 bp *argP* fragment cloned in pCL1920.
[d] The predicted amino acid substitutions were arrived at by translating *in silico* the mutated *argP* alleles, using the software Clone Manager version 5.

5.3.3 Sequence analysis of the Can^R *argP* alleles:

To molecularly characterize the mutation in each of the Can^R *argP* alleles, sequencing of these alleles was undertaken. For this, two primers were designed internal to the 1.86 kb *Sal*I fragment of the *argP* gene. Primer ARGP1 was from 481 to 501 nucleotides relative to the *Sal*I site whereas primer ARGP2 was (in the reverse orientation, complementary to the non-coding strand) 1343 to 1323 nucleotides relative to the *Sal*I site. Since the *argP* alleles were cloned in pCL1920 which contains the MCS from plasmid pUC18, universal M13 forward and reverse primers complementary to the sequences upstream of MCS were used to sequence either end of the 1.86 kb insert.

The sequencing results (Table 5.8 and Fig. 5.4) indicated that Can^R *argP* alleles harbored mutations either in the putative N-terminal DNA-binding domain or the central co-inducer recognition domain or in the C-terminal conserved domain (CTD). All the plasmids, except pHYD926, had only one mutation. In pHYD926, two mutations were revealed by sequencing; one in the *argP* gene at 296 nucleotides relative to the ATG start codon and another G.C to A.T mutation at –274 nucleotides relative to ATG. The latter mutation is unlikely to contribute to the Can^R phenotype, since it is predicted to be a synonymous codon substitution in an upstream ORF, *ygfE*.

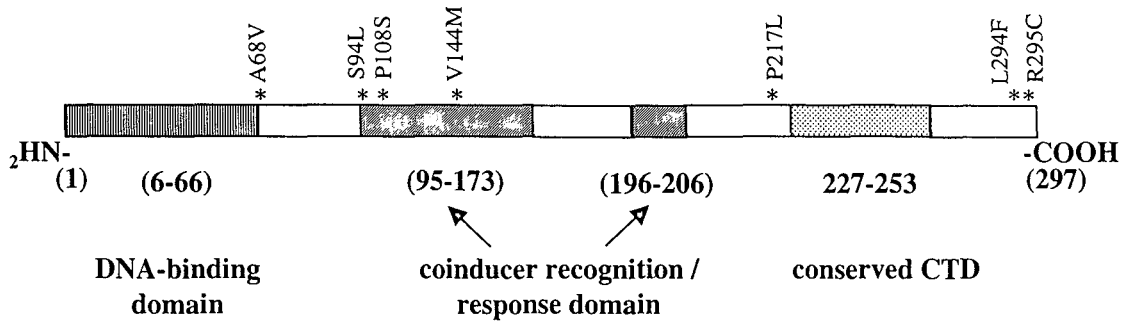


Fig. 5.4 Model for domain organization for ArgP and predicted amino acid substitutions in various MNNG-mutagenized Can^R *argP^d* alleles.
The domain organization of the ArgP protein was arrived at based on the conserved amino acid sequences between ArgP protein and other LysR-type of transcriptional regulators (Schell, 1993). The predicted amino acid substitutions, derived from DNA sequencing results of Can^R *argP^d* alleles, are depicted in relation to the domain organization of LysR-type of transcriptional regulators.

5.3.4 Effect of *gltBD* mutation on Can^R phenotype of *argP^d* alleles:

To examine whether the *gltBD* mutation affects the Can^R phenotype of the various *argP^d* alleles isolated in the present study, the plasmids harboring the various *argP* alleles were introduced into the Δ*gltBD* strain (GJ4652). Upon testing the resultant Spec^R transformants for Can^R phenotype, it was observed that all the transformants were Can^R and that they grew on par with the *gltBD$^+$* transformants. The result demonstrated that Can^R phenotype of *argP^d* alleles was not affected by the *gltBD* mutation.

5.3.5 Effect of *argP^d* alleles on osmosensitivity phenotype of WT, *gltBD* and *argP gltBD* strains:

In subsequent work described in the sections below, one representative *argP^d* allele (the S94L variant present on pHYD926) was chosen. Plasmids pCL1920 and

pHYD926 were introduced into WT, *gltBD* and *argP gltBD* strains, and the resultant $Spec^R$ colonies were tested for their ability to grow on media of high osmolarity. The results demonstrated that the presence of the Can^R $argP^d$ alleles did not appreciably affect the osmotolerance levels of WT and *gltBD* mutants. The *argP gltBD* mutant with the $argP^d$ allele grew to the same extent as the *gltBD* mutant with pHYD926, suggesting that pHYD926 did not increase the osmotolerance levels of the *argP gltBD* mutant. Similar results were observed when the S94L allele was introduced on a pBR329-based plasmid (pHYD953; ~40 copies per cell).

5.3.6 Effect of $argP^d$ alleles on NH_4^+-assimilation phenotype of *gltBD* and *argP gltBD* strains:

Plasmids pBR329 (vector) and pHYD953 (*argP* S94L) were introduced into the *gltBD* and *argP gltBD* strains in order to test the effect of the Can^R $argP^d$ allele on the NH_4^+-assimilation phenotype. The Amp^R transformants were streaked on W-salts + NH_4^+ (0.1 to 2.0 mM) plates. The results demonstrate that on all the concentrations of NH_4^+ tested, the *gltBD* strain (GJ4652) with pHYD953 was indistinguishable from that with pBR329. The *gltBD argP* strain (GJ4654) with pHYD953 grew similar to *gltBD* with either pBR329 or pHYD953 (i.e., the *argP* mutation was complemented by *argP* S94L), whereas GJ4654 with pBR329 grew poorly on lower NH_4^+ concentrations, consistent with the results discussed in Section 5.2.3 of this chapter. These results suggest that the Can^R $argP^d$ allele did not alter the NH_4^+ assimilation property of *gltBD* and *argP gltBD* mutants.

5.3.7 Arg cross-feeding ability of *argR* strain in the presence of a m.c. $argP^d$ allele:

The effect of $argP^d$ allele on Arg cross-feeding ability was studied by introducing plasmid pHYD953 or pBR329 (as vector control) into the *argR64* strain (GJ4748). The resultant Amp^R colonies were tested for their ability to cross-feed an *argE* auxotroph (strain 160.37), as mentioned in Section 5.2.10 of this chapter. The results show (Fig. 5.5) that the strains carrying the multicopy $argP^d$ allele excreted substantial amounts of Arg even by 12 h, as evidenced by the red halo around the strain, compared to the vector-bearing control. When the plate was incubated further for 48-60 h, the *argR64* strain with pBR329 also exhibited Arg cross-feeding of the *argE* auxotroph, and the differences between the two strains became less pronounced.

Fig. 5.5 Arg cross-feeding ability of *argR* strain in the presence of *argP*^{d}

5.3.8 Regulation of *argP*::*lacZ* fusion:

As explained in Section 3.2.5 of Chapter 3, the advantage of using λp*lac*Mu55 for random insertional mutagenesis was that it can form promoter-*lacZ* transcriptional (operon) fusions. In the present screen, the two *argP*::Kan insertions obtained were Lac^{+} demonstrating that the *argP* promoter sequences are regulating the transcription of the promoter-less *lacZ* gene of the transposon. One of them, *argP202*::*lac*Kan was used to study the regulation of the *argP* gene. Since the insertion had inactivated the chromosomal *argP* gene, it was decided to introduce *argP*$^{+}$ cloned in a low copy number plasmid vector (pCL1920), in order to examine autoregulation, if any. Also, the effect of a S94L *argP*^{d} allele on *argP*::*lacZ* expression was studied. For these purposes, GJ4536 was transformed with plasmids pCL1920 (vector control), pHYD915 (*argP*$^{+}$) and pHYD926 (*argP*^{d}). The resultant Spec^{R} strains were then assayed for β-galactosidase specific activity. To test the effect of *argR* mutation on *argP*::*lacZ* expression, the *argP202*::*lac*Kan was transduced into the *argR64* strain (GJ4631) by using the P1 lysate made on GJ4536 and selecting for Kan^{R} colonies. One such Kan^{R} colony was named as GJ4617 (*argP*::*lacZ argR64*). The assays were performed in the presence or absence of Arg, Orn and Lys.

The results, tabulated in Table 5.9, indicated the following:

1. The *argP* gene possesses a moderately strong promoter;
2. Null mutations in *argP* do not affect *argP*::*lacZ* expression (compare GJ4536 / pCL1920 with GJ4536 / pHYD915), suggesting that the *argP* gene is not autoregulated [contrary to the report by Celis (1999)];
3. Addition of exogenous Arg, Lys or Orn (whose transport into the cell was claimed to be regulated by ArgP) did not alter the *argP*::*lacZ* expression pattern;

4. The S94L $argP^d$ allele also did not alter the *argP*::*lacZ* expression, nor did the *argR* mutation.

Table 5.9 *argP*::*lac* expression under various conditions[i]

Recipient strain (genotype)	Plasmid (genotype)	β-galactosidase specific activity			
		–	Arg	Orn	Lys
GJ4536 *argP*::*lacZ*	pCL1920 (vector)[ii]	125	110	112	115
GJ4536 *argP*::*lacZ*	pHYD915 ($argP^+$)[ii]	107	109	101	110
GJ4536 *argP*::*lacZ*	pHYD926 ($argP^d$)[ii]	89	92	89	108
GJ4617 *argP*::*lacZ argR64*	—	62	59	56	63

[i] The indicated strains containing the chromosomal *argP*::*lacZ* fusion were grown in MA medium without (–) and with 1mM Arg, Orn or Lys at 37°C to mid-exponential phase for β-galactosidase assays. Enzyme specific activity values are reported in Miller Units (Miller, 1992).
[ii] These strains carry the pCL1920 vector with and without $argP^+$ and *trans*-dominant *argP* ($trans^d$ *argP*) inserts. Therefore, the medium was additionally supplemented with Spec.

Similar studies were undertaken with the second *argP*::*lac* fusion (*argP203*::*lac*), and the same pattern of results was obtained, although the *lac* expression values in general were lower than that for *argP202*.

5.3.9 Effect of exogenous Lys on osmosensitivity and NH_4^+-assimilation phenotypes of *gltBD* and *argP gltBD* mutants:

The rationale for this experiment was the observation (described later in Section 5.6.2 of this chapter) that exogenously-supplied Lys inhibited the ArgP-mediated induction of *yggA*::*lac* expression, presumably by modulating ArgP activity. Therefore, it was decided to examine the effect of Lys on osmosensitivity and NH_4^+-assimilation properties of *gltBD* and *gltBD argP* mutants.

5.3.9.1 Effect of Lys on osmosensitivity:

The effect of exogenous Lys on the osmosensitivity phenotype was tested for the *gltBD* (GJ4652 and GJ2529), *argP gltBD* (GJ4654), *glnE gltBD, thiI* (GJ4553), and WT

(MC4100) strains. The above strains were streaked on MA plates supplemented with 0.6 to 0.7 M NaCl, 1 mM Bet, and with and without 1 mM Lys. The effects of Thr and Met supplementation were also tested since Lys, Met and Thr share a common pathway in their biosynthesis. Testing Met and Thr effect on osmosensitivity would enable one to distinguish the specific effect of Lys from the nonspecific effects (due to perturbations in Lys, Met and Thr biosynthetic pathways).

Table 5.10 Effect of exogenous Lys on osmosensitivity of *gltBD* and *argP gltBD* mutants[a]

Strain	Genotype	MA + 1mM Bet + 0.6-0.7M NaCl				
		–	Lys	Thr	Met	Lys + Thr + Met
MC4100	WT	+++	+++	+++	+++	+++
GJ44553	*thiI*	+++	+++	+++	+++	+++
GJ4652	*gltBD*	++	–	++	++	–
GJ2529	*gltBD*	++	–	++	++	–
GJ4654	*argP gltBD*	–	–	–	–	–
GJ4663	*glnE gltBD*	–	–	–	–	–

[a] The indicated strains were streaked on MA + 1 mM Bet + 0.6-0.7 M NaCl plates supplemented with 40 μg/ml concentration of Lys, Thr, Met or a combination of Lys + Thr + Met, and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): — (no growth), ++ and +++ (full growth).

As can be gleaned from Table 5.10, Lys did not affect the osmosensitivity of *argP gltBD* and *glnE gltBD* strains, since they continued to be as sensitive as they were on unsupplemented plates. Thr and Met also did not affect their osmosensitivity phenotype. However, the *gltBD* mutants (GJ4652 and GJ2529) were severely growth-inhibited on high osmolarity plates supplemented with Lys, while Thr and Met did not inhibit the growth of *gltBD* mutants on similar plates. When the Lys + Thr + Met combination was added, the results were the same as that when only Lys was added i.e., no effect on *argP gltBD* and *glnE gltBD* strains but *gltBD* mutants were severely growth-inhibited. In other words, Lys-mediated inhibition of the *gltBD* strains on high-osmolarity medium was not reversed by Thr + Met supplementation. MC4100 (*gltBD*$^+$) grew well on the Lys-supplemented plates.

Table 5.11 Effect of exogenous Lys on NH_4^+ assimilation phenotype of *gltBD* and *argP gltBD* mutants[a]

Strain	Genotype	W-salts + NH_4^+											
		0.2mM				0.6mM				1.0mM			
		–	Lys	Met	Thr	–	Lys	Met	Thr	–	Lys	Met	Thr
MC4100	WT	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GJ2529	*gltBD*	±	–	±	±	+	–	+	+	++	–	++	++
GJ4652	*gltBD*	±	–	±	±	+	–	+	+	++	–	++	++
GJ4654	*argP gltBD*	–	–	–	–	–	–	–	–	–	–	–	–
GJ4663	*glnE gltBD*	–	–	–	–	±	–	±	±	+	–	+	+

[a] The indicated strains were streaked on W-salts plates containing 0.2 mM, 0.6mM and 1.0 mM NH_4^+ and additionally supplemented with 40 mg/ml concentration of Lys, Thr, Met or a combination of Lys + Thr + Met, and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): – (no growth), ±, +, ++, and +++ (full growth).

5.3.9.2 Effect of Lys on NH_4^+ assimilation:

To test the effect of exogenous Lys on the ability of strains to grow on medium of low NH_4^+, the strains *gltBD* (GJ4652 and GJ2529), *argP gltBD* (GJ4654), *glnE gltBD* (GJ4663) and WT (MC4100) were streaked on W-salts plates with 0.2 to 1.0 mM NH_4^+ supplementation. The results are shown in Table 5.11.

The results from the above experiment can be summarized as follows:

(i) As described above in Section 5.2.3, the *argP gltBD* strain (GJ4654) was severely compromised for growth on W-salts supplemented even with 1.0 mM NH_4^+. *gltBD* mutants (GJ4652 and GJ2529) grew well at that concentration;

(ii) *glnE gltBD* (GJ4663) was also compromised for growth on low NH_4^+ medium but was not as severely affected as *argP gltBD* double mutant;

(iii) Addition of exogenous Lys at 1 mM completely inhibited all the *gltBD* strains on W-salts plates supplemented with 1.0 mM NH_4^+, so that they became indistinguishable from the *argP gltBD* strain;

(iv) Addition of Met and Thr had no effect on the ability of the various strains to grow on W-salts supplemented with limiting NH_4^+;

(v) MC4100 (*gltBD*$^+$) was not affected for NH_4^+ assimilation by the above perturbations.

5.4 Identification of *yggA* null mutants as Can^{SS}

This section describes the unexpected finding that null insertions in a hitherto-uncharacterized ORF, *yggA*, confers Can^{SS}. *yggA* was identified in an experiment involving localized mutagenesis of the chromosome around *argP* and screening for mutants with altered Can^R phenotypes. This experiment was originally undertaken with the following objectives.

(i) To get additional null alleles of *argP* employing transposons carrying markers other than Kan^R;

(ii) *argK (ygfD)* gene is located at 66 min (2 kb away from *argP*). According to Celis' model, ArgP acts as an activator of *argK*, which then activates the transport of arginine into the cell. No phenotype has been described for an *argK* mutant. If Celis' hypothesis were to be true, then null insertions in *argK* would result in a Can^R phenotype;

(iii) Genes encoding the LysR-family of transcriptional regulators (of which ArgP is a predicted member) are often situated adjacent to their target genes with overlapping divergent promoters. Thus, the gene (*ygfE*) just upstream of *argP* but divergently transcribed might conceivably be one of the targets of ArgP.

5.4.1 Localized mutagenesis of the region around the *argP* locus:

Localized mutagenesis in the vicinity of *argP* was performed, as described in Chapter 2 (Section 2.12). Random transpositions of Tn*10*dTet were introduced using λNK1323 into a WT strain (MC4100) and a P1 lysate was prepared from a pool of such Tet^R colonies. This lysate was used to transduce a *serA* strain (GJ4674) simultaneously to $serA^+$ and Tet^R on MA Tet plate. (*serA* is located at 65.8 min, 2 kb away from *argP*.) The simultaneous double selection would ensure that Tet^R insertions occurring on either side of *serA* (invariant marker) within a 1-min region (approximately 45 kb) would be selected (Fig. 5.6). Since *argP*, *argK* and *ygfE* are located very close to *serA*, one can expect that some of the Tet insertions would result in knocking out the functions of these genes. After P1 transduction employing the lysate prepared on the pool of Tet^R colonies, the double-selected colonies were then screened for their sensitivity / tolerance to Can on MA Can plates. No Can^R colonies were obtained, but nine Can^{SS} colonies were identified and were designated as GJ4676 to GJ4684.

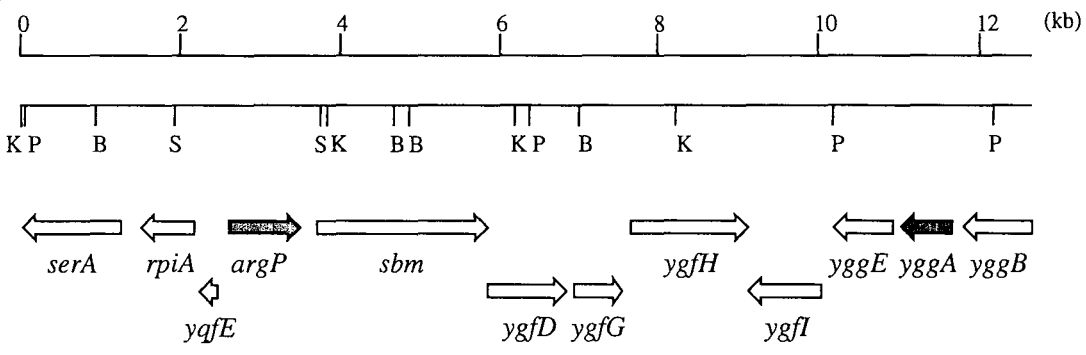


Fig. 5.6 Physical and gene map of the region around *argP* (65.9 min).
Below the kilobase scale is depicted the physical and gene map of the region around *argP* for the enzymes *Bam*HI (B), *Kpn*I (K), *Pst*I (P) and *Sal*I (S). The position of various genes and their transcriptional organisation (arrows) are marked.

5.4.2 Establishing 100% linkage between Tet^R and Can^{SS} phenotype:

To establish the hypothesis that the observed Can^{SS} phenotype is due to the Tet^R insertion and not due to any other mutation, P1 lysates were prepared on all the nine

Can^{SS} strains and GJ4652 (Δ*gltBD*) was transduced to Tet^R with each of them. All the tested Tet^R transductants were Can^{SS}, suggesting a 100% linkage between Tet^R and Can^{SS} phenotype in all nine strains. One colony from each transduction was selected for further work, and designated as GJ4691 to GJ4699. Growth of the Tet^R transductants on plates without Can was not different from that of the parent GJ4652.

5.4.3 Can^{SS} phenotype is *gltBD*-independent:

In order to check whether the Can^{SS} phenotype was dependent on the *gltBD* mutation, P1 lysates from two Can^{SS} strains, GJ4676 and GJ4677 were used to transduce MC4100 (WT) strain, and the resultant Tet^R colonies were designated GJ4822 and GJ4823. The latter strains were also Can^{SS}, indicating that the phenotype is *gltBD*-independent.

5.4.4 Genetic characterization of Can^{SS} Tet^R mutants:

To find whether the Can^{SS} Tet^R insertions were located in the *argP* gene, the P1 lysates made on all the nine Can^{SS} strains viz., GJ4676 to GJ4684 were used to transduce the *argP*::Kan strains (GJ4536 and GJ4540) to Tet^R. The resultant Tet^R colonies were screened for the presence of the Kan^R marker. If the Tet^R insertion was in the *argP* gene, then Tet^R insertion was expected to cross out the *argP*::Kan allele in the recipient strain at 100% frequency. The results showed, however, that the linkage between the Tet^R and Kan^R insertions was only 76% to 84% suggesting that the Tet^R insertion in none of the nine strains may be in the *argP* locus.

5.4.5 Tests of complementation with $argP^+$ and $argK^+$ genes:

To rule out the possibility that the Tet^R insertion is not in the *argP* gene, two representative Can^{SS} strains, GJ4691 and GJ4692 were transformed with pHYD915, which contains the 1.86 kb $argP^+$ fragment. The $Spec^R$ transformants when screened for Can^{SS} phenotype continued to be Can^{SS} (Table 5.12) indicating that the $argP^+$ gene did not complement these Tet^R strains for Can^{SS} phenotype. This result conclusively rules out the possibility that the Can^{SS} phenotype in these strains was due to an insertion in the *argP* gene.

Another candidate gene which was considered to be involved in Arg metabolism, an insertion in which might give a Can^{SS} phenotype was *argK (ygfD)*. *argK* is located ~5-

6 kb from *serA*. In order to test the hypothesis that these Can^{SS} Tet^{R} strains harbored null insertion in *argK*, the $argK^{+}$ gene was cloned from the Kohara λ-phage library (Kohara et al., 1987). Phage DNA was isolated from λ-471 and digested with *Bam*HI, which resulted in the generation of (among others) a 2.15 kb fragment that was expected to contain the $argK^{+}$ gene. This fragment was cloned at the *Bam*HI site of pCL1920 in either orientation to create the plasmids pHYD933 and pHYD934. These plasmids were then introduced into GJ4691 and GJ4692, and the resultant transformants were screened for Can^{SS} phenotype. The results (Table 5.12) show that the transformed strains continue to be Can^{SS}, establishing that the $argK^{+}$ insert did not complement the Tet^{R} insertions for Can phenotype.

Table 5.12 Tests of complementation of the Can^{SS} strains with $argP^{+}$ and $argK^{+}$ alleles[a]

Strain	MA Spec Can (20 μg/ml)		
	Vector (pCL1920)	*argP*+ (pHYD915)	*argK*+ (pHYD933)
GJ4652	+	+	+
GJ4691	–	–	–
GJ4692	–	–	–

[a] The indicated strains carrying the pCL1920 vector or $argP^{+}$ (in one orientation) and $argK^{+}$ (in either orientation) genes cloned in pCL1920 were streaked on MA + Spec plates supplemented with 20 μg/ml Can. The plates were incubated for 24 h at 37°C.
– (no growth), + (full growth).

5.4.6 Molecular characterization of Can^{SS} Tet^{R} mutants:

It was then decided to molecularly characterize the site of the Tet^{R} insertions in the Can^{SS} strains. Towards this end, inverse PCR strategy was adopted. Chromosomal DNA from one Can^{SS} mutant (GJ4691) was isolated and digested with *Sau*3AI; following circularization of the fragments by ligation at high dilution, inverse PCR was performed using AH1 and AH2 primers, according to the procedure outlined in Chapter 2. The ~0.3 kb amplicon obtained after inverse PCR was sequenced with TETF and TETR primers, and sequence analysis (BLAST analysis) showed that the gene disrupted in GJ4691 was *yggA*. *yggA* encodes a protein of 211 amino acids of unknown function and is located at

66.1 min, which is ~8kb away from *argP*. This result is in agreement with the 82% linkage between Tet^R of GJ4676 and *argP202*::Kan.

To determine whether the Tet^R insertion site in the other Can^{SS} strains was also in *yggA*, a PCR strategy was employed. With the aid of four primers, TETF and TETR, (two outwardly directed primers on either end of Tn*10*dTet), YGGAR (complementary to regions downstream of *yggA*), and YGGBF (complementary to regions upstream of *yggB* in the *fbi* gene), PCR was performed. Since *yggA* and *yggB* are adjacent to one another and transcribed in the same direction, the possibility was considered that they may be organized into an operon (but see also Section 5.5.7 below). For this reason, even insertions in *yggB* (which is upstream of *yggA*) might give a Can^{SS} phenotype. Therefore, primer YGGBF was designed upstream of *yggB* to identify insertions, if any, in the *yggB* gene. Using a combination of primers, viz., TETF + YGGAR, TETF + YGGBF, TETR + YGGAR and TETR + YGGBF, colony PCR was performed on strains GJ4692 to GJ4699 (with GJ4691 as well, included as control), according to the protocol described in Chapter 2. If there was a Tn*10*dTet insertion in *yggA* or *yggB*, only two of the above four combinations are expected to yield amplified products (see Fig. 5.7). The size of the amplicon and the combination of primers which successfully amplify the region would yield information about the insertion site and orientation of the Tet^R insertion.

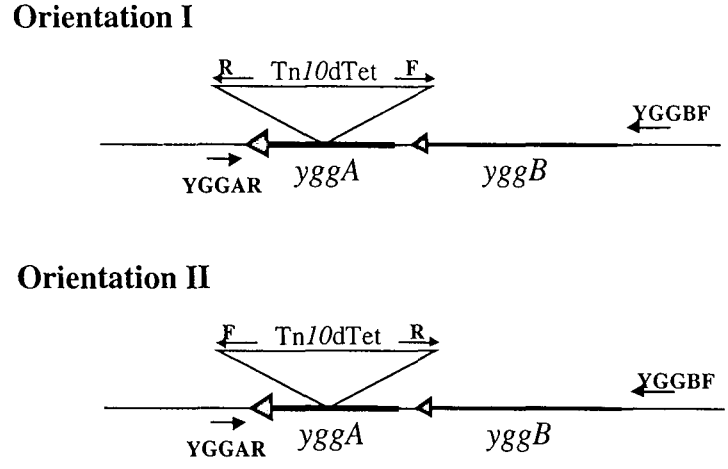


Fig. 5.7 Schematic representation of the strategy used for PCR amplification and sequencing of the *yggA* mutations from Can^{SS} strains.
Primers TETF and YGGBF (forward), and TETR and YGGAR (reverse) are shown. Depending on the orientation of the Tn*10*dTet insertion, only two of the four primer combinations would yield PCR products. For example, in Orientation I, TETF + YGGBF and TETR + YGGAR would yield PCR products (whose molecular weight depends on the insertion site in the *yggA* gene), but the other two primer combinations (i.e., TETF + YGGAR and TETR + YGGBF) would not result in any successful PCR amplification.

The PCR reactions generated fragments, as expected, in only two of the four PCR reactions for each of the eight strains and the sizes suggested that the Tet^R insertions were in *yggA*, but not in *yggB*. To further pinpoint the exact insertion site, the amplicon from each of the eight Can^{SS} Tet^R mutants was sequenced with YGGAR and either TETF or TETR primers. The sequencing results (Table 5.13, Fig. 5.8 and Fig. 5.9) suggest that there are at least seven independent insertions in the *yggA* gene which confer Can^{SS}. The possibility that GJ4696 and GJ4699 are siblings could not be ruled out. The Tet^R insertions in GJ4691 and GJ4693 were at the same site but the orientation of the Tn*10*dTet element was different, suggesting that these are independent insertions. The Tet^R insertions in GJ4694 and GJ4695 differ by one nucleotide.

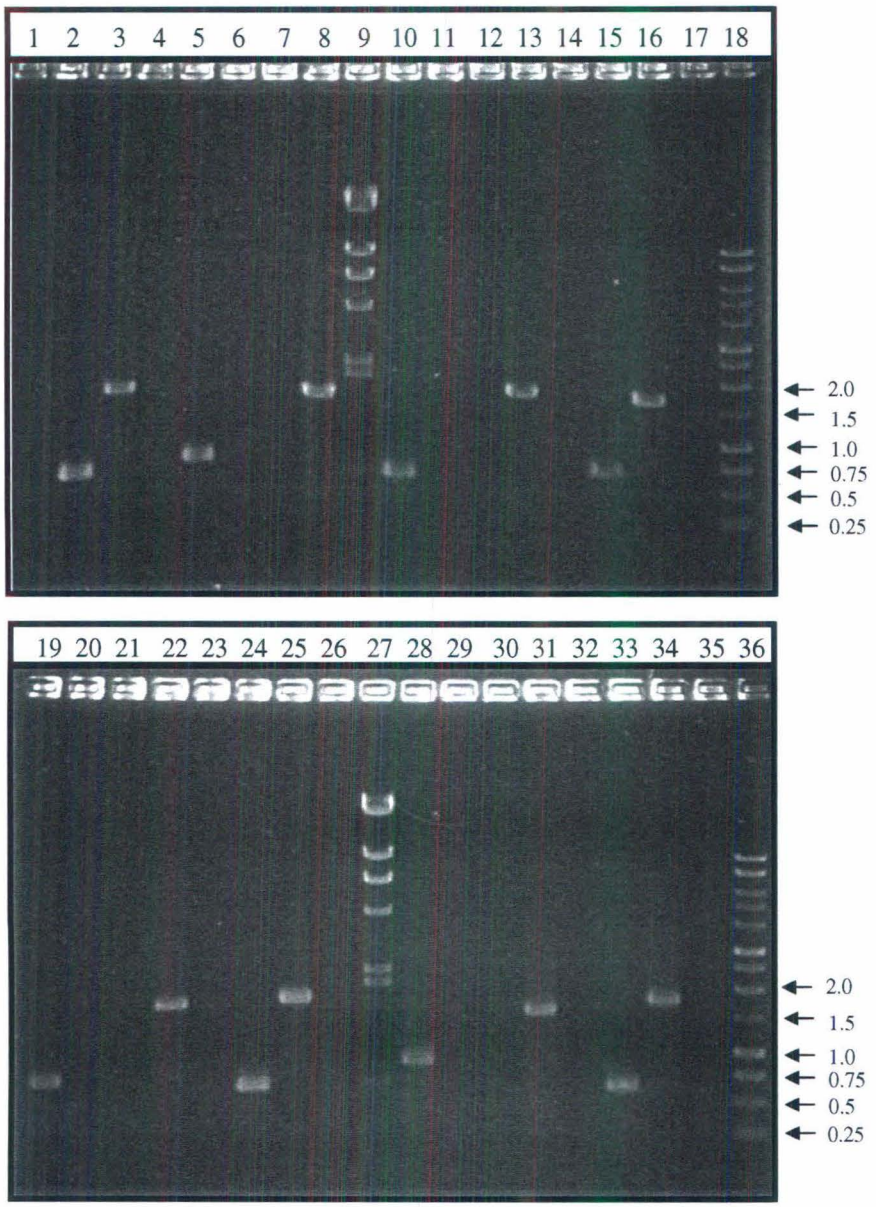


Fig. 5.8 PCR amplification of *yggA* mutations from Can^{SS} strains
The various lanes denote PCR reactions performed using the template from (Lanes 1 to 4) GJ4691, (Lanes 5 to 8) GJ4692, (Lanes 10 to 13) GJ4693, (Lanes 14 to 17) GJ4694, (Lanes 19 to 22) GJ4695, (Lanes 23 to 26) GJ4696, (Lanes 28 to 31) GJ4697 and (Lanes 32 to 35) GJ4699. For each strain, the primer combinations were YGGAR + TETF, YGGAR + TETR, YGGBF + TETF, YGGBF + TETR from left to right. Lanes 9 and 27 are λ / *Hin*dIII digest, and Lanes 18 and 36 are 1 kb Gene Ruler (Promega) molecular weight markers.

Table 5.13 Molecular analysis of *yggA*::Tn*10* mutants

Strain	Amplicon size (kb)				Insertion coordinates[a]	Preceding codon[b]	Orientation[c]
	TETF + YGGAR	TETR + YGGAR	TETF + YGGBF	TETR + YGGBF			
GJ4691	Nil	0.45	1.6	Nil	8934	W74	I
GJ4692	0.6	Nil	Nil	1.5	9054	Q34	II
GJ4693	0.45	Nil	Nil	1.6	8934	W74	II
GJ4694	Nil	0.55	1.5	Nil	9018	S46	I
GJ4695	0.55	Nil	Nil	1.5	9017	S46	II
GJ4696	Nil	0.45	1.6	Nil	8931	G75	I
GJ4697	0.7	Nil	Nil	1.3	9178	None	II
GJ4699	Nil	0.45	1.6	Nil	8931	G75	I

[a] Co-ordinates from AE000375 of Blattner's *E. coli* database represent nucleotides after which the Tn*10* element was inserted.
[b] Codon immediately upstream of which the insertion has occurred.
[c] Orientation of the Tn*10*dTet element is as shown in Fig. 5.7.

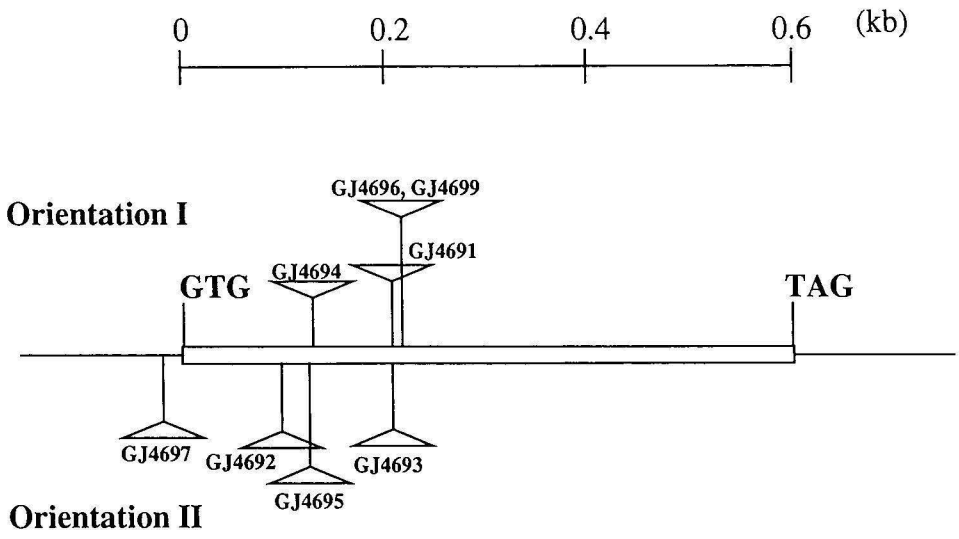


Fig. 5.9 Schematic representation of the Tn*10*dTet insertions in the *yggA* gene.
The inverted triangles represent the insertion sites in the *yggA* gene for each strain. Orientations I and II are as in Fig. 5.7.

5.5 Detailed phenotypic characterization of *yggA* mutants

5.5.1 Testing the water-stress sensitivity phenotype of Can^{SS} Tet^{R} mutants:

It was shown earlier in this chapter that *argP* mutation by itself confers Can^{SS} (Section 5.2.7) and that when combined with *gltBD* mutation, confers osmosensitivity (Section 5.2.1). It was decided to check whether the *yggA* Can^{SS} mutants would also be osmosensitive in the *gltBD* mutant background. To examine the water-stress sensitivity phenotype of the Can^{SS} *yggA*::Tn*10* mutants, all the nine strains carrying Tet^{R} insertions in the *gltBD* background (viz., GJ4691 to GJ4699), and two *yggA*::Tn*10* mutants in *gltBD*$^{+}$ background viz., GJ4822 and GJ4823 (as controls), were streaked on high osmolarity media containing various dissolved solutes viz., NaCl, sucrose and glucose. It was observed that the *yggA* strains were not sensitive to the dissolved solutes even in the *gltBD* background.

5.5.2 NH_4^+-assimilation property of *yggA gltBD* mutants:

The *yggA gltBD* strains (GJ4691 and GJ4692), when tested for their ability to grow on W-salts supplemented with low NH_4^+, grew on par with the *gltBD* mutant. This result suggested that *yggA* did not affect the NH_4^+ assimilation property of the *gltBD* mutant, unlike *argP* (see Section 5.2.3 where it was shown that the *argP gltBD* mutant

was severely reduced NH_4^+-assimilation property). This result reinforced the fact that *yggA* is like *argP* in conferring Can^{SS} but unlike the latter, is not associated with either an assimilation defect or osmosensitivity.

5.5.3 Effect of different amino acids on Can^{SS} phenotype of *yggA*::Tn*10*dTet mutants:

In order to check whether the Can^{SS} phenotype of *yggA* mutant strains could be modulated in the presence of various amino acids in a similar fashion as was observed with *argP* mutants, exogenous Arg, Lys, Orn and Cit were added to Can-containing media. The results showed that Arg reversed the Can^{SS} phenotype of *yggA* mutants, as previously observed for *argP* mutants (Section 5.2.8). However, Orn, Cit and Lys did not suppress the Can^{SS} phenotype of *yggA* mutants, unlike the case with *argP* mutants.

5.5.4 Effect of *argP* and *argR* mutations on the Can^{SS} phenotype of *yggA* mutants:

To determine the effect of other mutations (involved in Arg metabolism) on the Can^{SS} phenotype of *yggA* strains, *argP*::Kan and *argR64* mutations were introduced into the *yggA*::Tn*10* strains individually and in combination. P1 lysates prepared on *yggA301*::Tn*10*dTet (GJ4676) and *yggA302*::Tn*10*dTet (GJ4677) were used to transduce GJ4536 (*argP202*::Kan) to Tet^R and the resulting strains which harbor the two mutations viz., *yggA*::Tn*10*dTet and *argP202*::Kan were called GJ4706 and GJ4707, respectively. To construct *yggA argR* double mutant strain, GJ4748 (*argR64*) was transduced to Tet^R with the P1 lysates on GJ4676 and GJ4677, and one transductant from each cross was designated GJ4749 and GJ4750, respectively (*yggA argR64*). The triple mutant strains carrying *argP, argR* and *yggA* mutations were constructed by introducing *argP202*::Kan insertions into GJ4749 and GJ4750, employing P1 lysates prepared on GJ4536 and selecting for Kan^R. The resultant Kan^R transductants were christened GJ4790 and GJ4791. These strains were then streaked on MA Can plates with and without Ura.

The rank order of growth of the various strains on MA + Ura + Can (400 μg/ml to 1000 μg/ml) concentration was (from most sensitive to most tolerant): *argP yggA argR* = *yggA argR* < *argP argR* = WT << *argR*. The two *yggA* insertions behaved similar to one another in the various combinations tested. The data suggested that in the *argR* background, both *argP* and *yggA* decrease Can tolerance, with the latter being more

severe. The triple mutant *argR argP yggA* is no more Can^S than the double mutant *argR yggA*, suggesting that *yggA* and *argP* are not additive.

5.5.5 Tests of epistasis between *argP* and *yggA*:

In order to distinguish between the Can^{SS} limits of *argP* and *yggA*, these strains were streaked on varying concentrations of Can. It was observed that the *yggA* strain was more severely Can^{SS} than the *argP* strain; MIC for the *yggA* strain was 0.125 µg/ml Can whereas that for *argP202*:Kan was 2 µg/ml Can. Qualitatively, similar results were obtained in experiments designed to measure the zones of growth inhibition around Can placed in filter paper discs (Fig. 5.10).

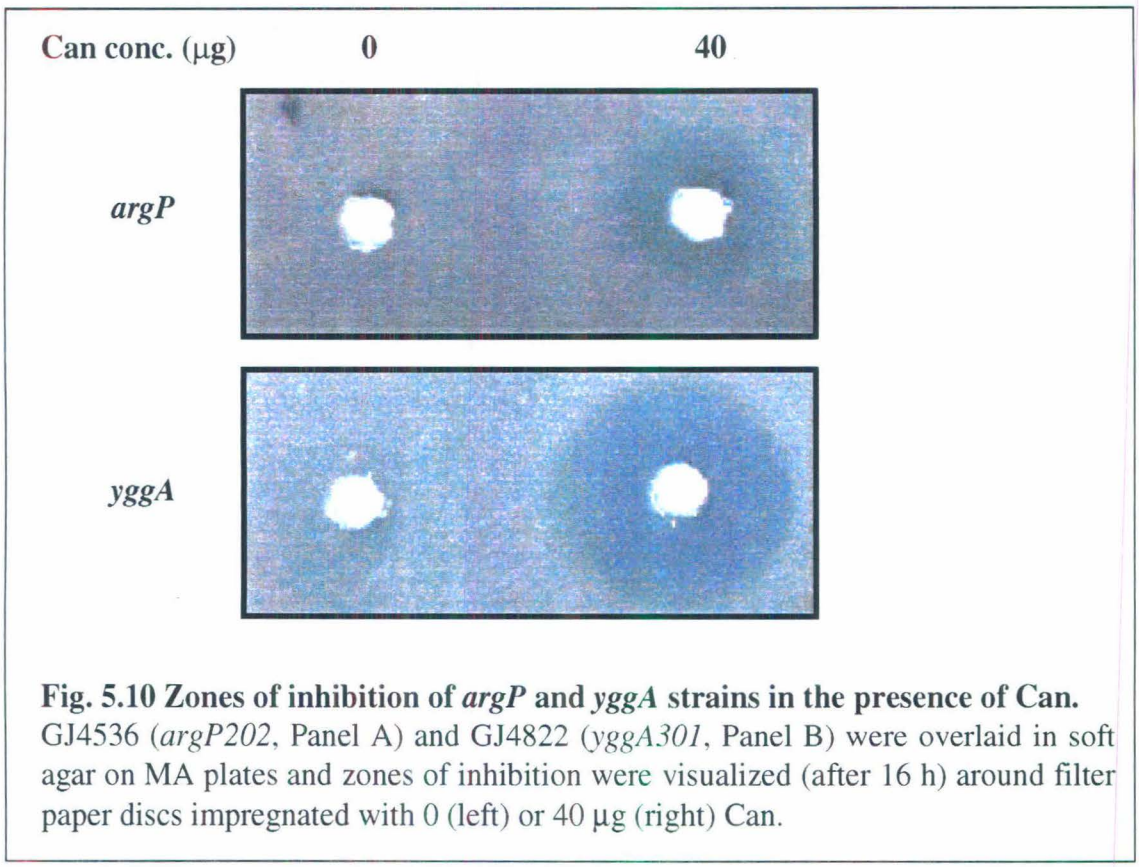


Fig. 5.10 Zones of inhibition of *argP* and *yggA* strains in the presence of Can. GJ4536 (*argP202*, Panel A) and GJ4822 (*yggA301*, Panel B) were overlaid in soft agar on MA plates and zones of inhibition were visualized (after 16 h) around filter paper discs impregnated with 0 (left) or 40 µg (right) Can.

Tests of epistasis were also performed employing *yggA*::Tn*10*dTet and an *argP^d* allele, in the following manner. It may be noted in this context that as single mutations, the former confer Can^{SS} and the latter Can^R. The plasmids pHYD926 and pHYD927 (which contain the *argP^d* alleles) were introduced into *yggA*::Tn*10*dTet strains (GJ4691 and GJ4692). The resulting transformants continue to remain Can^{SS}, whereas the same plasmids conferred Can^R in GJ4536 (*argP202*::Kan) and MC4100 (WT). This result indicates that *yggA* is epistatic to Can^R *argP^d*.

5.5.6 Testing *yggA* mutant phenotype in MG1655:

When the sequence of *in silico*-translated YggA was compared with that of other proteins in *E. coli* employing BLASTp search, a high degree of similarity (21% identity and 41% similarity) was observed between YggA and another ORF called YahN (Fig. 5.11). The corresponding gene (*yahN*) is located near *lacZ* (7.9 min) in MG1655, but absent in MC4100 (the WT strain used in the present study) because the latter carries the Δ(*argF-lac*) deletion. The possibility was therefore considered that YggA and YahN have redundant functions and that the reason why MC4100 *yggA* mutants are Can^{SS} is that they do not have both *yggA* and *yahN*.

```
YggA   -------------MFSYYFQGLALGAAMILPLGP-QNAFVMNQG---IRRQYHIMIALLC 43
YahN   MMQLVHLFMDEITMDPLHAVYLTVGLFVITFFNPGANLFVVVQTSLASGRRAGVLTGLGV 60
                    * . :   *::*  :*  :.*  * **: *      *:  :: .*

YggA   AISDLVLICAGIFG-GSLLMQSPWLLALVTWGGVAFLLWYGFGAFKTAMSSNIELASAEV 102
YahN   ALGDAFYSGLGLFGLATLITQCEEIFSLIRIVGGAYLLWFAWCSMRRQSTPQMSTLQQPI 120
       *:.* .    *:** .:*: *.  :::*:   * *:***:.: :::   :.::.  .  :

YggA   MKQGRWKIIATMLAVTWLNPHVYLDTFVVLG-SLGGQLDVEPKRWFALGTISASFLWFFG 161
YahN   SAP-WYVFFRRGLITDLSNPQTVLFFISIFSVTLNAETPTWARLMAWAGIVLASIIWRVF 179
            : ::   * .   **:. *  : ::. :*..:  . .:     * : **::* .

YggA   LALLAAWLAPRLRTAKAQRIINLVVGCVMWFIALQLARDGIAHAQALFS 210
YahN   LSQAFSLPAVRRAYGRMQRVASRVIGAIIGVFALRLIYEGVTQR----- 223
       *:   :  * *   .: **: . *:*.:: .:**:*  :*:::
```

Fig. 5.11 ClustalW alignment between the YggA and YahN proteins of *E. coli*. Similarity between the aligned polypeptide sequences (denoted in the one letter amino acid code) derived from the ORFs of *yggA* and *yahN* in *E. coli*. Amino acid numbering is indicated at the right end of each line. Each position of identity and of conservative substitution (within one or another of the following groups: D, E, N, Q; H, K, R; S, T; A, G; I, L, M, V; or F, W, Y) between the two sequences is represented respectively, by an asterisk or a colon. Where necessary, gaps have been introduced into the sequence to maximize the homology in alignment.

If this assumption were to be correct, the introduction of the *yggA* mutation into MG1655 should not make the strain Can^{SS}. To test this hypothesis, the *yggA* allele was transduced into MG1655 using the P1 lysate prepared on *yggA301*::Tn*10*dTet (GJ4676) and selecting for Tet^R colonies. All the Tet^R transductants tested were Can^{SS}, indicating that YahN does not substitute for YggA function in MG1655. The MG1655 *yggA* strains were as Can^S as MC4100 *yggA*.

5.5.7 A *yggB* deletion mutant is not Can^{SS}:

As mentioned above, the *yggB* ORF is present upstream of and in the same orientation as *yggA*. The predicted start codon of *yggA* from the stop codon of *yggB* is separated by 139 bp. *yggB* is predicted to encode a protein with sequence similarity to mechanosensitive channels, and the biochemical evidence for its channel properties has been obtained (Levina et al., 1999).

Two *yggB* deletion strains kindly provided by Ian Booth, MJF451 (Δ*yggB*) and MJF455 (Δ*yggB mscL*::Cm), were tested for their tolerance to Can. Neither strain was Can^{S}, establishing that *yggB* and *yggA* are not functionally related to one another.

5.5.8 Cloning of the *yggA* locus from Kohara phage λ-472 and complementation studies:

As described above in Section 5.4.6, the PCR strategy followed by sequencing of the amplicons had shown that insertions in the *yggA* gene conferred Can^{SS} phenotype. For complementation studies, *yggA* locus was first cloned in the following fashion. A 3.8 kb *Eco*RI fragment from phage λ-472 of the ordered library of Kohara *et al.* (1987), covering *yggA*, was initially cloned into pBS(KS) vector at its *Eco*RI site to generate pHYD944. In the next step, the insert DNA was released from pHYD944 following *Bam*HI / *Kpn*I digestion and cloned at the *Bam*HI / *Kpn*I site of pCL1920 to generate the plasmid pHYD945 (Fig. 5.12). Each of the nine *yggA*::Tn*10*dTet strains was then transformed with either pCL1920 or pHYD945, and the transformants were tested for growth on MA + Can (20 μg/ml) plates supplemented with Spec. Plasmid pHYD945, but not the pCL1920 vector, reversed the Can^{SS} phenotype of the *yggA* strains, indicative of positive complementation.

In addition to *yggA*, pHYD945 also carries the intact *yggB* and *fba* genes. A series of subcloning experiments was then performed, as depicted in Fig. 5.12 and described below:

(i) A 2.2 kb *Hin*dIII fragment cloned into the *Hin*dIII site of pCL1920 in either orientation (pHYD947 and pHYD948) was positive for complementation;

(ii) A 1.6 kb *Eco*RI-*Pst*I fragment cloned into the *Eco*RI-*Pst*I site of pBS(KS) (pHYD950) was also positive for complementation;

(iii) A 1.2 kb *Hin*dIII-*Pst*I fragment cloned into the *Hin*dIII-*Pst*I site of pCL1920, pBS(KS) and pBR329 (pHYD949, pHYD951 and pHYD952, respectively) was positive for complementation.

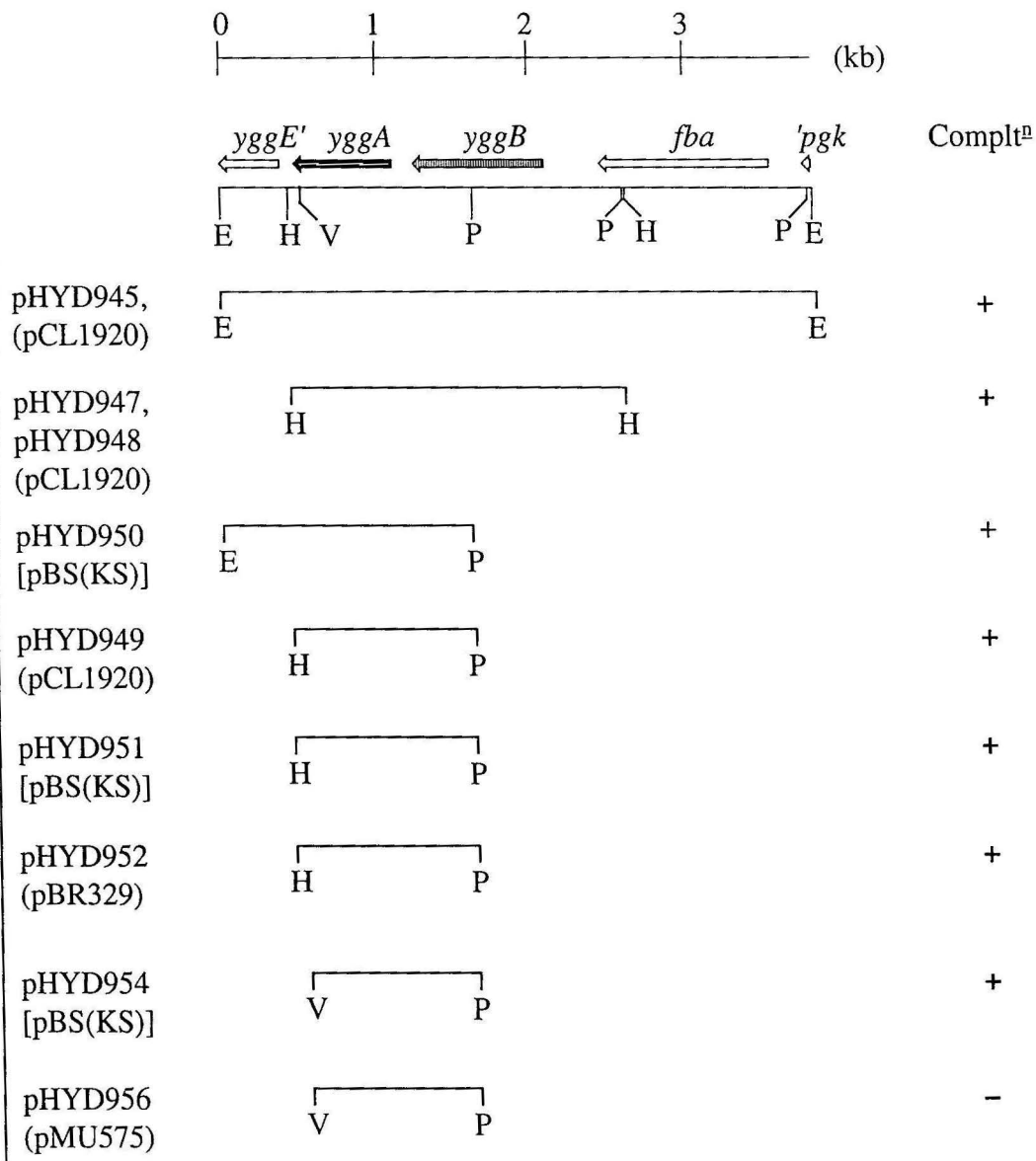


Fig. 5.12 Cloning of the *yggBA locus*, subcloning and complementation analysis. Below the kilobase scale is depicted the physical map of 3.8 kb *Bam*HI-*Kpn*I fragment from Kohara phage λ-472 for the enzymes *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), and *Pvu*II (V). The position of various genes and their transcriptional organization (arrows) are marked. Each line aligned beneath the physical map represents the extent of chromosomal DNA cloned into a plasmid, whose numerical pHYD designation is given alongside with the vector backbone in parantheses. Also indicated in the column marked Complt^{n} at right is the ability of the plasmid to complement (+) or not to complement (–) the yggA mutant for Can^{SS} phenotype.

The above results wherein, the 1.2 kb *Hin*dIII-*Pst*I fragment containing only the intact full length *yggA* gene along with N-terminal truncated *yggB* gene was shown to complement the Can^{SS} phenotype, indicated that *yggA* alone is necessary and sufficient for Can-tolerance phenotype in these strains.

In the subsequent experiments, a 1.1 kb *Pst*I-*Pvu*II fragment (which is capable of coding for YggA protein devoid of C-terminal 14 amino acids) was cloned into the *Pst*I-*Pvu*II site of pBS(KS) and pMU575 [an IncW-based single copy (s.c.) number plasmid] and designated pHYD954 and pHYD956, respectively. The truncated protein and gene would hereinafter be referred to as YggA' and *yggA'*, respectively. These plasmids were introduced into *gltBD yggA* (GJ4691) and MC4100 (WT) strains along with pBS(KS) and pMU575 as vector controls, and streaked on MA medium supplemented with 20 μg / ml

Table 5.14 Multicopy effects of *yggA*$^+$ and *yggA'* alleles on Can phenotype of WT, *argP* and *yggA* strains[a]

Strain (genotype)	Plasmid (genotype)	MA + Amp + Can (μg/ml) 0	20
MC4100 (WT)	pBR329 (vector)	++	++
MC4100 (WT)	pHYD952 (m.c. *yggA*$^+$)	++	++
GJ4654 (*gltBD argP*)	pBR329 (vector)	++	–
GJ4654 (*gltBD argP*)	pHYD952 (m.c *yggA*$^+$)	++	+
GJ4691 (*gltBD yggA*)	pBR329 (vector)	+	–
GJ4691 (*gltBD yggA*)	pHYD952 (m.c. *yggA*$^+$)	+	+
MC4100 (WT)	pBS(KS) (vector)	+	+
MC4100 (WT)	pHYD954 (m.c. *yggA'*)	+	+
GJ4820 (*gltBD argP*)	pBS(KS) (vector)	+	–
GJ4820 (*gltBD argP*)	pHYD954 (m.c. *yggA'*)	+	±
GJ4691 (*gltBD yggA*)	pBS(KS) (vector)	+	–
GJ4691 (*gltBD yggA*)	pHYD954 (m.c. *yggA'*)	+	+

[a] The indicated strains were streaked on MA + Amp plates supplemented with 0 and 20 μg/ml Can. The plates were incubated for 24 h at 37°C. Growth was scored on the following qualitative 4-point scale (in increasing order): — (no growth), ±, + and ++ (full growth).

Can. The results showed that multicopy (m.c.) *yggA'* (pHYD954) but not s.c. *yggA'* (pHYD956) was able to complement the mutant for Can^{SS} phenotype (Tables 5.14 and 5.15).

Table 5.15 Effect of single copy *yggA'* on Can^{SS} phenotype of *argP* and *yggA* strains[a]

Strain (genotype)	MA + Tp + Can	
	Vector (pMU575)	*yggA'* (pHYD956)
MC4100 (WT)	+	+
GJ4820 (*argP*)	–	–
GJ4691 (*yggA*)	–	–

[a] The indicated strains were streaked on MA + Tp plates supplemented with 20 μg/ml Can. The plates were incubated for 24 h at 37°C. Growth was scored on the following qualitative 2-point scale (in increasing order): – (no growth) and + (full growth).

5.5.9 Effect of m.c. *yggA*$^+$ and m.c. *yggA'* alleles on Can phenotype of the *argP* strain:

To test the effect of m.c. *yggA*$^+$ on Can^{SS} phenotype of an *argP* mutant, the strains GJ4654 (*gltBD argP*) and MC4100 (WT) were transformed with pBR329 (vector) or pHYD952 (1.2 kb full length *yggA* gene). On MA + Amp + Can (20 μg/ml), GJ4654 / pHYD952 grew on par with MC4100 / pBR329 and MC4100 / pHYD952 transformants (Table 5.14). This result showed that when *yggA*$^+$ was present on a m.c. plasmid, the Can^{SS} phenotype of the *argP* mutant was suppressed.

To test the effect of m.c. *yggA'*, plasmids pBS(KS) and pHYD954 were introduced into MC4100 (WT) and GJ4820 (*argP gltBD*), and streaked on MA + Amp + Can (20 μg/ml). The results demonstrated that m.c. *yggA'* suppressed the Can^{SS} phenotype of the *argP* mutant (Table 5.14), indicating that YggA' (in multiple copies) is able to function by bypassing the requirement of ArgP to confer Can-tolerance in an *argP* mutant.

5.5.10 Effect of s.c. *yggA'* on Can phenotype of the *argP* mutant:

To check the effect of s.c. *yggA'* on Can^{SS} phenotype of the *argP* mutant, plasmids pMU575 (vector control) and pHYD956 were introduced into *argP gltBD* (GJ4820). When the resultant Tp^R strains along with MC4100 / pMU575 and MC4100 / pHYD956 control strains were streaked on MA + Tp + Can (20 μg / ml), they continued to be Can^{SS}, demonstrating that s.c. *yggA'* did not suppress the Can^{SS} phenotype of the *argP* mutant (Table 5.15).

5.5.11 Effect of m.c. *yggA*$^+$ on Can^R phenotype of WT strain:

Since mutations in *yggA* confer Can^{SS} in *E. coli*, it was decided to check whether m.c. *yggA*$^+$ would confer Can^R in the WT strain (MC4100).

For this purpose, MC4100 / pBR329 and MC4100 / pHYD952 were streaked on MA + Ura + Amp plates supplemented with varying concentrations of Can. The results showed that WT strain carrying m.c. *yggA*$^+$ was Can^R, compared to the derivative carrying the vector alone.

5.5.12 Arg cross-feeding ability of *argR* strain harboring *yggA* mutation or m.c. *yggA*$^+$:

To test the effect of *yggA* mutation on the Arg cross-feeding ability of *argR* strain, the *argR yggA* strain GJ4894 was patched on the surface of MA + tetrazolium plates containing the *argE* recipient cells, according to the procedure described in Section 2.15.6 in Chapter 2. The results (Fig. 5.13, Panel A) show that the *yggA* mutation marginally decreased the cross-feeding ability of *argR* strain (compare *argR* with *argR yggA*), similar to that seen earlier when *argP* mutation was introduced into the *argR* strain (Fig. 5.2).

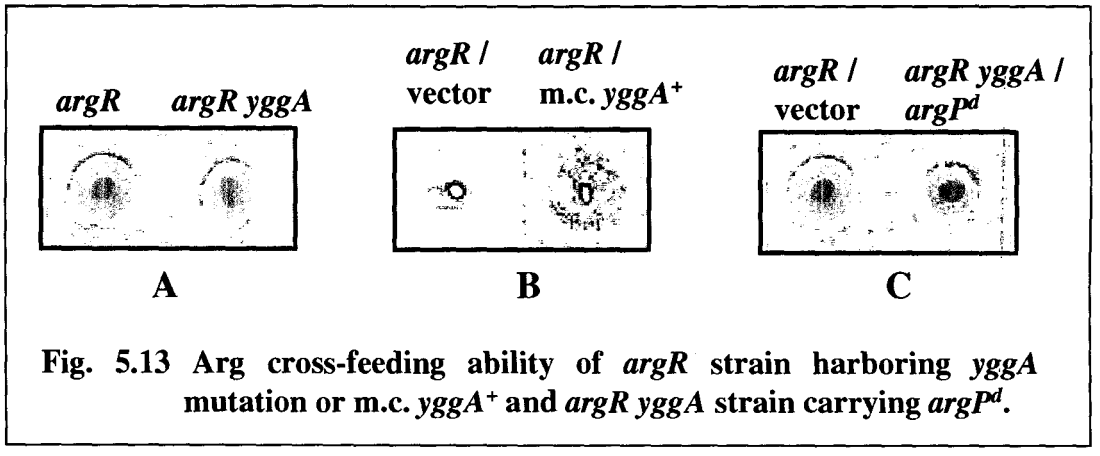


Fig. 5.13 Arg cross-feeding ability of *argR* strain harboring *yggA* mutation or m.c. *yggA*$^+$ and *argR yggA* strain carrying *argP*^{d}.

When plasmid pHYD926 (which carries the S94L $argP^d$ allele) was introduced into the *argR yggA* strain, the Arg cross-feeding ability of the strain was not altered at any time-point of incubation (tested up to 60 h; Fig. 5.13, Panel C). On the other hand, when the S94L $argP^d$ allele was introduced into the *argR* $yggA^+$ strain, it increased the cross-feeding ability of the strain massively (*argR* / $argP^d$) in less than 12 h (Fig. 5.5). This result showed that the $argP^d$-mediated cross-feeding in an *argR* strain was decreased by the *yggA* mutation.

The introduction of plasmid pHYD952 carrying m.c. $yggA^+$ also led to a substantial increase in the Arg cross-feeding ability of the *argR* strain (GJ4748) (Fig. 5.13 Panel B).

5.5.13 Inviability of *argR* strain containing m.c. $yggA^+$ and $argP^d$:

In order to check whether the presence of m.c. $yggA^+$ and $argP^d$ within the same cell would have an additive effect on the Arg cross-feeding ability of an *argR* strain, plasmids pHYD952 (pBR329 with $yggA^+$) and pHYD926 (pCL1920 with $argP^d$) were sought to be introduced sequentially into an *argR* strain (GJ4748). In the first step, GJ4748 was transformed with plasmid pHYD952 and one of the resultant Amp^R colonies was used as recipient in the subsequent transformation with pHYD926. Upon repeated attempts, no Amp^R $Spec^R$ colonies were obtained. The failure to obtain transformants may represent preliminary evidence for the possible incompatibility (that is, synthetic lethality) of m.c. $yggA^+$ with $argP^d$.

5.6 Studies on *yggA* transcriptional regulation

As described above in 5.5.8, the *yggA'* fragment had been cloned in the single copy vector, pMU575, to generate plasmid pHYD956. Plasmid pMU575 is also a promoter-probe vector carrying the *lacZ* reporter gene for generating operon fusions (Yang and Pittard, 1987) and accordingly, plasmid pHYD956 could also be used for studying the *yggA* transcriptional regulation. The 1.1 kb insert contains all the upstream regulatory elements of *yggA* and is truncated within the *yggA* ORF; therefore the *lacZ* expression profile from the plasmid is expected to reflect the *yggA* promoter activity and its regulation by various factors.

In order to test whether the 1.1 kb *Pst*I- *Hin*dIII insert contains the *yggA* promoter sequences, MC4100 (WT strain) was transformed with pMU575 (vector) and pHYD956 (with 1.1 kb *yggA'* insert) and the resultant Tp^R colonies were streaked on MA + Tp + Xgal with 1 mM Arg, LB + Tp + Xgal, and MacConkey + Tp indicator plates. The pHYD956-containing strains were intense blue on LB Xgal and MA + Xgal + Arg plates when compared with the pMU575 transformants, indicating that pHYD956 does contain promoter sequences that drive the expression of the *lacZ* gene present downstream. On MacConkey + Tp plates as well, the MC4100 / pHYD956 transformants were red while MC4100 / pMU575 colonies were white.

5.6.1 Construction of strains harboring various mutations to study *yggA::lacZ* expression:

To examine the effect of various mutations on the *yggA::lacZ* expression, the following derivatives with plasmids pMU575 (vector control) and pHYD956 (*yggA::lac*) were constructed.

(i) The plasmids were introduced into strains with mutations in *yggA* (GJ4822) and *argR* (GJ4748);

(ii) The *argR yggA* strain was constructed in the following fashion. GJ4748 (*argR64*) was transduced to Tet^R using the P1 lysate prepared on GJ4691 (*yggA*::Tn*10*). One of the resultant Tet^R colonies was selected for further work and designated GJ4894. Into this strain, pHYD956 was subsequently introduced. The resultant Tp^R colony was used for studying the *yggA::lacZ* regulation in the *argR yggA* background;

(iii) For studying the effect of *argP* mutations, however, a complicating factor was the fact that both the *argP*::*lac*Kan null insertions isolated in the present study were themselves Lac^+, which might interfere with the measurement of β-galactosidase specific activity of the *yggA::lac* fusion. To circumvent this problem, the *lacZ* gene in GJ4536 (*argP202*::*lac*Kan) was disrupted by P1 transduction employing a lysate prepared on a *lacZ*::Tn*10*dTet strain (GJ2269). The resultant Tet^R strain would inactivate the *lacZ* gene fused to *argP* without interfering with other features of the *argP*::Kan insertion. One such Tet^R colony (which was white on LB Xgal medium) was designated GJ4892 (*argP202*::Kan *lacZ*::Tn*10*dTet). This strain continued to be Can^{SS}, a behavior identical to its parent GJ4536. For examining the effect of the *argP* mutation on *yggA::lacZ* fusion, GJ4892 was transformed with pMU575 and pHYD956;

(iv) The *argP*::Kan lysate prepared on GJ4892 was used to transduce GJ4748 (*argR64*). The resultant Kan^R strain was designated GJ4895 (*argR argP*). Into this strain, both plasmids were introduced;

(v) To construct a strain carrying the $argP^d$ allele, MC4100 was transformed with plasmid pHYD926. As described above in Section 5.3, pHYD926 carries an $argP^d$ allele cloned in pCL1920. Plasmid pHYD915 (pCL1920 carrying $argP^+$) was introduced into MC4100, which served as a copy number control for the experiment. Into the above two strains, pHYD956 was subsequently introduced.

5.6.2 *yggA*::*lacZ* expression studies:

The above strains were grown in MA medium supplemented with Tp (and Spec when strains carried plasmids pHYD915 and pHYD926) till mid-exponential phase (A_{600} ~0.7-0.8) for β-galactosidase assays. To study the effect of various exogenous compounds on *yggA*::*lacZ* expression, Arg, Cit, Orn or Lys was added to the medium at 1 mM, and Can at 40 μg/ml concentrations. β-galactosidase specific activity was measured according to the procedure described in Chapter 2. The results from the various experiments on *yggA*::*lacZ* transcriptional regulation are shown in Table 5.16 and Fig. 5.14 and the salient features of the findings are summarized as follows. In all cases, derivatives transformed with plasmid vector pMU575 exhibited a background β-galactosidase activity of around 9 units, which value has been subtracted from the values obtained for the pHYD956 transfromants in the results described below.

The most prominent findings from all the experiments taken together were that:

1. Arg, or other intermediates in the Arg pathway, as well as Can, moderately induced *yggA*::*lac* in the WT strain;
2. *yggA*::*lac* was also induced substantially (11-fold) in the *argR* mutant, which could be due to the increased intracellular Arg pools in the strain;
3. Most importantly, *yggA* expression was absolutely dependent on $argP^+$. When the *argP* mutation was introduced into an *argR* strain carrying the *yggA*::*lacZ* fusion (GJ4895), the expression was completely abolished, irrespective of whether Arg, Orn, Cit, Can or Lys was added or not. This result provided the first *in vivo* evidence that ArgP might be the transcriptional activator of *yggA*;
4. A mutation in *yggA* had a moderate inducing effect on *yggA*::*lacZ* expression in the presence or absence of Arg, Cit or Orn. However, there was a tremendous (~9.5-fold)

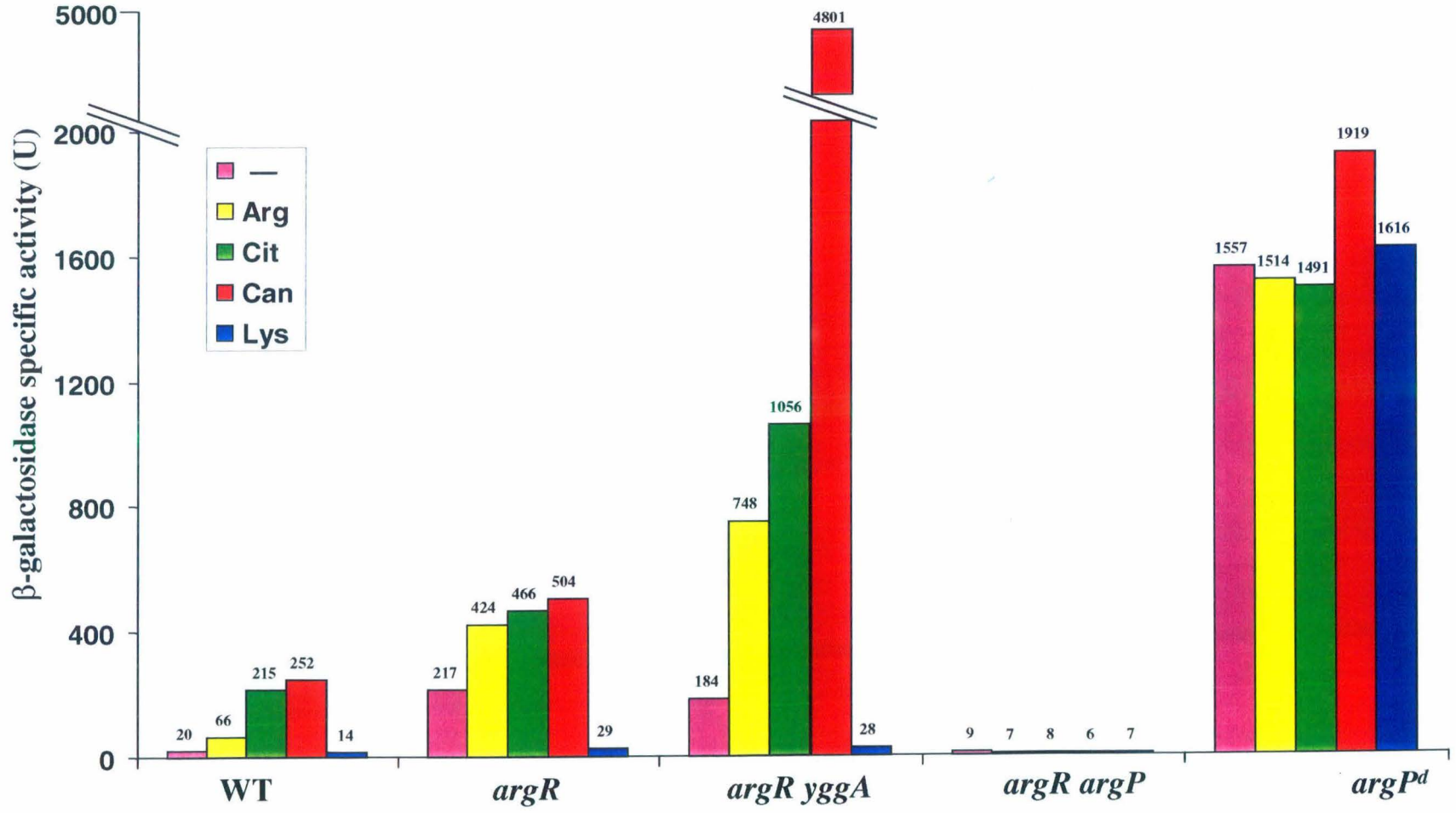


Fig. 5.14 *yggA*::*lac* expression in various mutants in the presence of different amino acids.

induction of the *yggA*::*lacZ* fusion with Can in the *argR yggA* strain (GJ4894), when compared to *argR* strain (GJ4748) (compare 4801 U in the former with 504 U in the latter);

5. Lys seems to repress the *yggA*::*lacZ* expression in an *argR* strain (GJ4748; compare 217 U with 29 U in the absence and presence of Lys, respectively). Lys also inhibited the *yggA*::*lacZ* expression in *argR yggA* (GJ4894) background, similar to that observed in an *argR* strain (compare 28 U in the former with 29 U in the latter). In other words, Lys supplementation was also like the *argP* mutation in abolishing the *argR* (or Arg addition) effect in inducing *yggA* expression;

6. The *yggA*::*lacZ* fusion was massively upregulated in the presence of $argP^d$ alleles (MC4100 / pHYD926). The induction ratio in the absence of any additive was ~173-fold when compared to MC4100/pHYD915 (carrying $argP^+$; compare 1557 U with 9 U). In the presence of Arg, Cit, Orn and Can, the induction ratios were 72-, 18-, 37- and 11-fold, respectively. In the presence of Lys, the $argP^d$ allele induced the *yggA*::*lacZ* expression about 538-fold, as compared to $argP^+$ allele (compare 1616 U in the former with 3 U in the latter). In other words, *yggA*::*lacZ* expression had become co-effector independent in the presence of S94L *argP* allele.

Table 5.16 *yggA*::*lac* expression in various mutants in the presence of different amino acids[a]

Strain (genotype)	β-galactosidase specific activity (U)					
	—	Arg	Cit	Orn	Can	Lys
MC4100 (WT)	20	66	215	70	252	14
GJ4892 (*argP*)	17	16	ND	ND	ND	ND
GJ4822 (*yggA*)	26	92	ND	ND	ND	ND
GJ4748 (*argR*)	217	424	466	439	504	29
GJ4894 (*argR yggA*)	184	748	1056	973	4801	28
GJ4895 (*argR argP*)	9	7	8	9	6	7
MC4100 / pHYD915[b] (WT / $argP^+$)	9	21	81	41	176	3
MC4100 / pHYD926[b] (WT / $argP^d$)	1557	1514	1491	1505	1919	1616

[a] The indicated strains containing the *yggA*::*lacZ* plasmid pHYD956 were grown in Tp-supplemented MA medium without (—) and with 1 mM Arg, Cit, Orn, and Lys, and Can at 40 μg/ml at 37°C to mid-exponential phase for β-galactosidase assays. Enzyme specific acitivity values are reported in Miller Units (Miller, 1992), determined after correction for the background level of β-galactosidase expression from the pMU575 vector.

[b] The medium was additionally supplemented with Spec.

ND = not determined

Given that Arg (or Cit) and Lys supplementation had opposing effects on *yggA* transcription (induction and repression, respectively), the effect of Lys combined with either Arg or Cit was also tested. In the *argR* (GJ4748) and *argR yggA* (GJ4894) strains, the induction observed was intermediate to that obtained when either Lys or Arg or Cit was added individually (Table 5.17).

Table 5.17 *yggA*::*lac* expression in *argR* and *argR yggA* mutants in the presence of Arg, Cit and Lys[a]

Strain[b]	Genotype	β-galactosidase specific activity (U)				
		Arg	Cit	Lys	Arg + Lys	Cit + Lys
GJ4748	*argR* / *yggA*::*lacZ*	424	466	29	121	92
GJ4894	*argR yggA* / *yggA*::*lacZ*	748	1056	28	205	137

[a] The indicated strains containing the *yggA*::*lacZ* plasmid pHYD956 were grown in MA medium supplemented with Tp and with 1 mM Arg, Cit, or Lys at 37°C to mid-exponential phase for β-galactosidase assays. Enzyme specific activity values are reported in Miller Units (Miller, 1992), determined after correction for the background level of β-galactosidase expression from the pMU575 vector.
[b] Both strains contain pHYD956.

5.6.3 Growth phase-dependent expression of *yggA*::*lacZ* expression in the presence of Arg:

Expression of *yggA*::*lacZ* at various steps of growth was studied in cultures grown in the presence of Arg. This experiment was performed to test whether the expression of *yggA* was growth phase-dependent. The results showed that the *yggA*::*lacZ* fusion was expressed moderately and was not affected by the growth phase of the cultures.

5.7 Activator titration experiments

If the hypothesis that ArgP is the activator of *yggA* were to be true, then multiple copies of *yggA* promoter sequence elements would titrate the ArgP protein in an *argP*$^+$ strain, thus rendering the strain phenotypically null for *argP* gene. In order to test this activator titration model, the *yggA'* gene cloned in m.c. pBS(KS) (pHYD954) with its promoter sequences intact was used. The pHYD954 was introduced into MC4100 and GJ4652 (*gltBD*), and also into control strains, GJ4654 (*gltBD argP*), GJ4820 (*gltBD argP*

lacZ::Tet), and GJ4691 (*gltBD yggA*::Tet) and the transformants were tested for the following three phenotypes of *argP*: (a) growth on low-ammonium medium, (b) osmosensitivity, and (c) Can^{SS} phenotype.

5.7.1 Effect of m.c. of *yggA'* on NH_4^+-assimilation phenotype in *gltBD*$^-$ *argP*$^+$ strain:

As described above in Section 5.2.3, the *gltBD argP* double mutant was severely compromised for its ability to grow on low- NH_4^+ medium. *gltBD* strains grow on W-Salts + 0.2-0.4 mM NH_4^+ by 42 to 48 h while the *gltBD argP* mutant fails to grow even at 0.6-0.8 mM NH_4^+.

To test whether multiple copies of *yggA'* would affect the NH_4^+ assimilation phenotype of *gltBD*$^-$ *argP*$^+$ strain, pHYD954 and pBS(KS) plasmids were introduced into MC4100 (WT) and GJ4652 (*gltBD*), and the resultant Amp^R colonies were streaked on W-salts + Amp + NH_4^+ (0.6 mM and 1.2 mM). As controls, the two plasmids were also introduced into GJ4820 (*gltBD argP*) and GJ4691 (*gltBD yggA*), and the resultant derivatives were similarly tested.

Table 5.18 Effect of m.c. *yggA'* on ammonia-assimilation phenotype in *argP*$^+$ *gltBD* strains[a]

Strain (genotype)	W-Salts + Amp			
	0.6 mM NH_4^+		1.2 mM NH_4^+	
	Vector	m.c. *yggA'*	Vector	m.c. *yggA'*
MC4100 (WT)	++	++	++	++
GJ4652 (*gltBD*)	–	–	+	–
GJ4820 (*gltBD argP*)	–	–	–	–
GJ4691 (*gltBD yggA*)	–	–	+	–

[a] The indicated strains carrying pBS(KS) or pHYD954 (*yggA'*) were streaked on Ap-supplemented W-salts plates with either 0.3 or 0.6 mM $(NH_4)_2SO_4$. The plates were incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): – (no growth), + and ++ (full growth).

The results, summarised in Table 5.18, show that GJ4652 (*gltBD*$^-$ *argP*$^+$) with vector grows moderately on 1.2 mM [NH_4^+], but that the same strain with pHYD954 does not grow on this medium. In other words, the latter strain behaves like GJ4820 (*gltBD*$^-$

argP$^-$) with either pBS(KS) or pHYD954, suggesting that *gltBD*$^-$ *argP*$^+$ strain in presence of m.c. *yggA* promoter sequences phenocopies *gltBD*$^-$ *argP*$^-$ strain.

The *yggA gltBD argP*$^+$ strain (GJ4691) in the presence of multiple copies of *yggA* promoter sequences (pHYD954) was also crippled for growth on W-Salts + 1.2 mM NH_4^+ plates, whereas the same strain with pBS(KS) grew moderately well on such a plate. This result can also be interpreted as being a manifestation of ArgP titration in the following fashion. Assuming that the role of ArgP as activator in NH_4^+ assimilation is independent of its role in *yggA* activation, the presence of multiple copies of the *yggA* promoter would be expected to titrate out the ArgP protein required for the cells' ability to grow on W-Salts + low ammonium plates. Thus, the strain would behave like *argP*$^-$ for NH_4^+ assimilation, even though it is chromosomally *argP*$^+$.

5.7.2 Effect of m.c. *yggA'* on osmosensitivity phenotype in *gltBD*$^-$ *argP*$^+$ strain:

From the above result, it was clear that m.c. *yggA* promoter sequences (*yggA'*) phenocopied the *argP* mutation in a *gltBD* strain for the NH_4^+-assimilation phenotype. Since the *gltBD*$^-$ *argP*$^-$ double mutant is also osmosensitive, it was decided to check whether the multiple copies of *yggA'* would make a *gltBD*$^-$ *argP*$^+$ strain osmosensitive. For this, the various strains carrying *yggA'* were streaked on MA + NaCl + Bet + Amp plates and the results are summarized in Table 5.19.

Table 5.19 Effect of m.c. *yggA'* on osmosensitivity phenotype in *argP*$^+$ *gltBD* strains[a]

Strain (genotype)	MA + Amp + NaCl + Bet			
	0.4 M		0.5 M	
	vector	m.c. *yggA'*	vector	m.c. *yggA'*
MC4100 (WT)	+++	+++	+++	+++
GJ4652 (*gltBD*)	+++	++±	++±	+
GJ4691 (*gltBD yggA*)	+++	+	++±	+
GJ4820 (*gltBD argP*)	–	–	–	–

[a] The indicated strains carrying pBS(KS) or pHYD954 (*yggA'*) were streaked on Amp-supplemented MA + 1 mM Betaine plates with either 0.4 or 0.5 M NaCl. The plates were incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 5-point scale (in increasing order): – (no growth), +, ++±, +++± and +++ (full growth).

The results demonstrate that m.c. *yggA* promoter made the *gltBD*$^-$ *argP*$^+$ strain partially NaCl-sensitive. Based on these results, it can be argued that multiple copies of ArgP-binding sequences made the *gltBD*$^-$ *argP*$^+$ strain osmosensitive, by titrating out the ArgP protein.

5.7.3 Effect of m.c. *yggA'* on Can^{SS} phenotype in *argP*$^+$ strain:

To test the effect of m.c. *yggA'* on Can^{SS} phenotype plasmids pBS(KS) (vector control) and pHYD954 (m.c. *yggA'*) were introduced into MC4100 (WT) and GJ4820 (*gltBD argP* as control), and the resulting colonies were streaked on MA + Amp + 20 μg / ml Can plates. Plasmid pHYD954 did not alter the Can phenotype of the *argP*$^+$ strain.

5.8 Discussion:

In the present chapter, results were presented implicating *argP* in ammonia assimilation and osmoadaptation in *E. coli*. The results suggest that *argP* may be involved in *gltBD*-independent NH_4^+-assimilation. Mutations in *argP* appear to act synergistically with *gltBD* to reduce the Glu pools inside the cell, leading to osmosensitivity. Evidence was presented to explain the Can^{SS} phenotype of *argP* null mutants. A hitherto uncharacterized ORF (*yggA*) has been identified, null mutations in which confer Can^{SS} and its transcription *in vivo* was shown to be positively regulated by ArgP and intracellular Arg pools. Furthermore, the results indicate that YggA may be involved in the export of Arg (and related compounds) and accordingly, the name ***argO*** for (Arg outward transport) is proposed for *yggA*.

5.8.1 *argP* acts synergistically with *gltBD* to confer osmosensitivity:

In the screen for insertion mutations conferring NaCl-sensitivity following λp*lac*Mu55 insertional mutagenesis and ampicillin selection, two independent insertions in *argP* were obtained, which displayed three novel phenotypes, osmosensitivity, reduced NH_4^+-assimilation (both also needing an additional *gltBD* mutation) and Can^{SS} (*gltBD*-independent). The *argP* and *gltBD* mutations were shown to act synergistically in conferring sensitivity to dissolved impermeable solutes which are either ionic (NaCl, KCl, Na_2SO_4, K_2SO_4) or nonionic (glucose and sucrose). The *argP gltBD* mutant grew better than the nonspecific sick mutant (*gltBD recA* and *gltBD ruvABC*) strains on MA

medium without osmotic stress; but on MA medium with osmotic stress, the *argP gltBD* mutant was shown to be severely growth-inhibited. These results supported the notion that the *argP gltBD* mutant was osmosensitive. This was a novel phenotype ascribed to *argP* based on the present study. Earlier, *argP* had been suggested to be involved in Arg transport and inhibition of chromosome replication initiation from *oriC*.

5.8.2 Impaired NH_4^+-assimilation in the *argP gltBD* mutant as a cause of osmosensitivity phenotype:

The growth promotion of *argP gltBD* by Asp (which is readily converted to Glu within cells) on media of low a_w pointed to the probable impairment in the double mutant of Glu synthesis and ammonia assimilation (Section 5.2.3) since the latter two phenomena are intimately connected to one another in *E. coli*. It is known that osmotically-stressed cells accumulate Glu, and that such accumulation is necessary for osmotolerance. A *gltBD* single mutant can synthesize Glu only via the glutamate dehydrogenase (GDH)-catalyzed reaction, which might be just sufficient for it to survive under conditions of osmotic stress. It has been previously reported that Glu levels in a *gltBD* mutant are low under high-osmotic growth conditions, and it was found in this study that such a strain is slightly osmosensitive.

The fact that the *argP gltBD* mutant is severely compromised for NH_4^+-assimilation even in the absence of osmotic stress provides strong support to the hypothesis that it is an inability to synthesize sufficient Glu that is responsible for the osmosensitive phenotype of the double mutant.

argP encodes a putative LysR-type of regulator and, as discussed below, there is substantial evidence that it is a transcriptional activator of *yggA*. One can therefore postulate that it is also involved as a transcriptional activator in promoting NH_4^+-assimilation in a *gltBD*-independent fashion. As described in Chapter 1, in *E. coli*, there are only two known pathways by which NH_4^+-assimilation and Glu biosynthesis are accomplished (Chapter 1; Section 1.2). One is GOGAT (encoded by the *gltBD* genes) and the other is GDH (encoded by *gdhA*). Thus, the likely target of ArgP in a *gltBD* mutant strain could be *gdhA* or some of the intricate pathways of the nitrogen regulation (Ntr) system involved in the control of NH_4^+-assimilation and Glu biosynthesis.

5.8.3 Null mutations in *argP* confer Can^{SS} phenotype:

The two independent *argP*::Kan null mutants obtained in the present study were extremely Can^{SS} [sensitive to 2 μg/ml (*argP202*::Kan) and 20 μg/ml (*argP203*::Kan)], and as expected, the mutations were recessive to *argP*$^{+}$. According to Celis' model, ArgP activates Arg transport by activating the transcription of *argK* and thus, regulating the levels of the LAO- and AO-binding proteins (Celis *et al.*, 1973; Celis, 1977a, b; 1990). Point mutations in either *argP* or *argK* were shown to confer Can^{R}, and it was argued that these mutations rendered the proteins nonfunctional, which resulted in the inability of the cells to transport Arg or its toxic analog Can, thus conferring the Can^{R} phenotype to the strain (Celis, 1973; Rosen, 1973b). By *in vitro* run-off transcription assays employing purified ArgP and promoter sequences of *argK* and *argP*, it was reported that ArgP activates the expression of *argK* and represses the transcription of *argP* gene in the presence of Arg (that is, negative autoregulation; Celis, 1999). This earlier reported work mentions only about the trans-dominant allele of *argP* (presumed to be negative dominant) but the *argP* null mutant phenotype was not determined.

If the hypothesis that ArgP activates genes involved in Arg transport were true, then loss-of-function null mutations in *argP* should also give rise to Can^{R}. But the fact that both the *argP* null mutants obtained in the present study were Can^{SS} suggests that the model put forth by Celis for the role of ArgP in Arg uptake is incorrect. The claim by Celis that ArgP activates *argK* transcription *in vitro* may also have to be reviewed since the template used in his studies would not, on analysis of the subsequently published genome sequence of *E. coli*, be expected to carry the *argK* promoter at all.

Another result from the present study which is not consistent with the previously reported findings is the autoregulation of the *argP* gene. The results in Section 5.3.8 demonstrate that *argP* expression was not altered in the presence of the *argP*::Kan mutation or *argP*^{d} Can^{R} allele (pHYD926), indicating that there might not be any autoregulation of *argP* transcription. Also, it was demonstrated that in the present studies that Lys, Arg and Orn, which are reported to bind to LAO- and AO- binding proteins, did not alter the expression of *argP*::*lacZ*. Since these results were obtained employing the *argP*::*lacZ* fusion in an *in vivo* system, they are likely to reflect the physiological expression more accurately than the previously reported *in vitro* run-off transcription assays (Celis, 1999).

5.8.4 Identification of *yggA* null mutants conferring Can^{SS}:

In attempts to obtain additional *argP* alleles and to investigate the possible role of null mutations in *argK* in Can phenotype, localized mutagenesis around the *argP* locus was performed. No Can^R mutants but instead nine Can^{SS} mutants were obtained in the screen, none of which was in *argP, argK* or *ygfE* (Section 5.4.5). Neither $argP^+$ nor $argK^+$ complemented the Can^{SS} phenotype. Molecular characterization of these mutants employing inverse PCR technique followed by sequencing unexpectedly revealed that all the Tn*10*dTet insertions were in *yggA*, an hitherto uncharacterized ORF located 8 kb away from *argP*. The Can^{SS} phenotype of both the *argP* and *yggA* mutants was abolished by mutation in *argR*, presumably by decreasing the intracellular Can to Arg ratio.

5.8.5 *yggA* mutations do not affect osmotolerance and NH_4^+ assimilation property in *gltBD* mutants:

The *yggA* gene, like *argP*, is non-essential. *yggA* mutations by themselves or in combination with *gltBD* mutation did not confer water-stress sensitivity (unlike the *argP gltBD* mutant), suggesting that *yggA* represents a novel locus which contributes to Can-tolerance but not to osmosensitivity phenotype (Section 5.5.1). The *yggA* mutation did not also alter the NH_4^+-assimilation property of *gltBD* mutant either (Section 5.5.2). These two results suggested that *argP* and *yggA* might act in similar fashion in conferring Can^{SS} phenotype, but that the former might have additional roles in conferring osmotic and NH_4^+-assimilation phenotypes.

5.8.6 *yggA* and *yggB* do not constitute an operon:

$yggA^+$ gene was cloned from the Kohara *E. coli* library and was shown to complement all the nine *yggA*::Tn*10*dTet mutants for Can^{SS} phenotype. A closer examination of the *yggA* locus revealed that *yggB* and *yggE* genes are located immediately upstream and downstream of *yggA*, respectively (Fig. 5.6). The presence of very short regions of intervening sequence between *yggB* and *yggA* pointed to the possibility of their being part of an operonic arrangement. To test this hypothesis, *yggA* alone, along with the C-terminal region of *yggB* without its promoter and majority of the N-terminal region, when introduced into all the nine *yggA* mutants, complemented for Can^{SS} (Section 5.5.8). This result suggested that *yggA*, but not *yggB*, was necessary and sufficient for conferring Can-tolerance in *E. coli*. This result also demonstrated that *yggA* and *yggB* might not be arranged in an operon. By employing deletion mutants of the

upstream gene, *yggB* (which is believed to encode a mechanosensitive channel), it was shown that Δ*yggB* does not confer Can^{SS} (Section 5.5.7).

5.8.7 *yggA* is epistatic to *argP* in conferring Can^{SS} phenotype:

In an effort to get a closer insight into the mechanism of ArgP in Can-tolerance, allelic variants of the *argP* gene were generated following mutagenesis with MNNG and selecting for Can^{R} colonies. MNNG treatment results in G.C → A.T transition mutations. Seven Can^{R} *argP* alleles were obtained, of which six were shown to be dominant over *argP*$^{+}$ allele (Section 5.3.2). The sequencing of these various gain-of-function *argP*^{d} alleles revealed that the mutations spanned the length of the *argP* gene (Fig. 5.4). One representative Can^{R} *argP* allele was introduced into *yggA* null mutants and the resultant colonies continued to be Can^{SS}, demonstrating that *yggA* is epistatic to *argP* (Section 5.5.5). Such epistatic behaviour implies that *yggA* acts downstream of *argP* in a common pathway, and that the phenotype conferred by the dominant *argP* alleles requires a functional *yggA*$^{+}$ gene. As further discussed below, this conclusion is strongly corroborated by *in vivo* studies on *yggA*::*lac* regulation.

argP gltBD osmosensitivity was shown to be unlinked to the role of *argP* in Can phenotype since Can^{R} *argP* alleles did not affect NaCl-tolerance appreciably and *yggA gltBD* double mutants were not osmosensitive.

5.8.8 *yggA* is the transcriptional target of ArgP:

ArgP-like orthologs (belonging to the LysR-family of transcriptional regulators) are present in most of the microbes sequenced so far and there is a high degree of conservation of amino acid sequences amongst the members (Fig. 5.15). This similarity at the protein level suggests functional conservation of ArgP in prokaryotes. Before the studies on *yggA*::*lac* were initiated, the following lines of preliminary evidence had indicated that ArgP could be the transcriptional regulator of *yggA* and that the Can^{SS} phenotype of *argP* null mutants is a consequence of the lack of expression of *yggA*.

(a) *argP* and *yggA* null insertions both conferred Can^{SS} phenotype, with *yggA* being more sensitive than *argP*. This could mean that *argP* and *yggA* act in the same pathway in conferring Can^{SS} phenotype;

(b) *yggA*::Tn*10*dTet mutation was epistatic to an *argP*^{d} allele in conferring Can^{SS} phenotype. This result argues that *yggA* acts downstream of *argP* in conferring Can^{SS} phenotype;

(c) *yggA* and *argP* are homologous, respectively, to *lysE* (encoding a membrane-localized lysine exporter) and *lysG* (encoding LysR-type of transcriptional regulator which controls the expression of *lysE*; Bellmann *et al.*, 2001) respectively in *Corynebacterium glutamicum* (Fig. 5.15 and 5.16);

(d) m.c. *yggA*$^{+}$ suppressed the Can^{SS} phenotype of the *argP* mutant, presumably by nonphysiological expression of *yggA* bypassing the requirement for ArgP (Section 5.5.9).

(e) Presence of m.c. *yggA* promoter sequences (m.c. *yggA'*) in a *gltBD*$^{-}$ *argP*$^{+}$ strain phenocopied *argP* mutation for NH_4^+- and osmosensitivity- phenotypes (Section 5.7), possibly by titrating the activator protein ArgP.

Based on these findings, and in order to test directly the hypothesis that ArgP is the transcriptional activator of *yggA*, the *yggA*::*lacZ* transcriptional fusion was constructed and the effect of *argP* on its expression was studied. The *yggA*::*lacZ* expression *in vivo* was shown to be abolished in an *argP* mutant, providing strong evidence that ArgP is the transcriptional activator of *yggA* (Section 5.6). (The results were most clear in an *argR* background.) This is the first *in vivo* evidence for the activator role of the previously predicted transcriptional regulator ArgP.

In the WT strain, *yggA*::*lacZ* fusion was induced ~3-fold in the presence of exogenous Arg or Orn and 10-fold by Cit. Orn and Cit are converted to Arg within the cell, suggesting that Arg may be the co-effector of ArgP-mediated transcriptional activation of *yggA*.

The *yggA*::*lacZ* fusion was also induced about 9-fold in an *argR* strain. This result can be explained by either of two ways. In an *argR* strain, the Arg biosynthetic pathway is derepressed, resulting in high endogenous levels of Arg that may induce the *yggA*::*lacZ* expression. Alternatively, ArgR may directly regulate *yggA*::*lacZ* expression as a repressor. While the latter explanation has not completely been ruled out by the data in the present study, it is rendered less likely by the fact that the increase in *yggA* expression in the *argR* mutant was abolished upon introduction of an *argP* null mutation.

```
E.c.          ----------------MKRPDYRTLQALDAVIRERGFERAAQKLCITQSAVSQRIKQLEN 44
Y.p.          MWHSVVSIYYFGEMTPMKRPDYRTLQALDAVIRERGFERAAQKLCITQSAVSQRIKQLEN 60
V.c.          ----------------MRGLDYRWIEALDSVVSKGSFERAAEQLFISQSAVSQRIKQLEK 44
P.s           ------------------MFDYKLLSALAAVIEQAGFERAAQVLGLSQSAVSQRIKLLEA 42
C.g.          --------------------MNPIQLDTLLSIIDEGSFEGASLALSISPSAVSQRVKALEH 41
M.t.          --------------MVDPQLDGPQLAALAAVVELGSFDAAAERLHVTPSAVSQRIKSLEQ 46
                                :   : :* :::   .*: *:  * :: ******:* **

E.c.          MFGQPLLVRTVPPRPTEQGQKLLALLRQVELLEEEWLGD---EQTGSTPLLLSLAVNADS 101
Y.p.          LFGQPLLVRTVPPRPTEQGQKLLALLHQVELLEEEWLGN---DNGVDTPLLLSLAVNADS 117
V.c.          YLAQPVLIREQPPRPTLVGKKLLGLYRRVCLIEQELVPEL-TNQEHVRPVSMSIATNADS 103
P.s           RIGQPVLVRATPPSPTDIGRRLLNHVQQVRLLERDLQSQVPALDEEGMPERLRIALNADS 102
C.g.          HVGRVLVSRTQPAKATEAGEVLVQAARKMVLLQAETKAQ-LSG--RLAEIPLTIAINADS 98
M.t.          QVGQVLVVREKPCRATTAGIPLLRLAAQTALLESEALAE-MGGNASLKRTRITIAVNADS 105
               ..: :: *  *  .*  *  *:    :  *:: :   :             : :* ****

E.c.          LATWLLPALAPVLADSPIRLNLQVEDETRTQERLRRGEVVGAVSIQHQALPSCLVDKLGA 161
Y.p.          LATWLLPALKPVLADLPIRLNLQVEDETRTQERLRRGEVVGAVSIQPQPLPSCLVDQLGA 177
V.c.          LATWLLPALDKVMKSRQVELNLVIYGESRTLDKLKNGEVVGAISLEPQPITGCSAEYLGQ 163
P.s           LATWWAPAIGDFCTQHRLLLDLVVEDQDVGLKRMRAGEVAACLCGSERPVAGARSQLLGA 162
C.g.          LSTWFPPVFNEVASWGGATLTLRLEDEAHTLSLLRRGDVLGAVTREANPVAGCEVVELGT 158
M.t.          MATWFSAVFDGLG---DVLLDVRIEDQDHSARLLREGVAMGAVTTERNPVPGCRVHPLGE 162
              ::**  ..:  .       * : : .:      :: * . ..:  . ..:...    **

E.c.          LDYLFVSSKPFAEKYFPNGVTRSALLKAPVVAFDHLDDMHQAFLQQNFDLPPGSVPCHIV 221
Y.p.          LDYLFVASKAFAERYFPNGVTRSALLKAPAVAFDHLDDMHQAFLQQNFDLSPGSVPCHIV 237
V.c.          MEYLCVASPEFYQKYFAKGVTPRSLIKAPAVSYDQYDELHNKFLWDYFAVPRDKVINHTV 223
P.s           MRYRALASPAFIARHFADGVSPEKLVRSAALVYGPDDLLQHRYLAL-LGVQDG-FEHHLC 220
C.g.          MRHLAIATPSLRDAYMVDG--KLDWAAMPVLRFGPKDVLQDRDLDGRVDGPVGRRRVSIV 216
M.t.          MRYLPVASRPFVQRHLSDGFTAAAAAKAPSLAWNRDDGLQDMLVRKAFRRAITR-PTHFV 221
              : :  ::: :   :: .*        . : :.  * ::.  :   .

E.c.          NSSEAFVQLARQGTTCCMIPHLQIEKELASGELIDLTPGLFQRRMLYWHRFAPESRMMRK 281
Y.p.          NSSEAFVQLARQGTTCCMIPHLQIEKELASGELIDLTPGLLQRRMLFWHRFAPESRTMRK 297
V.c.          GSSEAFVRLALSGAAYCLIPRLQIISELESGALINMTPDFMLSYPIFWHHWQLETGVLLE 283
P.s           PSSEGFIRMIEAGMGWGLAPELQVREQLRSGTLLELLPDRCIDVPLYWHHWRNGGQLLTR 280
C.g.          PSAEGFGEAIRRGLGWGLLPETQAAPMLKAGEVILLD-EIPIDTPMYWQRWRLESRSLAR 275
M.t.          PTTEGFTAAARAGLGWGMFPEKLAASPLADGSFVRVC-DIHLDVPLYWQCWKLDSPIIAR 280
               ::*.*      *    : *.      *  * .: :         ::*: :      : .

E.c.          VTDALLDYGHKVLRQD------- 297
Y.p.          VTDALLSYGRQVLRQDSFIGQ-- 318
V.c.          ISEAITAYAKSVLPQ-------- 298
P.s           LTGHLAQHAAHWLVPLSDE---- 299
C.g.          LTDAVVDAAIEGLRP-------- 290
M.t.          ITDTVRAAASGLYRGQQRRRRPG 303
              ::  :   .
```

Fig. 5.15 ClustalW alignment between the ArgP proteins of *E. coli* and other microbes. Similarity between the aligned polypeptide sequences (denoted in the one letter amino acid code) derived from the ORFs of *argP* in *E. coli* (E.c.) and related sequences in *Yersinia pestis* (Y.p.), *Vibrio cholerae* (V.c.), *Pseudomonas syringae* pv. *syringae* (P.s.), *Corynebacterium glutamicum* (C.g.) and *Mycobacterium tuberculosis* (M.t.). Amino acid numbering is indicated at the right end of each line. Each position of identity and of conservative substitution (within one or another of the following groups: D, E, N, Q; H, K, R; S, T; A, G; I, L, M, V; or F, W, Y) between the two sequences is represented respectively, by an asterisk or a colon. Where necessary, gaps have been introduced into the sequence to maximize the homology in alignment.

```
E.c.          ----------------------------MFSYYFQGLALGAAMILPLGPQNAFVMNQGIR 32
Y.p.          ----------------------------MLAVYLHGFILSAAMILPLGPQNVFVMNQGIK 32
V.c.          ----------------------------MNWWILLQGFSLGATMIIPIGAQNAYVLNQGIK 33
P.s.          MRDDDYWFYLYRFNIESRHLNLLDGNVFMWQSYFNGLLIAAGLIMAIGSQNAFVLAQGLR 60
M.t.          ---------------------------------------MMTLKVAIGPQNAFVLRQGIR 21
C.g.          ----------------------------MVIMEIFITGLLLGASLLLSIGPQNVLVIKQGIK 34
                                                     : :.:*.**. *: **::

E.c.          RQYHIMIALLCAISDLVLICAGIFGG-SLLMQSPWLLALVTWGGVAFLLWYGFGAFKTAM 91
Y.p.          RQHHLMSASLCALSDIILICAGIFGGSALLSRSPLLLALVTWGGVAFLMWYGWGALMAAW 92
V.c.          RHHHLTTAATCGVLDMIFITLGIFGGGALISQNTSLLIGVTLAGILFLCGYGFLSLRAAL 93
P.s.          REHHVSVAMLCIICDAILVAAGVFGLANVLAQNPTLLAVARWGGVLFLSWYGVQALRRAC 120
M.t.          REYVLVIVALCGIADGALIAAGVGGFAALIHAHPNMTLVARFGGAAFLIGYALLAARNAW 81
C.g.          REGLIAVLLVCLISDVFLFIAGTLGVDLLSNAAPIVLDIMRWGGIAYLLWFAVMAAKDAM 94
              *.  :     * : *  :.  *  *   :    . :      .*  :*  :.  :   *

E.c.          SS-----------------NIELASAEVMKQGR---------------WKIIATMLAVTW 119
Y.p.          RG-----------------DGVASSATSVTQGR---------------WRILVTLLAVTW 120
V.c.          KPP----------------QASESTANPMAAGR---------------KAVIFGAFAVTV 122
P.s.          SK-----------------QSLEHGAAAGKRSR---------------RTVLLSALAVTL 148
M.t.          RP-------------------SGLVPSESGPAAL---------------IGVVQMCLVVTF 108
C.g.          TNKVEAPQIIEETEPTVPDDTPLGGSAVATDTRNRVRVEVSVDKQRVWVKPMLMAIVLTW 154
                                    :                              :   :.:*

E.c.          LNPHVYLDTFVVLGSLGGQLDVEPKRWFALGTISASFLWFFGLALLAAWLAPRLRTAKAQ 179
Y.p.          LNPHVYLDTFVVLGSLGGQLLPDIRPWFALGAVTASIVWFFALALLAAWLSPWLNRPVAQ 180
V.c.          FNPHLYLDTVVILGSIGGQFQGDERISFAIGTILASFVWFFTLSLGAAKLSTTLSKPRVR 182
P.s.          LNPHVYLDTVLLIGSLGAQQSVPG--AYVAGAASASLLWFSCLAIGAAWLAPWLARPATW 206
M.t.          LNPHVYLDTVVLIGALANEE-SDLRWFFGAGAWAASVVWFAVLGFSAGRLQPFFATPAAW 167
C.g.          LNPNAYLDAFVFIGGVGAQYGDTGRWIFAAGAFAASLIWFPLVGFGAAALSRPLSSPKVW 214
              :**: ***:.:.:*.:. :        :  *:  **.:**  :.: *. *   :  . .

E.c.          RIINLVVGCVMWFIALQLARDGIAHAQALFS--- 210
Y.p.          RIINLFVGGVMGFIAFQLARQGFGL--------- 205
V.c.          QVIDMAVAAMMFIIAFALTKN-LYLNHWLN---- 211
P.s.          RLLDVMVAVMMFSVAWQLIRSA------------ 228
M.t.          RILDALVAVTMIGVAVVVLVTSPSVPTANVALII 201
C.g.          RWINVVVAVVMTALAIKLMLMG------------ 236
              : ::  *.  *  :*  :
```

Fig. 5.16 ClustalW alignment between the YggA proteins of *E. coli* and other microbes. Similarity between the aligned polypeptide sequences (denoted in the one letter amino acid code) derived from the ORFs of *argP* in *E. coli* (E.c.) and related sequences in *Yersinia pestis* (Y.p.), *Vibrio cholerae* (V.c.), *Pseudomonas syringae* pv. *syringae* (P.s.), *Corynebacterium glutamicum* (C.g.) and *Mycobacterium tuberculosis* (M.t.). Amino acid numbering is indicated at the right end of each line. Each position of identity and of conservative substitution (within one or another of the following groups: D, E, N, Q; H, K, R; S, T; A, G; I, L, M, V; or F, W, Y) between the two sequences is represented respectively, by an asterisk or a colon. Where necessary, gaps have been introduced into the sequence to maximize the homology in alignment.

It was identified in this study that Arg, Cit, Orn and Can induce and Lys repress *yggA* expression, all via ArgP and it remains to be determined what is (or are) the true co-effector(s) for ArgP. The protein may have a single co-effector binding site, in which case the other identified molecules modulate the concentration of the co-effector or serve as its analogs or competitors for binding; alternatively, the protein may have more than one binding site for different co-effectors.

5.8.9 Possible molecular explanations for *trans*-dominance of various *argP* alleles:

As described in Section 5.3, dominant *argP* mutations conferring Can^R were identified and molecularly characterized. The *yggA::lacZ* expression data presented in Section 5.6 showed that one of them (S94L) had increased the transcription of the *yggA* gene 80 to 90-fold, and rendered it constitutive of any co-effectors, supporting the hypothesis that this and other $argP^d$ alleles of *argP* confer Can^R through increased transcription of *yggA*. The simplest interpretation is that the $argP^d$ alleles encode variant ArgP proteins that are able to constitutively activate *yggA* transcription, and this needs to be further studied by *in vitro* experiments.

The dominance of the various Can^R *argP* alleles over WT *argP* gene can be explained if one assumes that ArgP acts as a homo-oligomer, and that the hetero-oligomers of the Can^R ArgP variant with WT Arg continue to be constitutive activators. It has been shown previously that the LysR-type of transcriptional regulators form dimers, and that it is the dimeric form which is active in gene regulation function (Schell, 1993). It was previously shown in *in vitro* assays that ArgP (IciA) binds to the *dnaA* promoter 1P element as a dimer and such a binding enhances the binding of RNA polymerase to the *dnaA* promoter 1P. Also, it was reported that ArgP (IciA) behaves as dimers in solution (Hwang and Kornberg, 1990).

Of the seven Can^R *argP* alleles obtained during the screen, only one (i.e., R295C) was recessive to $argP^+$ allele. This mutant protein might be able to activate the *yggA* gene constitutively in the absence of WT ArgP. The recessivity of ArgP(R295C) to WT ArgP can be explained in either of two ways. One is that the stability of ArgP(R295C)-ArgP heterodimers might be less, which results in decreased transcription of *yggA* by the heterodimer as compared to ArgP(R295C) homodimer. The other possibility is that the ArgP(R295C)-ArgP heterodimer is not proficient in binding to the operator sequences of *yggA*, unlike ArgP(R295C) homodimers. Understanding the molecular basis of recessivity of ArgP(R295C) may be useful in deciphering the ArgP-mediated transcriptional activation of the *yggA* gene in *E. coli*.

The various point mutations conferring Can^R were obtained in different predicted domains of ArgP, and it would be interesting to delineate the mechanisms by which each of them renders ArgP constitutive for the transcription of *yggA*.

5.8.10 Does *yggA* encode an efflux system for Arg?

Based on the homology between YggA and other members of the membrane-associated efflux systems, it is tempting to suggest that *yggA* encodes a membrane protein involved in efflux of basic amino acids like Arg. Thus, the Can^{SS} phenotype of *argP* and *yggA* can be explained based on the assumption that *yggA* is involved in the efflux of Arg and its analog, Can. That YggA might act as an exporter of Arg is suggested also by the diametrically opposite effects of *yggA* and *argP* null mutations on the one hand, and m.c. $yggA^+$ or $argP^d$ alleles on the other, on Can-tolerance and / or Arg cross-feeding ability (in an *argR* background). Loss-of-function mutations in *argP* and *yggA* decreased the Arg cross-feeding ability when introduced into an *argR* strain (Sections 5.2.10 and 5.5.12). On the other hand, when $yggA^+$ was introduced into an *argR* strain on a m.c. plasmid, it increased the cross-feeding ability substantially (Section 5.5.12). Similarly, $argP^d$ also increased the cross-feeding ability of an *argR* strain (Section 5.3.7). These results suggest that by increasing the copies of *yggA* (m.c. $yggA^+$) or by introducing *argP* alleles which constitutively activate *yggA* transcription, the cell was excreting more Arg. Likewise, the Can^R phenotype of the various $argP^d$ alleles could be a result of increased efflux of Can through YggA, following constitutive transcription of the *yggA* gene in these strains.

Another result which hinted that *yggA* might encode an exporter was the increase in Can-tolerance levels in a WT strain, conferred by introduction of m.c. $yggA^+$. In a related context, it had been previously reported that overproduction of YdeD and YfiK, which function as exporters of cysteine and its intermediate *O*-acetylserine, confer increased azaserine-resistance (Franke *et al.*, 2003).

The third result which supports such an hypothesis was the finding of increased *yggA*::*lacZ* expression in an *argR yggA* strain (GJ4894), as compared to *argR* alone (GJ4748). If the hypothesis that *yggA* encodes an exporter involved in efflux of Arg and related compounds from the cell is correct, then mutations in *yggA* would result in accumulation of such compounds (that are inducers of ArgP-mediated activation of *yggA*::*lac*) inside the cell, particularly in a strain harboring the *argR* mutation (which results in derepression of the Arg biosynthetic pathway).

5.8.11 YggA in relation to other exporter systems in bacteria:

The results obtained in the present study therefore add YggA as a novel example to a large family of membrane proteins that play a crucial role in the efflux of compounds from the cytoplasm. The capacity of microorganisms to export compounds into the

extracellular space is well known and has been connected mainly with the protection of cells against toxic compounds. For instance, resistance to antibiotics, heavy metal ions or organic acids may reside in the ability to specifically or non-specifically expel such compounds (Paulsen *et al.*, 1996; Nikaido, 1996). The mechanisms involved range from the change of membrane permeability via coupling to osmotic pressure or the inversion of uptake systems to the existence of specific exporters (Broer and Kramer, 1991a,b; Luntz *et al.*, 1986).

In recent years, reports have accumulated on efflux / export systems for substrates, intermediates or end products of normal cellular metabolic pathways. These include sugar exporters of the 'Set' family (for Sugar Efflux Transporter), with three examples in *E. coli* and related proteins in *Yersinia pestis* and *Deinococcus radiodurans* (Liu *et al.*, 1999). Two of the sugar exporters belong to systems catalyzing proton-coupled lactose export while the third one is YdeA from *E. coli*, which was identified as an efflux system for L-arabinose (Bost *et al.*, 1999).

Recent work has also revealed the existence of many exporters of amino acids, such as members of RhtB family, which are differentiated into RhtB and LysE subfamilies. In *E. coli*, RhtB is involved in excretion of homoserine and homoserine lactone, both of which are metabolic regulators, and RhtC is involved in threonine export (Zakataeva *et al.*, 1999). LysE subfamily is similar to the RhtB subfamily in that (a) they encode hydrophobic proteins with similar predicted transmembrane segments; (b) three motifs are significantly conserved between them; and (c) a conserved glycine residue is present 16 residues N-terminal to the second motif. However, it differs from the RhtB subfamily in that the LysE family contains PXGP motif near the N-terminus, instead of PGP found in the RhtB subfamily.

LysE from *C. glutamicum* is a paradigmatic example and one of the biochemically best-studied systems. The export is energized by proton-antiport (Bellman *et al.*, 2001; Vrljic *et al.*, 1996, 1999). The expression of *lysE* is regulated by the LysR-type of trancriptional regulator LysG in *C. glutamicum* with Lys, Arg, Cit or His as co-regulator. Studies on the physiological function of *lysE* gene showed that, in the absence of the carrier, Lys can reach an intracellular concentration of more than 1100 mM, which prevents cell growth (Vrljic *et al.*, 1996). Its physiological function is suggested to be the export of Lys and / or Arg after growth on oligopeptides rich in these amino acids. Therefore, in addition to regulation of the levels of this amino acid at the stage of synthesis, its export apparently also represents a possible means of regulating its

intracellular concentration. Thus, LysE serves as a valve for exporting excess Lys that might be harmful to the cells when grown in the environment of lysine-containing peptides (Erdmann *et al.*, 1993). Therefore, by analogy, YggA of *E. coli* might also have such a protective function of exporting Arg and / or its intermediates when their concentration builds up to high levels intracellularly. But unlike *lysE* expression in *C. glutamicum*, Lys inhibits the *argP*-mediated expression of *yggA*, raising the possibility that YggA may be an Arg efflux system, which is physiologically downregulated by Lys.

The physiological advantage of the presence of amino acid uptake systems for bacterial cells is obvious. Externally available amino acids can be used directly for protein synthesis or as carbon, energy and / or nitrogen source without spending energy for anabolism. In contrast, the physiological significance of amino acid excretion in microorganisms is less obvious. Three different models have been developed to explain amino acid excretion from a physiological point of view: (i) overflow metabolism (e.g., Glu efflux in *C. glutamicum*); (ii) limited catabolism (e.g., Lys efflux in *C. glutamicum*); and (iii) derepressed anabolism (e.g., Thr efflux in *E. coli*) (Kramer, 1994; Burkovski and Kramer, 2002).

5.8.12 Predictions on YggA topology:

The amino acid sequence of YggA revealed that YggA-like homologs are present in most of the bacterial species sequenced so far (Aleshin *et al.*, 1999). YggA shows high similarity with LysE of *C. glutamicum* (Fig. 5.16). A more detailed analysis reveals that the positions of charged amino acids in these proteins are conserved, indicating an important role in the topology of the proteins or in their putative participation in the binding and transport of charged compounds like Arg, Cit, Orn etc.

YggA protein is an extremely hydrophobic protein with the average of hydrophobicity being 0.833 (Fig. 5.17 A,B). Analysis of the YggA amino acid sequence for hydrophobic domains predicted that it may contain six transmembrane helices (http://sosui.proteome.bio.tuat.ac.jp), each spanning a length of 23 amino acids (Table 5.20). Of these six helices, four are of primary type and the other two are of secondary type. The helical wheel diagram of the YggA protein is in agreement with the above prediction of six transmembrane helices (Fig. 5.18). The prediction that it is a membrane protein is consistent with its postulated role in the efflux of Arg and its analogs.

Table 5.20 Predicted transmembrane spanning helices of YggA.

No.	N-terminal	Transmembrane region	C-terminal	type	length
1.	5	YFQGLALGAAMILPLGPQNAFVM	27	SECONDARY	23
2.	37	IMIALLCAISDLVLICAGIFGGS	59	PRIMARY	23
3.	66	PWLLALVTWGGVAFLLWYGFGAF	88	PRIMARY	23
4.	102	EVMKQGRWIIATMLAVTWLNPH	124	SECONDARY	23
5.	149	ALGTISASFLWFFGLALLAAWLA	171	PRIMARY	23
6.	178	KAQRIINLVVGCVMWFIALQLAR	200	PRIMARY	23

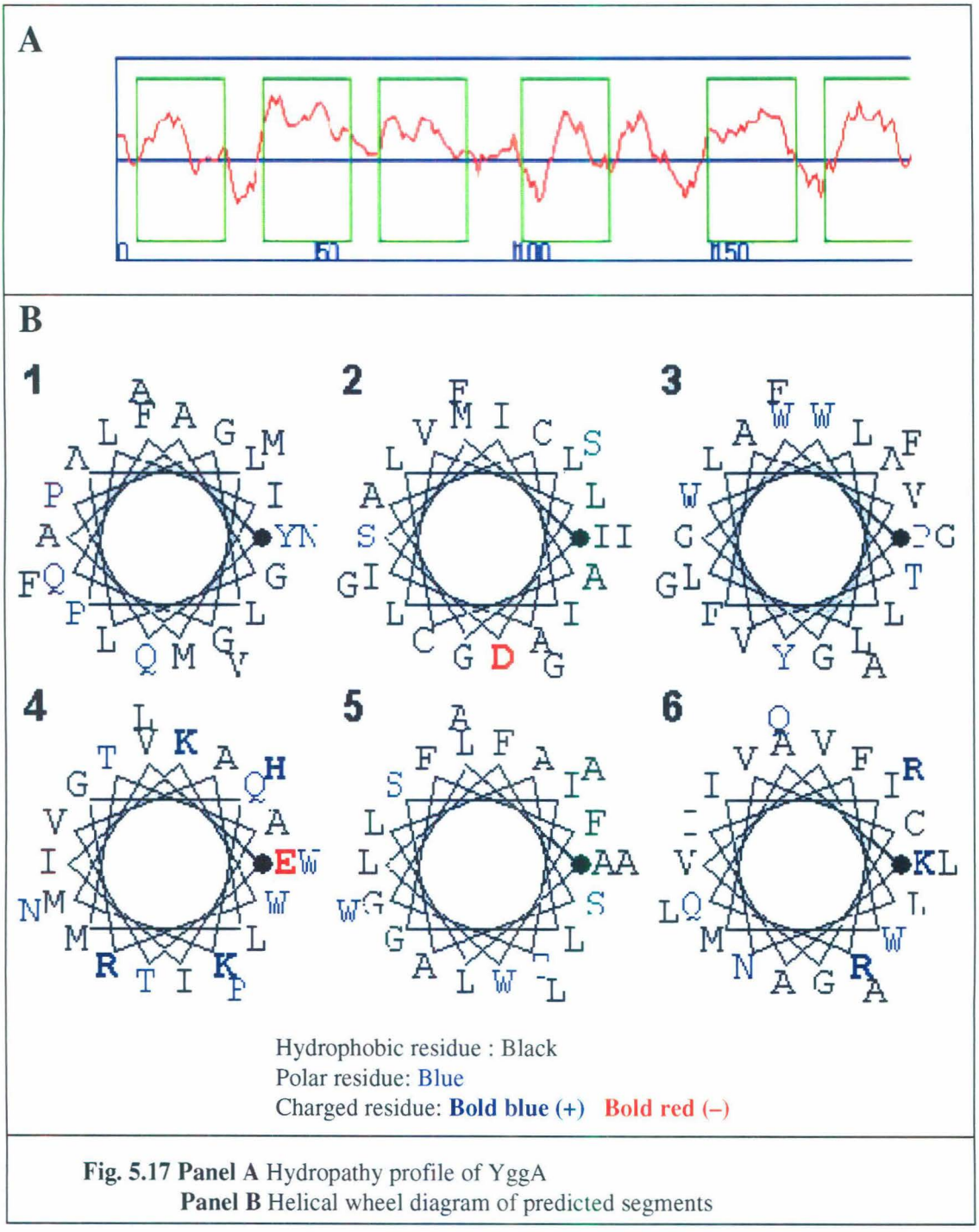


Fig. 5.17 Panel A Hydropathy profile of YggA
Panel B Helical wheel diagram of predicted segments

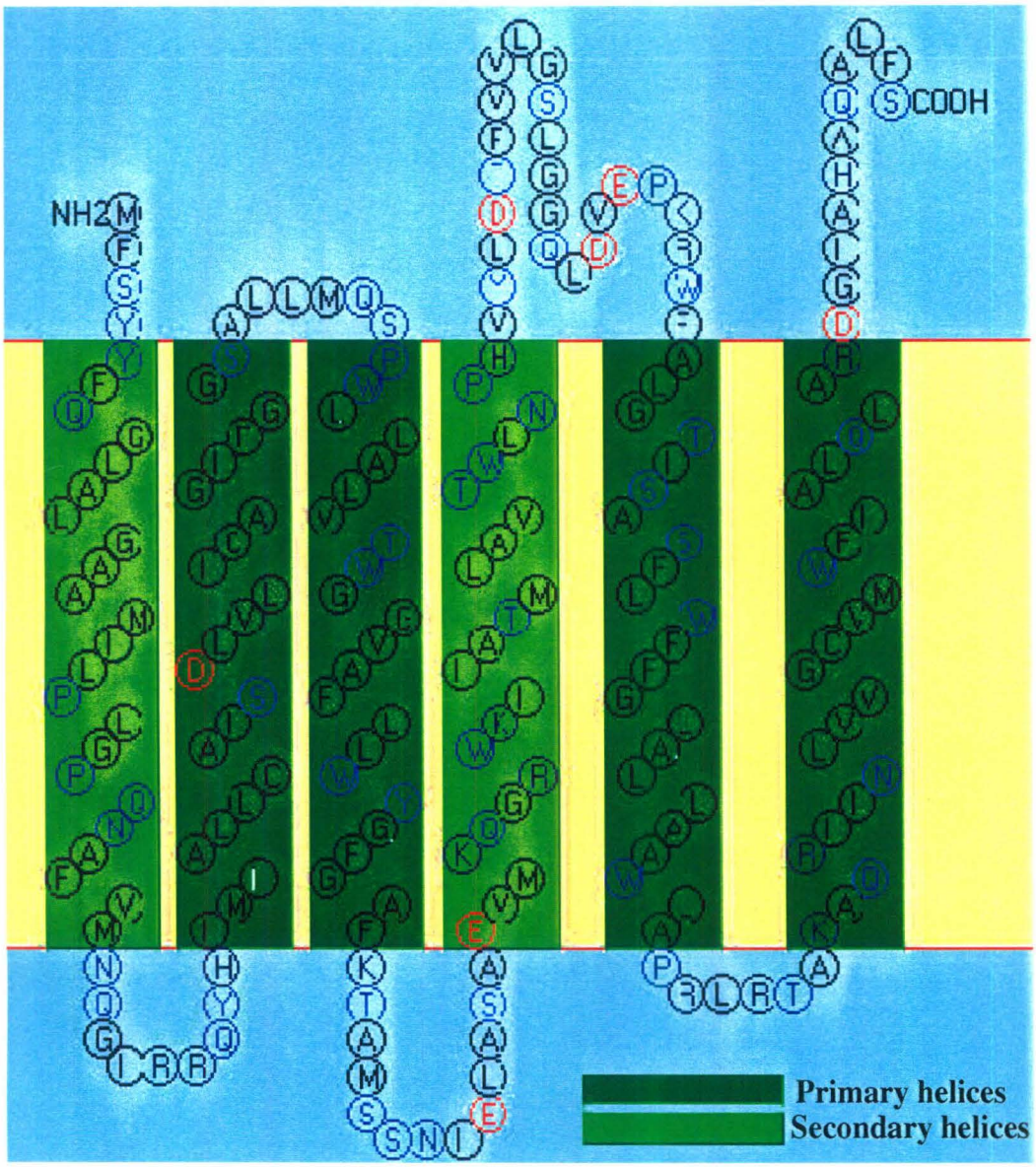


Fig. 5.18 Predicted membrane-spanning helices of YggA.

5.8.13 Applied significance of Arg export:

In an *argR* strain, the massive excretion of Arg in the presence of m.c. *yggA*$^{+}$ or of *argP*^{d}, as evidenced by their ability to cross-feed the strain 160-37 (*argE* mutant) suggests that the overproduced compounds of the Arg pathway were continuously excreted (Sections 5.3.7 and 5.5.12). This result raised the possibility of exploiting such a system in industrial overproduction of Arg (biotechnological application). One of the crucial metabolic steps on the way to achieving high-yield production in Arg-producing fermentation process lies in the enhancement of the export of the amino acid out of the overproducing cell. The amino acid exporter for lysine (LysE in *C. glutamicum*), cysteine

(YdeD in *E. coli*; Winterhalter and Leinfelder, 1997; European patent) and threonine (ThrE in *C. glutamicum*) were patented by other groups with an intention of exploiting the system for overproduction of the respective amino acids. Based on the results obtained from the present investigations, a system consisting of *argR* strain with m.c. *yggA*$^+$ and/or transdominant *argP* gene has the potential of becoming a good candidate for increasing the yield of Arg production on an industrial scale.

5.8.14 Mechanism of Lys-mediated repression of *yggA* expression:

Lys had been shown to repress the *yggA*::*lacZ* expression via ArgP (Section 5.6.2 above). But such a repression was lost in the *argP*^{d} (S94L ArgP) background. To explain the Lys-mediated repression of *yggA* expression, two mechanisms can be envisaged, which are not mutually exclusive. In the first mechanism, Lys binding to ArgP alters its conformation in such a way that it no longer activates *yggA* transcription. Alternatively, Lys addition may alter the intracellular concentration of Arg (by competing for the binding site of the LAO binding protein), which is the true co-effector of ArgP. The fact that Lys repressed *yggA* expression even in an *argR* strain could be explained based on the assumption that the increased Arg pools in an *argR* strain have to be re-captured following their efflux. Thus, during the uptake of Arg, Lys may again compete with Arg for the same binding site in the transport protein. This might result in the repression of *yggA* expression.

5.8.15 Is Lys addition phenocopying *argP* mutation? Antagonistic actions of Arg and Lys on *argP* function:

It has been shown in Section 5.3.9 that the addition of exogenous Lys reduced the NH_4^+-assimilation and conferred osmosensitivity in a *gltBD* mutant. To explain the Lys-mediated osmosensitivity in the *gltBD* mutant, two alternative models can be invoked.

Model 1: This model assumes that Lys addition phenocopies the mutation in *lysC*. It is known from previous studies that Lys both represses the transcription of the *lysC* gene which encodes Aspartokinase III (AK-III) and feedback inhibits the enzymatic activity of AK-III (Section 4.5.1, Chapter 4). Thus, one way Lys might exert its effect is through repression and inhibition of AK-III. However, unlike the *lysC* mutation, Lys addition decreases NH_4^+-assimilation in the *gltBD* mutant. Also, osmosensitivity in the *lysC* mutant is *gltBD*-independent (Section 3.2.11.2 of Chapter 3), whereas Lys addition does

not confer osmosensitivity in a *gltBD*$^+$ strain (Section 5.3.9). These observations do not support the hypothesis that Lys addition phenocopies *lysC* mutation.

Model 2: The alternative model is that Lys-addition phenocopies the mutation in *argP*. The results that support this model are:

(i) both Lys addition and *argP* mutation decrease NH_4^+ assimilation and confer osmosensitivity only in the *gltBD* background (Section 5.3.9) and,

(ii) *yggA*::*lacZ* expression data suggest that Lys addition antagonizes the activator activity of ArgP (Section 5.6.2). When Arg or Lys alone was present in the medium, they activated and repressed the *yggA*::*lacZ* expression, respectively. However, when both were present simultaneously in the medium, the *yggA*::*lacZ* fusion was expressed at intermediate levels to those seen when either amino acid was added individually. These results suggested that in the presence of Lys, the cell behaves as though *argP* is knocked out i.e., Lys-addition phenocopies *argP* mutation.

At the same time, one observation made in this study is not adequately explained by the second model. In an *argP* dominant mutant, Lys-addition did not inhibit *yggA* expression but it did block NH_4^+ assimilation and osmotolerance (in a *gltBD* background). The Lys concentrations used in these two sets of experiments were different (0.3 mM and 1 mM, respectively) and whether this accounts for the discrepancy needs to be investigated.

5.8.16 What is the physiological role of *yggA*?

The physiological function of YggA in *E. coli* is not known, but one can speculate as follows. The role of YggA may be to ensure a correct balance of intracellular Arg and Lys pools. When the former goes up, Arg efflux is activated, while when the latter goes up, it is inhibited. Lys might have a repressing role on *yggA* expression to fine-tune the regulation of levels of basic amino acids (like Lys and Arg) inside the cell.

An alternative possibility for the physiological function of YggA is as follows. *yggA* is expressed moderately in *E. coli* cells and the expression remained unchanged in the different growth phases of the cells. *yggA*::*lacZ* expression was induced by Can 12-fold in the WT strain and 27-fold in an *argR yggA* strain. The high induction of *yggA*::*lacZ* fusion by Can as compared to that by other molecules (Arg, Orn or Cit) suggests that Can might be a better co-effector molecule and possibly could also be a better substrate for YggA-mediated export. The extreme Can^{SS} phenotype of *argP* and *yggA* null mutants also indicates that YggA exporter has more affinity for Can than for

Arg. This raises the possibility that the physiological function of YggA protein is to protect the cells from the toxic effects of Can, which is a natural (plant-derived) anti-bacterial agent. Thus, under conditions of growth in the presence of Can, the YggA protein may function to efflux the Can molecules which enter the cell through Arg uptake systems.

Chapter 6

Isolation and characterization of suppressors of NaCl-sensitive mutations

6.1 Introduction:

In Chapter 3, the isolation of NaCl-sensitive mutants was described. Several novel loci were shown to be involved in osmoregulation, and Chapters 4 and 5 described their detailed characterization. This chapter describes the isolation and partial characterization of second-site (extragenic) point mutations which suppress the water-stress sensitivity of various mutants. Characterization of the second-site suppressor was expected to give greater insights into the mechanisms by which these genes might act in conferring low a_w stress adaptation.

6.2 Strategy:

Localized P1 mutagenesis approach was employed to isolate NaCl-tolerant suppressors of the various mutants. Random transposon-induced mutagenesis followed by localized P1 mutagenesis would generate a population of phages, each containing a point mutation linked to a selectable marker. The recipient strain can be transduced with this lysate to generate the required mutants. The advantages of this method of mutagenesis are (i) multiple hits in the same cell are avoided; (ii) mutations other than null are also obtained; (iii) mapping of the point mutation is facile as it is linked to a selectable marker within a two-minute region [since each P1 phage head can package 2 min length of *E. coli* chromosome (i.e., 80 kb DNA) and the mutation is expected to be linked to the antibiotic marker within this region].

6.3 Results:

6.3.1 Isolation of suppressors of NaCl-sensitive mutants:

Random transpositions were performed on MG1655 using λNK1324 (which harbors Tn*10*dCm), as described in Section 2.9.2. Approximately 10^5 to 10^6 independent Cm^R colonies were obtained, which were pooled after overnight growth on LB Cm Cit and a P1 lysate was prepared on the pooled colonies. This lysate was then treated with hydroxylamine (which causes G.C → A.T transitions) for a period of 15-16 h (as described in Chapter 2, Section 2.8.3) to kill ~99% of phages in the population.

Using this mutagenized lysate, various NaCl-sensitive mutants viz., *argP gltBD* (GJ4654), *thiI gltBD* (GJ4659), *lysC gltBD* (GJ4660), *speC gltBD* (GJ4662) and *glnE gltBD* (GJ4663) were transduced simultaneously to Cm^R and NaCl-tolerance by selecting on MA plates supplemented with Cm and 0.6 to 0.7 M NaCl + 1 mM Bet. This double

selection would ensure that Cm^R transductants carrying a suppressor mutation would appear as colonies on the plate after 48 to 60 h. Approximately 20 to 100 Cm^R $NaCl^T$ colonies were obtained for each strain from ~10^4-10^5 Cm^R colonies that were plated. These colonies were retested for their $NaCl^T$ phenotype and only those that were consistently Cm^R and $NaCl^T$ were chosen for further analysis.

6.3.2 Characterization of $NaCl^T$ Cm^R colonies of various mutants:

The $NaCl^S$ strain could become $NaCl^T$ Cm^R during the screening either by second site extragenic suppression (the desired event) or due to any of the following three reasons:

(i) By crossing out the Kan^R insertion in the respective genes of the parental $NaCl^S$ strain with the cognate WT gene linked to the Cm^R marker. This would result in $NaCl^T$ Cm^R colonies which are Kan^S;

(ii) By crossing out the Δ*gltBD* mutation in the recipient with *gltBD*$^+$ linked to Cm^R from the donor. Since the suppressor analysis was performed in a Δ*gltBD* background and some of the mutations show synergism / additivity with *gltBD* for water-stress-sensitive phenotype, it was reasoned that if the incoming fragment with Cm^R crossed out the Δ*gltBD* locus, such colonies would come up as $NaCl^T$ colonies during the screen;

(iii) By harboring mutations in *argR*. It was shown that exogenous Arg had a slight growth-promoting ability. *argR* mutations also marginally promoted the growth of various NaCl-sensitive mutants. Thus, these $NaCl^T$ Cm^R colonies would be Kan^R but Can-resistant.

All the suppressor colonies were therefore tested for their growth on LB Kan, W-salts + <1 mM NH_4^+ and MA + Uracil + Can in order to score, respectively for the above three causes of putative $NaCl^T$.

6.3.2.1 Suppressors of *gltBD argP202*::Kan (GJ4654):

All the 24 $NaCl^T$ Cm^R colonies obtained from the *gltBD argP* recipient in the above screen were Kan^R, inferring that the NaCl tolerance in these colonies was not due to crossing out of *argP*::Kan with *argP*$^+$ linked to the incoming Cm^R marker.

Subsequently, it was observed that all the 24 colonies grew on par with the WT strain on W-salts with low [NH_4^+], suggesting that these colonies had become $NaCl^T$ by crossing out the *gltBD* deletion with the *gltBD*$^+$ locus linked to Cm^R. It was therefore

concluded that there were no true second-site suppressors of *argP* (unlinked to *gltBD*) obtained in this screen.

6.3.2.2 Suppressors of *gltBD glnE* (GJ4663):

One out of 24 suppressor colonies tested had become Kan^S i.e., the *glnE*::Kan mutation was crossed out by $glnE^+$ allele. Of the remaining colonies, 21 grew on par with the WT strain on low-[NH_4^+] plates, suggesting that they had become $gltBD^+$ by crossing out the *gltBD* deletion. Neither of the remaining strains harbored an *argR* mutation, suggesting that in these two mutants, the suppressor mutation was unlinked to *gltBD, argR* or *glnE*. To characterize the sup^+ locus, P1 lysates were grown on these two strains and the parental GJ4663 strain was transduced to Cm^R. The resultant transductants were tested for their ability to grow on media of high osmolarity. The result demonstrated that in one case (designated GJ4898), there was a linkage of ~22% between Cm^R and $NaCl^T$ (*sge-18* for suppressor of *glnE*), while in the other, no linkage (between Cm^R and $NaCl^T$) could be established.

6.3.2.3 Suppressors of *gltBD lysC* (GJ4660):

Amongst the 24 suppressors of *lysC gltBD*, 17 had become Kan^S, indicative of the fact that the *lysC*::Kan mutation in them was crossed out with the Cm^R-linked $lysC^+$ gene. None of the remaining colonies, when tested for *gltBD* and *argR* phenotypes had become $gltBD^+$ or harbored an *argR* mutation. This could mean that suppression in these strains might be due to an extragenic suppressor mutation (sup^+). To test whether the sup^+ mutations were linked to Cm^R and if so, to characterize them, P1 lysates made on these seven strains were used to transduce the parental $NaCl^S$ *lysC gltBD* (GJ4660) to Cm^R. ~30 colonies from each transduction were tested for their NaCl-tolerance phenotype. Six out of the seven transductions yielded no linkage of $NaCl^T$ to Cm^R. In the seventh case (designated GJ4899), there was ~10% linkage between $NaCl^T$ (*slc-12* for suppressor of *lysC*) and Cm^R. Since the linkage value of 10% would mean the sup^+ mutation may lie ~40-45 kb from the Cm^R on either side, molecular characterization of such a large region for a point mutation would be difficult. Therefore, no further work was carried out on this strain.

6.3.2.4 Suppressors of *gltBD speC* (GJ4662):

In the suppressor analysis of the *speC* Δ*gltBD* strain, eight $NaCl^T$ Cm^R colonies were obtained. None of them was Kan^S i.e., the *speC*::Kan mutation had not been crossed out by the incoming Cm^R marker. Subsequently, these eight colonies were tested for *gltBD* and *argR* phenotypes, as described in Chapter 2. The results demonstrated that one of the colonies had become $gltBD^+$ and five harbored an *argR* mutation. To find the linkage, if any, between the Cm^R and sup^+ mutation in the remaining two strains, P1 lysates were grown on them and the parental GJ4662 strain was transduced to Cm^R. Upon testing 25 to 30 Cm^R transductants from each cross, none was $NaCl^T$, suggesting that there was no linkage between Cm^R and NaCl-tolerance phenotype in these two strains.

6.3.2.5 Suppressors of *gltBD thiI* (GJ4659):

Twenty one of the 24 $NaCl^T$ Cm^R suppressors of *gltBD thiI* had become Kan^S, indicating that they had lost the *thiI*::Kan marker. It was also shown that all the Kan^S strains were Thi^+ prototrophs, while the Kan^R strains remained Thi^-.

Since the osmosensitivity of *thiI* mutants was shown to be *gltBD*-independent, the remaining three suppressors were not tested for their *gltBD* phenotype. When these colonies were tested for their *argR* phenotype, they were all $argR^+$. It was concluded that the three suppressors might represent true second site (extragenic) suppressors. Therefore, in order to characterize the suppressor mutations in these strains, P1 lysates were prepared and used to transduce the parental *gltBD thiI* strain (GJ4659) to Cm^R. The resultant Cm^R colonies were tested for $NaCl^T$ phenotype to establish the linkage between $NaCl^T$ and Cm^R. In one suppressor (designated GJ4723), there was 85% linkage between $NaCl^T$ (*sti-1* for suppressor of *thiI*) and Cm^R, while in the other two, linkage between $NaCl^T$ and Cm^R could not be established. Detailed characterization of this strain will be discussed later in Section 6.3.4 of this chapter.

At the end of the suppressor screening (summarized in Table 6.1), only three linked suppressors were obtained, one each for *thiI, lysC* and *glnE*, with varying degrees of linkage between Cm^R and $NaCl^T$ (sup^+).

Table 6.1 Summary of NaCl-tolerant suppressors of various strains

	argP gltBD	*glnE gltBD*	*lysC gltBD*	*speC gltBD*	*thiI gltBD*
No. of suppressors	24	24	24	8	24
No. of Cm^R Kan^S	0	1	17	1	21
No. Cm^R gltBD$^+$	24	21	0	0	0
No. Cm^R argR$^-$	0	0	0	5	0
No. of linked extragenic suppressors	0	1	1	0	1

6.3.3 Altered strategy for the isolation of suppressors of NaCl-sensitivity:

In the sections described above, it was observed that the majority of the "suppressors" had become $NaCl^T$ by crossing out of the original Kan insertion. In order to circumvent this problem, it was decided to repeat the suppressor screening experiment with the modification that Kan would be added to the NaCl Cm selection plates; thus, a triple selection for Cm^R Kan^R $NaCl^T$ colonies would be imposed after transduction with the mutagenized P1 lysate. Two NaCl-sensitive mutants, *argP gltBD* (GJ4654) and *thiI gltBD* (GJ4659) were used as recipients for the P1 transduction, which was performed as described above. The resultant Kan^R colonies would be tested only for *gltBD* and *argR* strains before attempts to molecularly characterize them.

6.3.3.1 Suppressors of *gltBD argP202* (GJ4654):

Thirty-six $NaCl^T$ Cm^R Kan^R colonies were obtained out of 10^4-10^5 Cm^R transductants that were subjected to triple selection following infection with the mutagenized P1 lysate. All of them had become *gltBD*$^+$, as judged by their ability to grow on W-Salts + < 1mM NH_4^+, indicating that, as on the previous occasion, the $NaCl^S$ *gltBD argP* mutant had become $NaCl^T$ by crossing out of the Δ*gltBD* allele. Thus, in the second round of suppressor analysis also, no new loci could be identified which suppressed the NaCl-sensitivity of *argP gltBD*.

6.3.3.2 Suppressors of *gltBD thiI* (GJ4659):

When ~10^4 Cm^R colonies of a transduction of GJ4659 with the mutagenized P1 lysate of pooled Cm^R colonies were plated for triple selection on MA NaCl Bet Cm Kan plate, ~20 colonies were obtained. These colonies were subsequently shown to be $NaCl^T$ and

thiamin auxotrophs confirming that they all have the *thiI*::Kan insertion. In order to ascertain the linkage between (*sup*$^+$) NaCl^T and Cm^R, P1 lysates were made on a few of the above colonies and the Cm^R was transduced into the *gltBD thiI* strain (GJ4659). Two colonies which showed a high linkage between the NaCl^T suppressor mutation and Cm^R were selected for further analysis and were designated GJ4739 (Cm^R to *sti-47* linkage is 90%) and GJ4740 (Cm^R to *sti-49* linkage is 80%).

6.3.4 Characterization of NaCl^T suppressors of *thiI* (GJ4723, GJ4739 and GJ4740):

The three NaCl^T suppressors of *thiI* (GJ4723 from the first screen, and GJ4739 and GJ4740 from the second screen) were tested for their ability to grow on low a_w media containing other dissolved solutes (Table 6.2). The *sup*$^+$ mutation in each of these suppressors was shown to confer upon the *gltBD thiI*::Kan strain the ability to grow on plates containing other solutes like KCl, $(NH_4)_2SO_4$ (ionic, impermeable), and sucrose, glucose (nonionic, impermeable). (GJ4740 was better than GJ4723, which in turn was better than GJ4739.) Thus, the extragenic suppressor mutation in these strains was able to suppress the growth inhibition of GJ4659 imposed by all kinds of dissolved solutes. Therefore, characterizing the *sup*$^+$ mutation in these three strains might be useful in understanding the mechanism of water-stress adaptation in a *thiI* mutant.

Table 6.2 Growth of *sup*$^+$ mutants of *thiI* at high osmolarity[a]

Strain	MA + Bet				
	NaCl (0.6-0.7 M)	KCl (0.6-0.7 M)	$(NH_4)_2SO_4$ (0.5-0.6 M)	Glucose (0.9-1 M)	Sucrose (0.8-0.9 M)
MC4100	+++	+++	+++	+++	+++
GJ4652	++±	++±	++±	++±	++±
GJ4659	—	—	—	—	—
GJ4723	+++	+++	+++	+++	+++
GJ4739	++±	++±	++±	++±	++±
GJ4740	+++	+++	+++	+++	+++

[a] The indicated strains were streaked on MA + 1 mM Bet plates supplemented with NaCl, KCl, $(NH_4)_2SO_4$, glucose or sucrose and incubated for 42-48 h at 37°C. Growth was scored on the following qualitative 3-point scale (in increasing order): — (no growth), ++±, and +++ (full growth).

6.3.5 Genetic mapping of *sup*$^+$ mutations:

In order to map the *sup*$^+$ mutation in the suppressor strains, it was decided to first map the Cm^R marker (which was shown to be linked 80 to 90% to the *sup*$^+$ mutation). In the first step, one of the suppressors, GJ4723 was chosen for genetic mapping of the Cm^R. It involved the conjugation experiments to roughly map the Cm^R marker, followed by phage P1-mediated transduction mapping experiments to fine-map the Cm^R locus.

6.3.5.1 Conjugation experiments:

GJ4723 (which is Str^R because of an *rpsL* mutation) was used as recipient for mating with the six Tet^R-Hfr strains from the CSH strain kit (Miller, 1992). Each strain in this panel has a Tet^R insertion approximately 20 min away from the point of origin (Fig. 6.1). The points of origin are distributed at various sites around the chromosome in the six Hfr strains. Tet^R exconjugants were selected in each cross and Str^R was used for counterselection against the donor.

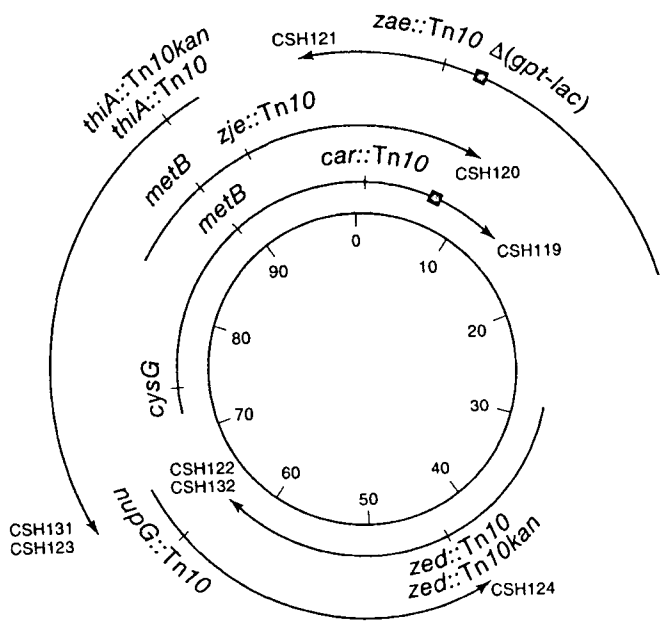


Fig. 6.1 The set of Hfr mapping strains used for mapping the Cm^R marker (Miller, 1992). Arrow heads indicate the origin and direction of transfer for each strain; bars indicate the position of Tet^R insertions used as selective markers. Strain genotypes are listed in Table 2.1.

Exconjugants were scored for loss of the Cm^R marker (which in turn was 85% linked to the mutation of interest) in the recipient strain. 27% of the Tet^R exconjugants

obtained from the cross with Hfr strain, CSH123 (which has a clockwise direction of chromosome transfer and in which the Hfr point of origin and Tet^R insertion site are at 66 min and 90.3 min, respectively) were Cm^S and 6% of the Tet^R exconjugants in the cross with CSH124 (with a clockwise direction of transfer and in which the Hfr point of origin and Tet^R insertion site are at 42 min and 64 min, respectively) were also Cm^S (Table 6.3). With each of the other four Hfr strains, none of the Tet^R exconjugants was Cm^S. In particular, the negative result with CSH122 (Hfr point of origin at 64 min and Tet^R at 43 min) indicated that the Cm^R insertion was located after 64 min on the chromosome. Thus, the conjugation results placed the Cm^R between 64 min and 90 min on the *E. coli* linkage map.

Table 6.3 Conjugational mapping of the Cm^R marker in GJ4723

GJ4723 x	No. of Cm^S colonies / total Tet^R Str^R colonies (%)
CSH119	0 / 41 (0)
CSH120	0 / 42 (0)
CSH121	0 / 45 (0)
CSH122	0 / 44 (0)
CSH123	12 / 44 (27)
CSH124	3 / 44 (7)

6.3.5.2 Transductional fine mapping experiments:

Further refinement of the map position of Cm^R insertion was achieved by P1 transduction experiments. In the initial set of experiments, P1 lysates and strains carrying markers at different loci which were readily available in the lab were used. P1 lysates which were grown on each of the three strains, GJ629 (*zhc-904*::Tn*10*), CAG12072 (*nlp*::Tn*10*) and GJ966 (*zha-6*::Tn*10*), carrying Tn*10* (Tet^R) markers at ~74, 71.8 and 72.3 min respectively, were used to transduce the Cm^R-carrying strain GJ4723 to Tet^R, and the Tet^R transductants were then screened for Cm^S. Additionally, a P1 lysate prepared on GJ4723 was used to transduce CAG18556 (*cysG*::Tn*10*Kan at 75.3 min) and MG1655 (which is Str^S because of $rpsL^+$) to Cm^R. The Cm^R transductants were screened respectively, for Kan^S and Str^R.

Table 6.4 Transductional fine mapping of the Cm^R marker in GJ4723

Transductional cross	Marker on the chromosome (min)	Resultant transductants	Linkage (%)
CAG18556 x P1 (GJ4723)	75.3	0 / 48 Kan^S Cm^R	0
MG1655 x P1 (GJ4723)	74.4	0 / 85 Str^R Cm^R	0
GJ4723 x P1 (GJ629)	~74	0 / 42 Cm^S Tet^R	0
GJ4723 x P1 (CAG12072)	71.8	18 / 46 Cm^S Tet^R	39
GJ4723 x P1 (GJ966)	72.3	45 / 45 Cm^S Tet^R	100

The linkage of the Cm^R locus with *cysG*::Tn*10*Kan (75.3 min), *rpsL* (74.4 min) and *zhc-904*::Tn*10* (74 min) was found to be nil, whereas with *nlp*::Tn*10* (71.8 min) and *zha-6*::Tn*10* (72.3 min), the linkage was 40% and 100% respectively (Table 6.4). Thus, the above linkage values suggested that the Cm^R insertion was near 72.3 min on the *E. coli* linkage map.

6.3.5.3 Mapping the Cm^R marker in GJ4739 and GJ4740:

Since the Cm^R insertion in GJ4723 was shown to be near 72.3 min on the *E. coli* chromosome, it was decided to test if the other two extragenic suppressors viz., GJ4739 and GJ4740 also had the Cm^R insertion near 72.3 min. A similar P1 transduction approach was taken. P1 lysate of GJ966 (Tn*10* at 72.3 min, which had yielded 100% linkage with Cm^R of GJ4723) was used to transduce GJ4739 and GJ4740 to Tet^R, and the resultant transductants were screened for Cm^S colonies. The results showed that the linkage between the Tn*10* of GJ966 and Cm^R of GJ4739 and GJ4740 was 0% and 62% respectively, suggesting that GJ4723, GJ4739 and GJ4740 are all independent mutants and not siblings.

In order to map the *sti* mutation in these three strains more precisely and to ascertain whether the *sti* mutation is similar in these strains, the following P1 transductional mapping approach was taken. The P1 lysate grown on CAG12072 (*nlp*::Tn*10* at 71.8 min) was used to transduce the suppressor strains to Tet^R and the colonies were then tested for their $NaCl^T$ phenotype. The results are summarized in Table 6.5. *nlp*::Tn*10* showed a linkage of 39%, 0% and 93% with the Cm^R marker in GJ4723, GJ4739, and GJ4740 respectively, suggesting that the Cm^R in GJ4739 was away from

71.8 min region. When the Tet^R Cm^R colonies of the above cross involving GJ4739 were tested for $NaCl^T$ phenotype, it was observed that 34% of them had become NaCl-sensitive, i.e., the *nlp*::Tn*10* is moderately linked 34% to the *sti-47* locus. This result demonstrated that *sti-47* mutation in GJ4739 was also located near 71-73 min region of *E. coli* chromosome.

Table 6.5 Transductional mapping of the CmR marker in GJ4723, GJ4739 and GJ4740

Transductional cross	Tet^R map position (min)	Cm^S to Tet^R (linkage %)
GJ4723 x P1 (GJ966)	72.3	45 / 45 (100)
GJ4739 x P1 (GJ966)	72.3	0 / 46 (0)
GJ4740 x P1 (GJ966)	72.3	28 / 45 (62)
GJ4723 x P1 (CAG12072)	71.8	18 / 46 (39)
GJ4739 x P1 (CAG12072)	71.8	0 / 45 (0)
GJ4740 x P1 (CAG12072)	71.8	43 / 46 (93)

Since the Cm^R insertion in GJ4723 and GJ4740 is near *gltBD* (72.3 min), it was decided to check the *gltBD* phenotype of these three suppressors on W-Salts + <1 mM NH_4^+ plates. The results showed that GJ4739 is *gltBD*$^-$ while GJ4723 and GJ4740 had become *gltBD*$^+$. It once again demonstrated that the Cm^R in GJ4740 is away from the *gltBD* locus. To determine whether the above *sup*$^+$ mutations of the three strains could also suppress growth inhibition of the *gltBD*$^+$ *thiI*::Kan strain, the P1 lysates of GJ4723, GJ4739 and GJ4740 were used to transduce GJ4533 (*thiI*) to Cm^R. The resultant Cm^R transductants were tested for their ability to grow on media of low a_w imposed by various dissolved solutes. It was observed that a proportion of the transductants in each cross (85%, 90%, and 80% respectively), as expected for the linkage between Cm^R and the suppressor locus, was able to grow on these plates at an expected frequency suggesting that the *sup*$^+$ mutation suppressed the $NaCl^S$ phenotype even in the *gltBD*$^+$ *thiI*::Kan background.

6.4 Discussion:

In the present chapter, suppressors of various osmosensitive mutants were sought and characterized. Isolation and characterization of extragenic suppressors were expected to give clues to the understanding of the mechanisms of water-stress adaptation in *E. coli*. Towards this goal, localized P1 mutagenesis approach was standardized and employed for obtaining $NaCl^T$ suppressors.

6.4.1 Suppressors of *argP gltBD* and *glnE gltBD*:

When the $NaCl^T$ suppressors of *argP gltBD* and *glnE gltBD* were analyzed, it was found that for *argP*, all fifty suppressors (100%) had acquired the ability to grow on low-[NH_4^+] medium, suggesting the crossing out of the Δ*gltBD* mutation in the strain with the Cm^R linked to *gltBD*$^+$ locus; similarly for *glnE*, 87% of the suppressors had become *gltBD*$^+$ by this test. An alternative but less likely possibility is that at least some of the suppressors are not *gltBD*$^+$ but are able to suppress both the low-[NH_4^+] growth defect as well as the osmosensitivity in the mutants. In any case, these results suggest a strong interaction between *gltBD* on the one hand and *argP* or *glnE* on the other in conferring $NaCl^S$. As described in Section 3.2.11.2 of Chapter 3, neither *argP* nor *glnE* single mutant is osmosensitive, but when combined with *gltBD*, the mutations show a synergistic effect on NaCl-sensitivity phenotype. The present result therefore again lends support to the hypothesis that *argP* and NH_4^+ assimilation / glutamate biosynthesis are intimately connected.

In the screen with the *glnE gltBD* strain, one colony was obtained that showed a 22% linkage between Cm^R and $NaCl^T$ suppressor mutation. Further characterization of this suppressor would give insights into *glnE*-mediated osmosensitivity in *E. coli*. It would be interesting to see if the suppressor maps to *glnA*, which encodes glutamine synthase (GS), involved in the conversion of glutamate to glutamine. In Chapter 4, it was hypothesized that the *glnE gltBD* mutant is osmosensitive because of the constitutive activity of GS which converted the glutamate pools to glutamine within the cell. GS may be constitutively active because of the absence of ATase (GlnE), which controls the catalytic activity of GS. Therefore, a suppressor of *glnE gltBD* could have mutations in GS which decrease its catalytic activity so that the conversion of glutamate to glutamine would be reduced. Another possible locus is the *gdhA* gene, which encodes the glutamate dehydrogenase (GDH). GDH converts NH_3 plus 2-ketoglutarate to glutamate, and is thus

the only route through which glutamate is made in a Δ*gltBD* strain. Therefore, a mutation which increases the transcription of the *gdhA* gene or increases the activity of the GDH enzyme might suppress the osmosensitivity of *glnE gltBD* strain.

6.4.2 Suppressors of *lysC*:

In the effort to screen for suppressors of *lysC gltBD* for osmotolerance, it was found that ca. 70% (17/24) of the colonies had crossed out *lysC*::Kan, with Cm^R linked to $lysC^+$ gene. This result can be interpreted as a strong genetic evidence to implicate *lysC* in water-stress adaptation in *E. coli*. The fact that in the absence of any selection for *lysC*::Kan crossouts, $NaCl^T$ suppressors were obtained which had become $lysC^+$ suggests that the *lysC* gene product is very critical for the survival of *E. coli* under osmotic stress conditions. Although the screen was performed in a Δ*gltBD* background, none of the suppressors had become $gltBD^+$. This result confirms the results obtained in Chapter 3 that *lysC* does not require *gltBD* to confer osmosensitivity. Of the remaining suppressors, only one had shown a modest linkage of 11% between $NaCl^T$ and Cm^R.

6.4.3 Suppressors of *speC*:

Upon screening for suppressors of NaCl-sensitivity in *speC gltBD,* five of the eight colonies isolated were shown to harbor mutations in *argR*. It has been shown in Chapter 4 that *argR* mutations abolish the NaCl-sensitivity in a *speC* mutant, possibly by increasing the flux through the Arg biosynthetic pathway. Therefore, obtaining suppressors in *argR* locus bolstered the hypothesis that increasing the Arg levels inside the cell either by *argR* mutations or by exogenous addition of Arg alleviates the growth inhibition of the *speC* mutant in low a_w medium. As suggested in Section 4.7.3 of Chapter 4, increased Arg pools may contribute to the increase in Put / Spd levels in the *speC* mutant through the alternate *speAB* pathway, for which Arg is the starting substrate (Fig. 1.4 of Chapter 1). Since Put excretion plays a major role in K^+ accumulation during osmoadaptation, replenishing the Put levels in a *speC* mutant through the *speAB* route might alleviate the growth sensitivity on high osmolarity plates.

6.4.4 Suppressors of *thiI*:

In the first round of the screening for suppressors of NaCl-sensitivity in *thiI*, 21 of the 24 $NaCl^T$ Cm^R colonies had become Kan^S by crossing out the *thiI*::Kan with Cm^R

linked to *thiI*$^+$. This result demonstrated that *thiI* has a major role to play in osmoadaptation in *E. coli* since in an unselected screen for suppressors of *thiI*, *thiI*$^+$ revertants were obtained. One true suppressor linked to Cm^R was obtained. In the subsequent screening for suppressors employing continued selection for *thiI*::Kan, two linked suppressors were obtained. These three suppressors, upon further characterization, were found to carry the *sti* mutation linked to Cm^R by about 80 to 90%. The Cm^R in two of the suppressors (GJ4723 and GJ4740) was mapped near the *gltBD* locus, while in the third (GJ4739), the Cm^R could not be accurately mapped, but the *sti-47* locus was shown to be linked ~35% to *nlp*::Tn*10* (71.8 min). GJ4723 and GJ4740 had also become *gltBD*$^+$ during the selection process, but it was shown that crossing out of Δ*gltBD* with *gltBD*$^+$ linked to Cm^R might not be the sole reason for suppression of NaCl-sensitivity of the *thiI* mutant, since the same *sti* mutations conferred $NaCl^T$ in a *gltBD*$^+$ *thiI* mutant also.

It has been previously shown that glutamate synthase (GOGAT) encoded by *gltBD* is an Fe-S-containing protein and that in an *iscS* mutant, its activity is decreased to 34% of that in an *iscS* mutant (Schwartz et al., 2000). From the results in Chapter 4 (Section 4.6.3), it was speculated that *thiI* might be involved in *de novo* assembly or repair of Fe-S clusters and that the absence of such an activity might inhibit cell growth under water-stress conditions. One of the proteins whose Fe-S cluster assembly is compromised might be GOGAT. Since glutamate pools play an important role in the osmoadaptation process in *E. coli*, a plausible explanation is that GOGAT functions less efficiently in a *thiI* mutant. Thus, the *sti* mutation in the GJ4723 and GJ4740 suppressors might have resulted in the overexpression of *gltBD* or might have altered the protein structure so as to become a more efficient enzyme (hypermorph). But in the case of GJ4739, this may not be the case since it has remained *gltBD*$^-$.

The alternative possibility is that any of the other genes present in the 71 to 73 min region in *E. coli* harbors the *sti* mutation. Some of the genes in this region are yet to be assigned functions. It would be interesting to examine whether any of these ORFs encode proteins involved in Fe-S cluster assembly or by themselves contain Fe-S clusters for their activity. ThiI has also been shown to be involved in near-UV (NUV) phenotype through its s^4U (thiolation) modification activity on tRNAs (Section 4.6.1 of Chapter 4). If thiolated tRNAs are assumed to be essential for the cell to survive under high osmolarity conditions, then in a *thiI* mutant, the *sti*$^+$ mutation may provide the functions of the ThiI protein. This would mean that one of the ORFs present in this region

possessing tRNA modification activity is the candidate for harboring the *sti* mutation. Future work on the molecular identification of the *sti* mutation would be useful in understanding the basis of the role of *thiI* in osmoadaptation.

6.5 Summary

In summary, broadly three categories of suppressors of the various osmosensitive mutants were obtained: one class was the crossing out of the Kan^R insertion with the Cm^R-linked WT gene (for *thiI, glnE, lysC*); the second class was the crossing out of Δ*gltBD* with the *gltBD*$^+$ genes (for *argP glnE*); and the third category represented mutations specific to each suppressor (including *argR* for *speC*). These results in one way serve to bolster the confidence with which one can implicate these genes in water-stress adaptation. Especially for *thiI* and *lysC*, the fact that 70% and 85% of the $NaCl^T$ colonies respectively, had swapped the Kan^R insertion for the WT gene talks of the necessity of these genes in low a_w stress adaptation. For genes like *argP* and *glnE*, the fact that 100% and 87% of the suppressors have become *gltBD*$^+$, respectively suggests a strong synergism between these genes in conferring water-stress sensitivity.

Appendix

DNA-microarray-mediated global transcriptional profiling of the *E. coli* response to water stress.

INTRODUCTION:

DNA microarray technology provides a powerful tool for the analysis of global transcriptional responses elicited by various physical and chemical stresses. This approach of parallel study of the expression of every gene in an organism has already been successfully used in studying *E. coli* gene expression under a number of different growth conditions viz., tryptophan metabolism (Khodursky, et al., 2000), minimal versus rich media (Tao *et al.*, 1999; Selinger *et al.*, 2000), heat shock , hydrogen peroxide (Zheng *et al.*, 2001), pH response (Tucker *et al.*, 2002) etc. There are no reports of transcriptional profiling of the *E. coli* response to low a_w stress employing DNA microarrays, except a limited macroarray-mediated profiling reported recently (Weber and Jung, 2002).

This appendix describes the genome-wide transcriptional profiling of the WT strain and osmosensitive mutants of *E. coli* in response to low a_w stress. Analysis of the results revealed many genes involved in iron uptake and cysteine biosynthesis to be differentially regulated under water-stress conditions. In an *argP* mutant, the levels of genes involved in Arg / Orn catabolism and Arg transport were differentially expressed.

RESULTS AND DISCUSSION:

Isolation of total RNA and labelling of cDNA:

Total RNA was isolated from the various strains according to Aiba et al. (1981). For comparing the genes differentially regulated under water stress, total RNA was isolated from MC4100 (WT) grown to early stationary phase following subculturing into MA or MA + 0.5M NaCl Bet. The strains GJ2581 (Δ*gltBD* Δ*speC*), GJ4597 (Δ*gltBD apaH*), GJ4654 (Δ*gltBD argP*) and GJ4553 (*thiI*) were subcultured into MA + 0.4 M NaCl Bet, and total RNA was isolated once they reached stationary phase.

The subsequent labeling of cDNA with cy3 and cy5, and hybridization of the labeled cDNA populations was performed by Dr. J. Gowrishankar in Dr. Akira Ishihama's lab at the National Institute of Genetics, Japan. To measure gene expression under water-stress conditions, total RNA was labeled by reverse transcriptase in the presence of either cy3-dCTP (MA + NaCl Bet) or cy5-dCTP (MA) in reactions primed with random hexamers. The resulting cDNAs were hybridized to glass slide microarrays, and the relative fluorescence of the two fluorophores was measured. The resulting data are expressed as the fold induction or repression in the accompanying tables.

There were two caveats in the present experimental procedure: One was that the transcriptional profiling of the strains under two different conditions was performed only once. Normally, any microarray experiment needs to be repeated, by reversing the cy3 and cy5 labeling, to discount artifactual results. Secondly, in order to check the differential transcription of various genes in osmosensitive mutants, fluorescently labeled cDNAs from one mutant were hybridized with fluorescently labeled cDNA preparation of another mutant. This was contrary to the normal way of comparing labeled cDNA preparations of any one mutant with that of the WT (and not another mutant), so as to get a direct measurement of transcript levels in the presence or absence of a particular gene product. In the present studies, cDNA preparations from two osmosensitive mutants were compared because of cost considerations and limitation of the numbers of microarray slides available. Nevertheless, some meaningful conclusions (albeit, tentative) could still be drawn from these experiments based on the following assumptions:

Table App. 1 Different mutants and culture conditions used for gene profiling

Slide No.	cy3 labeled RNA sample	cy5 labeled RNA sample
A	WT; MA + NaCl + Bet	WT; MA
B[a]	Δ*gltBD speC*	Δ*gltBD apaH*
C[a]	*thiI*	Δ*gltBD argP*

[a] These cultures were grown in MA supplemented with 0.4 M NaCl and 1 mM Bet

Genes upregulated or downregulated in slide A constitute osmoresponsive (more strictly, NaCl-responsive) genes (for definition of slides A, B, and C, see Table App.1). If a gene has high cy5/cy3 ratio in slide A, it means that it is repressed under NaCl conditions or activated in MinA conditions. Since slides B and C represent experiments performed with various mutants grown in the presence of NaCl, the results from these two slides have to compared with results from slide A.

Case I:

(i) If gene X has high cy5/cy3 ratio in Slide A and no difference in slides B (or C), then the interpretation is that gene X is normally repressed by NaCl in WT, and that gene X is

normally repressed by NaCl in *speC* (or *thiI*), as well as in *apaH* (or *argP*), i.e., the expression of gene X is identical in these strains.

(ii) If gene X has high cy5/cy3 ratio in Slide A and high cy5/cy3 ratio in slide B (or C), then the interpretation is that gene X is normally repressed by NaCl in WT, and that gene X is normally repressed by NaCl in *speC* (or *thiI*), but fails to be repressed by NaCl in *apaH* (or *argP*).

(ii) If gene X has high cy5/cy3 ratio in Slide A and low cy5/cy3 ratio in slide B (or C), then the interpretation is that gene X is normally repressed by NaCl in WT, and that gene X is normally repressed by NaCl in *apaH* (or *argP*), but fails to be repressed by NaCl in *speC* (or *thiI*).

Case II:

(i) If gene X has low cy5/cy3 ratio in Slide A and no difference in slide B (or C), then the interpretation is that gene X is normally activated by NaCl in WT, and that gene X is normally activated by NaCl in *apaH* (or *argP*), as well as in *speC* (or *thiI*), i.e., the expression of gene X is identical in these strains.

(ii) If gene X has low cy5/cy3 ratio in Slide A and high cy5/cy3 ratio in slide B (or C), then the interpretation is that gene X is normally activated by NaCl in WT, and that gene X is normally activated by NaCl in *apaH* (or *argP*), but fails to be activated by NaCl in *speC* (or *thiI*).

(iii) If gene X has low cy5/cy3 ratio in Slide A and low cy5/cy3 ratio in slide B (or C), then the interpretation is that gene X is normally activated by NaCl in WT, and that gene X is normally activated by NaCl in *speC* (or *thiI*), but fails to be activated by NaCl in *apaH* (or *argP*).

Case III:

(i) If gene X has cy5/cy3 ratio of 1 in Slides A, B or C, then the interpretation is that gene X is expressed independent of NaCl stress, or of the presence or absence of the mutations.

(ii) If gene X has cy5/cy3 ratio of 1 in Slide A, and high cy5/cy3 ratio in Slide B (or C), then the interpretation is that gene X is equally expressed in both NaCl-stressed and nonstressed conditions and that gene X is repressed in *speC* (or *thiI*), or activated in *apaH* (or *argP*).

(iii) If gene X has cy5/cy3 ratio of 1 in Slide A, and low cy5/cy3 ratio in Slide B (or C), then the interpretation is that gene X is equally expressed in both NaCl-stressed and

nonstressed conditions and that gene X is activated in *speC* (or *thiI*), or repressed in *apaH* (or *argP*).

The salient features of the global transcriptional profiling of WT *E. coli* to low a_w stress can be summarized as follows:

(i) mRNA levels of 175 genes (4%) showed >3-fold induction under high NaCl growth conditions (Table App. 2A).

(ii) Approximately 103 genes (2.4%) showed >3-fold repression under high NaCl growth conditions (Table App. 2B).

(iii) Of all genes, those of the *proU* operon (*proV, proW*) were induced the maximum in NaCl-grown cultures, consistent with the previously reported studies.

(iv) *proP* was modestly upregulated under water-stress conditions, again consistent with previous studies (Weber and Jung, 2002).

(v) Expression of none of the other genes (*apaG, argP, glnE, lysC, speC* and *thiI*) identified in the present study was significantly affected in the WT strain by water stress.

(vi) Genes involved in iron uptake are repressed under water-stress conditions:

E. coli has various scavenging / uptake systems for transporting Fe into the cell. The microarray experiment revealed that many genes which are involved in siderophore-mediated Fe-transport are repressed under low a_w stress conditions. The repression was seen for various kinds of Fe-uptake systems viz., *ent, feo, fep,* and *exb* systems, suggesting that there is a general but strong repression of all genes involved in Fe-uptake.

The repression of siderophores under low a_w stress indicates either that the strain is experiencing iron-excess condition or that the Fur repressor is not functional under water-stress conditions, such that the strains are unable to produce one or more siderophores. Based on protein analysis, it was previously reported that in *Bacillus subtilis*, high salinity resulted in iron-limitation and that the cells responded by derepression of a variety of iron-controlled genes (Hoffmann et al., 2002). This apparent anomaly in the expression of iron-regulated genes of *E. coli* and *B. subtilis* under low water activity stress has to be studied further.

(vii) Upregulation of genes involved in cysteine (Cys) biosynthetic pathway under conditions of osmotic stress:

Various genes involved in biosynthesis of Cys in *E. coli* were upregulated in NaCl-grown cultures. Cys biosynthesis in *E. coli* is carried out by genes arranged in various operons at different locations of its genome. In the present study, the *cysHIJ, cysPUWAM, cysZK* and *cysDNC* operons were upregulated under high NaCl conditions. The fact that these genes are present as different operons on the genome and spotted at different locations on the microarray slide, but still show a common induction profile in the experiment (NaCl versus MA) suggests that Cys biosynthetic genes have an important role to play in osmotolerance mechanisms. It is worth mentioning here that in preliminary experiments, exogenous Cys supplementation alleviated the growth of WT and various water-stress mutants under conditions of low a_w stress (imposed by NaCl or sucrose). These results indicate that elevated levels of Cys might be essential for *E. coli* to survive conditions of low a_w activity stress. Cys under water-stress conditions may promote growth of *E. coli* by acting as a readily available donor of S-atoms for Fe-S cluster assembly or repair or by participating in the formation of GSH, a cellular redox component. But, previous studies have reported that *cysM, cysHIJ, cysD* and *cysK* genes were downregulated under osmotic upshift (Weber and Jung, 2002). Further work is needed to resolve these contradictory results.

Altered gene expression in various water-stress sensitive mutants:

Tables App. 3 and App. 4 summarize the genes which are differentially expressed in various water-stress sensitive mutants of *E. coli* (only the top 60 genes for each are reported here). Since comparisons were made between two mutants and the experiment was performed only once, firm interpretations from these results are not possible. Nevertheless, tentative conclusions can be drawn which could be useful in determining genes which have altered expression in these mutants. These results should only be treated as preliminary and have to be confirmed by direct *in vivo* promoter-reporter gene fusion analyses.

In the experiment involving the *argP* mutant (Slide C),

(i) The levels of *argT* (which encodes the LAO binding protein) and *artJ* (which encodes the Arginine-binding protein I) were upregulated;

(ii) The *yggA* (*argO*) levels in Slide C were not affected significantly, consistent with the results presented in Chapter 5, wherein ArgP was shown to act as an activator only in the presence of Arg;

(iii) The levels of *lysC* were 3.7-fold upregulated;

(iv) The upregulation of *proV* and *proP* and downregulation of *lamB* genes [which mirrors the behavior of osmotically-stressed WT cells (Gowrishankar, 1985)] in the *argP* mutant was indicative of the fact that the *argP gltBD* mutant is more osmotically stressed than *thiI*;

(v) The induction of *argB, argE, argG* and *argH* genes was suggestive of arginine starvation-mediated derepression of the arginine biosynthetic pathway;

(vi) The *astCBAD* operon involved in catabolism of Arg and Orn was downregulated.

In the experiment involving the *thiI* mutant (Slide C),

(i) The levels of *narG, narH, narI, narJ, narK, narX* and *nirB* genes, which encode proteins involved in electron transfer, were upregulated. Fe-S clusters have been shown to be essential for the functioning of some of these proteins. Their upregulation suggests that *thiI* might have a role in Fe-S cluster assembly in *E. coli*;

(ii) Some of the genes which encode ribosomal subunit proteins (*rplA, rplI, rplS* and *rplY*) were downregulated;

(iii) Genes encoding tRNA-modifying enzymes (namely, *trmD* and *tgt*) were downregulated.

Table App. 2A Genes upregulated in MA + NaCl

Gene name	Fold increase	Gene function / property
*proV**	29.5	High-affinity transport for glycine; glycine betaine-binding
*sdhB**	21.2	Succinate dehydrogenase iron-sulfur protein
*cysP**	18.4	Periplasmic sulfate binding protein
*ftn**	14.7	Ferritin
*sdhD**	14.7	Succinate dehydrogenase hydrophobic subunit
proW	13.7	High-affinity transport for glycine, betaine, and proline
sdhA	12.9	Succinate dehydrogenase flavoprotein subunit
*(gltD)**	12.4	Glutamate synthase, small subunit
fdoG	10.2	Formate dehydrogenase-O subunit, major
sucA	9.5	Alpha-ketoglutarate dehydrogenase (decarboxylase component)
bioC	9.1	Blocked prior to pimeloyl CoA formation
*gcvP**	8.8	Glycine dehydrogenase (decarboxylating)
*yhjX**	8.5	Function unknown
nrdF	8.5	Nonessential ribonucleoside-diP reductase 2, subunit beta, class I, function unknown
*dnaE**	8.0	DNA polymerase III, alpha-subunit; suppressor of dnaG mutation
*yedV**	7.6	Function unknown
cysU	7.6	Cysteine transport system; may also transport molybdate
nmpC	7.5	Outer membrane porin, in cryptic prophage DLP12; interrupted by IS5B
rbsA	7.5	D-ribose high-affinity transport system (may have chemotaxis function)
malT	7.1	Lambda sensitivity; positive regulator for mal regulon
aldA	7.1	Aldehyde dehydrogenase, NAD linked
cysJ	6.9	Sulfite reductase, beta-subunit
yehU	6.5	Function unknown
yciW	6.5	Function unknown
malQ	6.2	Amylomaltase
yciE	6.0	Function unknown
ybgP	5.9	Function unknown
nupC	5.8	Transport of nucleosides, except guanosine
bioB	5.7	Biotin synthetase; dethiobiotin to biotin pathway
recG	5.5	Branch migration of Holliday junctions, junction-specific DNA helicase
fumA	5.1	Fumarase A, aerobic
rfaS	5.1	LPS core, not affecting attachment of O antigen
hisD	4.8	Histidinol dehydrogenase
nrdA	4.7	Ribonucleoside diphosphate reductase subunit B1; class I enzyme, aerobic, physiologically active
malP	4.7	Maltodextrin phosphorylase
hisQ	4.7	Histidine transport gene

Table App. 2A Genes upregulated in MA + NaCl

Gene name	Fold increase	Gene function / property
malF	4.6	Maltose transport complex, inner membrane-spanning subunit
246#1	4.5	ycgS
carA	4.4	Carbamoylphosphate synthase (glutamine-hydrolysing) light subunit
ygiM	4.4	Function unknown
ugd	4.3	UDP-glucose 6-dehydrogenase
ilvB	4.3	Acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I); valine sensitive; large subunit
yddX	4.3	Function unknown
malE	4.3	Maltose-binding protein, periplasmic; substrate recognition for transport and chemotaxis
argA	4.3	N-acetylglutamate synthase; growth on acetylornithine
argI	4.3	Ornithine transcarbamylase
yidC	4.2	Function unknown
yeiB	4.2	Function unknown
ppsA	4.2	Phosphoenolpyruvate synthase
cyoA	4.2	Cytochrome o oxidase subunit II; cytochrome bo3 ubiquinol oxidase subunit II
ygaH	4.2	Function unknown
hisG	4.1	ATP phosphoribosyltransferase
yfjG	4.1	Function unknown
yraM	4.1	Function unknown
yhdR	4.1	Function unknown
ycfC	4.1	Function unknown
ygaZ	4.0	Function unknown
ylaC	4.0	Function unknown
gspC	4.0	General secretory pathway genes of unknown function
gatR	4.0	Repressor for gat operon, interrupted by IS3E
flu	3.8	Antigen 43, phase-variable bipartite outer membrane protein; affects surface properties, piliation, colonial morphology;
kgtP	3.8	Alpha-ketoglutarate permease
purB	3.8	Adenylosuccinate lyase
dmsB	3.8	DMSO reductase subunit B; apparent Fe-S binding domain; anaerobic
tsx	3.8	T6, colicin K resistance; nucleoside channel
421#19	3.8	Function unknown
gcvH	3.8	Glycine cleavage, carrier of aminomethyl group
stpA	3.7	Hns-like protein, suppresses T4 td mutant
hofD	3.7	Type 4 prepilin-like proteins leader peptide processing enzyme

* the fold increase indicated for these genes is only an approximation since the corresponding mRNA concentration in the culture grown without NaCl was too low to be quantitated accurately.

App. 2B Genes downregulated in MA + NaCl

Gene name	Fold decrease	Gene function / property
entC	79.5	Isochorismate synthetase
fhuF	57.5	Ferric hydroxamate transport
bglJ	35.4	Mutation bglJ4 activates silent bgl operon, allowing arbutin and salicin transport and utilization
ycdO	33.0	Function unknown
entB	29.6	2,3-Dihydro-2,3-dihydroxybenzoate synthetase
fepB	26.3	Periplasmic component of ferrienterobactin transport system
entA	25.7	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase
(ydcY)	19.3	Function unknown
entE	18.0	Enterochelin synthetase, component E
feoB	16.9	Membrane protein of ferrous iron uptake system
ybdB	14.9	Function unknown
feoA	14.7	Ferrous iron uptake system
entF	14.5	Enterochelin synthetase, component F
fepC	14.5	Cytoplasmic membrane component of ferrienterobactin
fhuA	13.6	OMP receptor for ferrichrome, colicin M, and phages T1, T5, and phi80; energy-coupled transport of Fe3+ via ferrichrome; mutants albomycin resistant
ybdA	13.4	Function unknown
241#2	12.0	Function unknown
sodA	10.3	Member of SoxRS regulon; superoxide dismutase, Mn
yjbI	10.3	Function unknown
yiaU	10.2	Function unknown
ycfR	10.0	Function unknown
yagB	9.5	Function unknown
yahB	9.3	Function unknown
ydfZ	8.8	Function unknown
fecR	8.6	Regulatory gene mediating induction by iron
yhgG	8.3	Function unknown
yrhA	8.2	Function unknown
tauA	8.2	Uptake of taurine (probable S source)
yjhA	8.1	Function unknown
tnaB	7.9	Low-affinity Trp permease
tsr	7.8	Serine chemoreceptor; methyl accepting chemotaxis protein II, membrane receptor
exbD	7.8	Uptake of enterochelin; resistance or sensitivity to colicins; similarity with TolR
yagJ	7.5	Function unknown
yjfL	7.5	Function unknown
lacZ	7.4	Beta-D-Galactosidase
yjdE	7.2	Function unknown
fepA	7.2	Outer membrane component of ferribactin transport system

App. 2B Genes downregulated in MA + NaCl

Gene name	Fold decrease	Gene function / property
(rtcA)	7.1	RNA 3'-terminal phosphate cyclase; physiological role unknown
ygdB	7.0	Function unknown
fhuE	6.9	Outer membrane receptor for ferric-rhodotorulic acid
cspE	6.9	High copy promotes or protects chromosome condensation; cspA homolog
yrfC	6.9	Function unknown
yihN	6.7	Function unknown
229#1	6.7	Function unknown
exbB	6.6	Uptake of enterochelin; resistance or sensitivity to colicins; similarity with TolQ
yhiK	6.4	Function unknown
fliD	6.4	Hook-associated protein 2, axial family; flagellar regulon member
fecI	6.3	Transport gene mediating induction by iron
tonB	6.3	Sensitivity to T1, phi80, and colicins; uptake of chelated Fe and cyanocobalamin; energy transducer
appY	6.3	Regulates hya and appA operons; induced by PO4 starvation and stationary phase
ydiT	6.1	Function unknown
ygcW	6.1	Function unknown
ybfA	6.1	Function unknown
dgoR	5.9	Regulatory; growth on 2-keto-3-deoxygalactonate
kdgT	5.8	Ketodeoxygluconate transport system, structural gene
ycdB	5.8	Function unknown
sgaE	5.7	Putative sugar isomerase
frvR	5.7	Putative frv operon regulator
yjhP	5.6	Function unknown
yqgA	5.6	Function unknown

Table App. 3A Genes upregulated in *argP* or downregulated in *thiI*

Gene name	Fold difference	Gene function / property
narH	46.3	Nitrate reductase beta-subunit
*yhjX**	36.9	Function unknown
*narJ**	39.5	Nitrate reductase delta-subunit; chaperone
feoB	36.4	Membrane protein of ferrous iron uptake system
*aroA**	35.8	3-Enolpyruvylshikimate-5-phosphate synthetase
*yfiD**	33.5	Function unknown
*fhuF**	31.5	Ferric hydroxamate transport
nirB	27.5	Nitrite reductase [NAD(P)H] subunit
metE	24.4	Tetrahydropteroyltriglutamate methyltransferase
*narK**	23.8	Nitrate/nitrite antiporter
*artJ**	20.7	Periplasmic binding protein of Arg transport system
cspA	20.5	Cold shock protein CS7.4; similar to Y-box DNA binding proteins of eukaryotes; transcription factor
*274#5**	19.4	Function unknown
thrA	19.2	Aspartokinase I-homoserine dehydrogenase I
*narG**	18.9	Nitrate reductase alpha-subunit
*narI**	18.9	Cytochrome bNR, structural gene; gamma-subunit
*bglJ**	17.2	Mutation bglJ4 activates silent bgl operon, allowing arbutin and salicin transport and utilization
nadA	14.3	Quinolinate synthetase, A protein
ydiH	14.3	Function unknown
oppB	13.3	Oligopeptide transport
proP	13.1	Low-affinity transport; proline permease, minor
hisI	12.9	Bifunctional enzyme PR-ATP pyrophosphatase PR-AMP cyclohydrolase
ilvM	12.2	Acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II); valine insensitive; small subunit
exbB	11.1	Uptake of enterochelin; resistance or sensitivity to colicins; similarity with TolQ
ilvD	10.9	Dihydroxyacid dehydrase
hisF	10.3	Cyclase component of IGP synthase complex
mrdA	9.8	Penicillin-binding protein PBP 2, mecillinam resistance
hisA	9.4	N-(5'-phospho-L-ribosylformimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide 636 isomerase
serA	9.3	Phosphoglycerate dehydrogenase
(ilvG)	9.2	Acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II); valine insensitive; large subunit
thrB	8.9	Homoserine kinase
ilvE	8.7	Branched-chain amino acid aminotransferase
thrC	8.6	Threonine synthase
glnA	8.2	Glutamine synthetase
hdeD	8.1	Periplasmic, unknown function, has sigma S-dependent promoter
pflB	8.1	Pyruvate formate lyase I; induced anaerobically
focA	7.9	Membrane protein

Table App. 3A Genes upregulated in *argP* or downregulated in *thiI*

Gene name	Fold difference	Gene function / property
ilvA	7.4	Threonine deaminase
lysS	7.3	Lysyl tRNA synthetase, constitutive
hisH	7.1	Amidotransferase component of IGP synthase
ynfM	7.0	Function unknown
rplY	6.9	50S ribosomal subunit protein L25
rplS	6.8	50S ribosomal subunit protein L19
proV	6.8	High-affinity transport for glycine; glycine betaine-binding protein
yhiF	6.8	Function unknown
trmD	6.7	tRNA (guanine-7)-methyltransferase
yefG	6.7	Function unknown
hdeB	6.6	Periplasmic, unknown function, has sigma S-dependent promoter
argB	6.6	Acetylglutamate kinase } indicative of Arg-starvation in *argP*, through
argH	6.5	Argininosuccinate lyase } increased efflux and/or decreased uptake
tgt	6.4	tRNA-guanine transglycosylase
serC	6.3	Phosphoserine aminotransferase
ptsG	6.3	Glucosephophotransferase enzyme II
yfiB	6.2	Function unknown
purB	6.2	Adenylosuccinate lyase
yefI	6.2	Function unknown
yhbC	6.1	Function unknown
rimM	6.1	21-kDa protein essential for 16S RNA processing
rplI	6.1	50S ribosomal subunit protein L9
prfA	6.0	Peptide chain release factor 1
rplA	6.0	50S ribosomal subunit protein L1
ompT	5.9	Outer membrane protein 2b; protease VII; cleaves T7 RNA polymerases, Ada, SecY
tolA	5.9	Tolerance to group A colicins, single-stranded filamentous DNA phage; required for OM integrity; membrane protein; bacteriocin tolerant
rnb	5.9	RNase II; mRNA degradation
narX	5.9	Nitrate sensor-transmitter protein; functional redundance with narQ
rplK	5.9	50S ribosomal subunit protein L11; kasugamycin sensitivity
(yefJ)	5.7	Function unknown
oppA	5.6	Oligopeptide permease
priB	5.6	Primosomal protein n
ycfU	5.5	Function unknown
wbbJ	5.5	Involved in lipopolysaccharide biosynthesis

* the fold increase indicated for these genes is only an approximation since the corresponding mRNA concentration in the *thiI* culture was too low to be quantitated accurately.

Table App. 3B Genes downregulated in *argP* or upregulated in *thiI*

Gene name	Fold difference	Gene function / property
304#1	57.3	Function unknown
aceA	45.5	Isocitrate lyase; acetate utilization
ydcW	36.7	Function unknown
malE	36.3	Maltose-binding protein, periplasmic; substrate recognition for transport and chemotaxis
agp	36.1	Periplasmic glucose-1-phosphatase, acidic
*argT**	35.0	Lysine, Arginine, Ornithine-binding protein
aceB	34.2	Malate synthase A
aldA	33.4	Aldehyde dehydrogenase, NAD linked
fumA	32.5	Fumarase A, aerobic
fadA	30.1	Thiolase I
ybaW	28.1	Function unknown
trpC	24.0	Bifunctional enzyme N-(5-phosphoribosyl)anthranilate isomerase, indole-3-glycerolphosphate synthetase
421#19	21.5	Function unknown
bfr	21.3	Bacterioferritin
pckA	21.1	Phosphoenolpyruvate carboxykinase [ATP]
ilvB	20.9	Acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I); valine sensitive; large subunit
sdhA	20.9	Succinate dehydrogenase flavoprotein subunit
*fadB**	20.4	3-hydroxyacyl-CoA dehydrogenase: 3-hydroxyacyl-CoA epimerase; dodecenoyl-CoA-delta-isomerase, enoyl-CoA hydratase; fatty acid oxidation complex, alpha-subunit; four activities
upo3	17.1	Unknown protein 2D_000B3L from 2D-page
*273#4**	16.9	Function unknown
ybeL	16.1	Function unknown
yceP	15.8	Function unknown
ybdQ	15.6	Function unknown
ychH	15.0	Function unknown
yeaG	14.5	Function unknown
fumC	14.4	Fumarase C, aerobic; member of soxRS regulon
trpA	14.3	Tryptophan synthase subunit A
sucA	13.9	Alpha-ketoglutarate dehydrogenase (decarboxylase component)
sseA	13.8	Enhances serine sensitivity (inhibits homoserine deHase) on lactate; rhodanese-like protein
ydcI	13.4	Function unknown
acnA	12.9	Aconitase A
yceH	12.7	Function unknown
345#5	12.6	Function unknown
cspD	12.6	Similarity to CspA but not cold shock induced
sdhB	12.1	Succinate dehydrogenase iron-sulfur protein
yedU	12.0	Function unknown

Table App. 3B Genes downregulated in *argP* or upregulated in *thiI*

Gene name	Fold difference	Gene function / property
malM	11.8	Periplasmic protein
yfiA	11.7	Function unknown
*yfcX**	11.6	Function unknown
319#10	11.5	Function unknown
qor	11.0	Quinone oxidoreductase, NADPH dependent
yeaH	10.8	Function unknown
mdh	10.6	Malate dehydrogenase
ycgB	10.5	Function unknown
gabD	10.4	Succinate-semialdehyde dehydrogenase; NADP dependent
rimL	10.3	Modification of 50S ribosomal subunit protein L7/L12; acetylation of N-terminal serine
ydbC	10.1	Function unknown
lamB	10.0	Phage lambda receptor protein; maltose high-affinity uptake system
576#14	10.0	Function unknown
sucC	9.4	Succinyl-CoA synthetase beta-subunit
280#3	9.1	Function unknown
325#1	9.0	Function unknown
yohC	8.9	Function unknown
dctA	8.9	Uptake of C-4 dicarboxylic acids; 3-fluoromalate resistance, D-tartrate resistant
ygdH	8.9	Function unknown
ygiW	8.8	Function unknown
358#3	8.8	Function unknown
ynfD	8.5	Function unknown
yedP	8.4	Function unknown
sgcX	8.2	Putative gene in sgc cluster, function unknown
galE	8.2	UDP-galactose 4-epimerase; hexose-1-phosphate uridylyltransferase
dps	8.2	Stress response DNA-binding protein; starvation induced resistance to H_2O_2 phase
yqeB	8.1	Function unknown
334#5.1	7.9	Function unknown
*yneB**	7.8	Function unknown
*glcB**	7.5	Malate synthase G
*cstA**	7.4	Starvation induced stress response protein
*acs**	6.7	Acetyl CoA synthetase 2

* the fold increase indicated for these genes is only an approximation since the corresponding mRNA concentration in the *argP* culture was too low to be quantitated accurately.

Table App. 4A Genes upregulated in *apaG* or downregulated in *speC*

Gene name	Fold difference	Gene function / property
*cspJ**	19.7	Function unknown
*ymgA**	19.5	Function unknown
leuC	11.7	Alpha-Isopropylmalate isomerase subunit
leuB	11.6	Beta-Isopropylmalate dehydrogenase
glcB	10.1	Malate synthase G
leuA	9.5	Alpha-Isopropylmalate synthase
leuD	9.1	Alpha-Isopropylmalate isomerase subunit
acs	8.9	Acetyl CoA synthetase 2 O6-methylguanine-DNA methyltransferase, inducible; DNA repair against methylating
*ycfR**	8.2	Function unknown
*242#1.4**	7.8	Function unknown
ddg	7.1	Acetyltransferase
yfjO	7.0	Function unknown
yjcG	6.8	Function unknown
*(yciG)**	6.4	Function unknown
*ydfR**	6.2	Function unknown
sodA	6.0	Member of SoxRS regulon; superoxide dismutase, Mn
cutC	5.7	Copper sensitivity
yabN	5.4	Function unknown
glcF	5.0	Glycolate oxidase subunit, FeS protein
*fhuF**	4.8	Ferric hydroxamate transport
soxS	4.8	Regulatory protein of soxRS regulon; induces nine-protein sox regulon when superoxide levels increase
280#1	4.6	Function unknown
(slp)	4.6	C starvation and stationary phase inducible; outer membrane lipoprotein
yabI	4.5	Function unknown
*cspI**	4.4	Cold shock protein homolog
cspA	4.4	Cold shock protein CS7.4; similar to Y-box DNA binding proteins of eukaryotes; transcription factor
*glcG**	4.3	Unknown function
ybeD	4.3	Function unknown
*cspB**	4.2	Cold shock protein with similarity to CspA
rpoH	4.1	RNA polymerase, sigma 32-subunit, heat-shock transcription
dppA	4.0	Uptake of dipeptides
uvrA	3.9	Excision nuclease subunit A; repair of UV damage to DNA; LexA regulon
yjcH	3.9	Function unknown
htrA	3.9	Serine protease that is required at high temperature. Involved in the degradation of damaged proteins. It can degrades ICIA, ADA, Casein and Globin. Shared specificity with DegQ.
araA	3.8	L-Arabinose isomerase
yhiE	3.8	Function unknown
yfjN	3.7	Function unknown

Table App. 4A Genes upregulated in *apaG* or downregulated in *speC*

Gene name	Fold difference	Gene function / property
ilvB	3.6	Acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I); valine sensitive; large subunit
sgcC	3.6	Putative phosphotransferase enzyme IIC
ynhC	3.6	Function unknown
yejF	3.5	Function unknown
sgcQ	3.5	Putative gene in sgc gene cluster, function unknown
ybaS	3.5	Function unknown
yiaG	3.4	Function unknown
phoH	3.4	Member of pho regulon, P starvation induced
664#6	3.3	Function unknown
yhiN	3.3	Function unknown
greA	3.2	Transcription elongation factor
msyB	3.2	In multicopy restores growth and protein export functions of secY and secA mutants
gadA	3.1	Glutamate decarboxylase
ybaT	3.1	Function unknown
ugpE	3.1	sn-Glycerol-3-P transport system; membrane protein
xasA	3.1	Glutamate-dependent enzyme, may function in protection against cytoplasmic acidification
ybbM	3.1	Function unknown
ybhE	3.1	Function unknown
yrfC	3.0	Function unknown
ytfR	3.0	Function unknown
leuO	3.0	Affects expression of small regulatory Dsr-RNA, translational regulation of rpoS, relieves bgl silencing
asnA	3.0	Asparagine synthetase A
fhuA	3.0	OMP receptor for ferrichrome, colicin M, and phages T1, T5, and phi80; energy-coupled transport of Fe3+ via ferrichrome; mutants albomycin resistant
yabJ	3.0	Function unknown
ylbC	3.0	Function unknown
aslA	3.0	Suppresses gpp mutants; putative arylsulfatase
arp	3.0	Ankyrin-repeat protein A
sbp	2.9	Periplasmic sulfate-binding protein
fixB	2.9	Related to carnitine metabolism; amino acid similarity to Rhizobium fix gene N-fixation genes
yaeF	2.8	Function unknown
yejG	2.8	Function unknown

* the fold increase indicated for these genes is only an approximation since the corresponding mRNA concentration in the *speC* culture was too low to be quantitated accurately.

Table App.4B Genes downregulated in *apaG* and upregulated in *speC*

Gene name	Fold difference	Gene function / property
gatD	34.7	Galactitol-1-phosphate dehydrogenase
ompF	30.4	Outer membrane protein 1a (Ia;b;F)
ptkB	20.9	Function unknown
(gatZ)	18.3	Function unknown
*yciD**	17.1	Function unknown
*dadA**	15.6	D-amino acid dehydrogenase subunit
*sdhA**	15.3	Succinate dehydrogenase flavoprotein subunit
*atoE**	12.8	Short-chain fatty acids transporter
ptkC	12.8	Function unknown
gatY	12.8	D-Tagatose-1,6-bisphosphate aldolase
gatR	11.6	Repressor for gat operon, interrupted by IS3E
ompC	11.6	Outer membrane protein 1b (Ib, c)
*rimL**	10.4	Modification of 50S ribosomal subunit protein L7/L12; acetylation of N-terminal serine
aceB	10.2	Malate synthase A
aceA	9.8	Isocitrate lyase; acetate utilization
*hisC**	9.5	Histidinol-phosphate aminotransferase
aspC	9.4	Aspartate aminotransferase
mdh	9.2	Malate dehydrogenase
*fba**	8.7	Fructose-bisphosphate aldolase class II
yneC	8.5	Function unknown
sdhD	8.1	Succinate dehydrogenase hydrophobic subunit
yhjK	7.8	Function unknown
yneB	7.7	Function unknown
*galE**	7.6	UDP-galactose 4-epimerase; hexose-1-phosphate uridylyltransferase
tpx	7.5	Thioredoxin-linked thiol peroxidase
pnp	7.3	Polynucleotide phosphorylase
*galT**	7.2	Galactose-1-phosphate uridylyltransferase
*yqeB**	7.2	Function unknown
icdA	7.0	Isocitrate dehydrogenase [NADP]
gatY	6.6	D-Tagatose-1,6-bisphosphate aldolase
ydeV	6.4	Function unknown
ybeQ	6.3	Function unknown
uidR	6.2	Regulatory gene for uid operon
sodB	6.1	Superoxide dismutase, Fe
rbsA	6.0	D-ribose high-affinity transport system (may have chemotaxis function)
minC	6.0	Inhibition of FtsZ ring at division site
gltA	5.8	Citrate synthase
yeaA	5.8	Function unknown

Table App.4B Genes downregulated in *apaG* and upregulated in *speC*

Gene name	Fold difference	Gene function / property
hns	5.7	DNA-binding protein, histone-like; diverse mutant phenotypes affecting transcription, transposition, inversion, cryptic-gene expression; involved in chromosome organization
agaZ	5.7	Putative tagatose 6-phosphate kinase
agp	5.7	Periplasmic glucose-1-phosphatase, acidic
yhaD	5.7	Function unknown
rplC	5.6	50S ribosomal subunit protein L3
pckA	5.6	Phosphoenolpyruvate carboxykinase [ATP]
ppsA	5.5	Phosphoenolpyruvate synthase
yeeX	5.4	Function unknown
ygfJ	5.3	Function unknown
sucC	5.2	Succinyl-CoA synthetase beta-subunit
aroG	5.2	DAHP synthetase; phenylalanine repressible; TyrR regulon
fdoG	5.1	Formate dehydrogenase-O subunit, major
345#5	5.1	Function unknown
yfiA	5.1	Function unknown
rbsD	5.0	D-ribose high-affinity transport system
fumC	4.9	Fumarase C, aerobic; member of soxRS regulon
sucA	4.9	Alpha-ketoglutarate dehydrogenase (decarboxylase component)
icc	4.9	Affects the expression of the lacZ gene
aco2	4.9	Function unknown
sucD	4.9	Succinyl-CoA synthetase alpha-subunit
yfcX	4.8	Function unknown
rbsC	4.8	D-ribose high-affinity transport system
nuoG	4.8	NADH dehydrogenase I subunit
rplB	4.7	50S ribosomal subunit protein L2
serS	4.6	Serine hydroxamate resistance; seryl-tRNA synthetase
sdhB	4.6	Succinate dehydrogenase iron-sulfur protein
330#9	4.6	Function unknown
sfcA	4.6	Putative malic enzyme, NAD linked
yhhR	4.6	Function unknown
yqfA	4.5	Function unknown
rbsK	4.4	Ribokinase

* the fold increase indicated for these genes is only an approximation since the corresponding mRNA concentration in the *apaH* culture was too low to be quantitated accurately.

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