Genetics and Physiology of Osmoregulation and Arginine Transport in *Escherichia coli*

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By

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DECLARATION

The research work presented in this thesis entitled **"Genetics and Physiology of Osmoregulation and Arginine Transport in *Escherichia coli* "**, has been carried out by me at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, under the guidance of Dr. J. Gowrishankar. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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CERTIFICATE

This is to certify that this thesis entitled, "**Genetics and Physiology of Osmoregulation and Arginine Transport in *Escherichia coli* "**, submitted by Mr. Laishram Rakesh Singh for the Degree of Doctor of Philosophy to Manipal University is based on the work carried out by him at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad. This work is original and has not been submitted in part or full for any degree or diploma of any other university or institution.

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Dedicated to

My sisters

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Rakesh S. Laishram

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Abbreviations

Abbreviations for standard (SI) units of measurement, and chemical formulae are not included in the list below:

A	-	Absorbance
Amp	-	Ampicillin
ATP, GTP, CTP,UTP	-	Adenosine 5'-,Guanosine 5'-,Cytidine 5'-,Uridine 5'-Triphosphates
cAMP	-	Cyclic adenosine-5' monophosphate
APS	-	Ammonium persulfate
bp	-	Base-pairs
^{o}C	-	Degrees in Centigrade
cfu	-	Colony-forming unit
Ci	-	Curie
cpm	-	Counts per minute
Da	-	Dalton
dATP, dCTP	-	Deoxyadenosine 5'-triphosphate
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetra acetic acid
hr	-	hour(s)
IPTG	-	Isopropyl-β-D-thiogalactoside
Kan	-	Kanamycin
kb	-	kilobase-pairs
K_d	-	Binding dissociation constant
M	-	Molar
MA	-	Minimal A medium supplemented with 0.2% glucose
MCS	-	Multiple cloning site
MIC	-	Minimal inhibitory concentration
min	-	minute
nm	-	Nanometers
NTP, dNTP	-	Nucleoside-, Nucleotide- Triphosphate
ONPG	-	Orthonitrophenyl β-D-Galactoside
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
pfu	-	Plaque-forming unit
PNK	-	Polynucleotide Kinase
Rif	-	Rifampicin
RNA, mRNA, tRNA	-	Ribonucleic acid, messenger RNA, transfer RNA
rpm	-	Revolutions per minute
RT	-	Room temperature
Spec	-	Spectinomycin

TEMED	-	N,N,N',N'-Tetramethylethylenediamine
Tet	-	Tetracycline
U	-	Units
UV	-	Ultra-violet
WT	-	wild-type
Xgal	-	5-Bromo 4-Chloro 3-Indolyl-□-D-thiogalactoside

The superscripts 'R' and 'S' and 'SS' represent respectively, 'resistance', 'sensitivity' and supersensitivity.

ABSTRACT

In contrast to the information available for the uptake systems, far fewer nutrient efflux systems or exporters in bacteria have been identified. One example of a nutrient exporter is that for arginine (Arg), ArgO (previously designated as YggA) which can also export its analogue canavanine (CAN, which is a plant derived naturally occurring antimetabolite), in *Escherichia coli*. ArgO shares considerable sequence similarity with an exporter LysE of *Corynebacterium glutamicum* which mediates both Arg and lysine (Lys) export. Both Arg and Lys serve as inducers of *lysE* transcription, and this regulation is mediated by the protein LsyG which shares sequence similarity with *E. coli* ArgP [members of the LysR family of regulator proteins].

In vivo studies with *argO-lac* fusions have established that *argO* is also under the strict transcriptional control of ArgP and that its expression is induced by 1 mM Arg as well as by its toxic analogue CAN. Unlike the situation with *C. glutamicum* LysE, transcription of *E. coli argO* in vivo is completely shut off upon addition of 1 mM Lys, and in the presence of both Arg and Lys, it is the Lys effect which dominates. Besides, dominant gain-of-function mutations in *argP* (*argP*^{d}), that confer both a CAN-resistant phenotype (CAN^{R}) and constitutively elevated *argO* transcription, have also been identified.

The studies reported in this thesis investigate the roles of ArgP in *argO* transcription in vitro. The primary objective of this project is to elucidate the mechanisms of ArgP- as well as co-effectors (Arg and Lys)-mediated *argO* transcriptional regulation, and to determine at what step(s) of transcription is *argO* transcription regulated. The strategy involved using in vitro biochemical approaches like DNA-protein interaction, ligand-

protein interaction, footprinting assays, enzyme protection assays, in vitro transcription assays and related techniques to study the interplay between RNAP and ArgP at *argO* promoter in presence and absence of Arg or Lys. Besides it was attempted to characterise the *argP*^{d} CAN^{R} mutants to determine how they rendered *argO* expression constitutive.

For use in the in vitro experiments, ArgP protein (33.4 KDa) was purified with a six-histidine (His_6) tag at its C-terminal end. The His_6-tag did not affect the function of ArgP. Interaction studies revealed that Arg and Lys compete with each other for binding to ArgP and that the K_ms of their binding are 150 μM and 80 μM respectively. However, the binding of the co-effectors does not affect the oligomeric state nor the thermodynamic properties of ArgP, although Lys but not Arg binding led to slight perturbations of the protein's secondary structure. Evidence for the binding of CAN to ArgP was also obtained.

To reconstitute the *argO* transcriptional regulation, the in vivo transcription start site of *argO* was first mapped by primer extension analysis on mRNA, to the A residue situated 28 nucleotides upstream of the GTG start codon of the *argO* structural gene. The –10 region of the promoter sequence was verified by site-directed mutagenesis experiments. Subsequently, a fragment of 427-bp long encompassing *argO* region from −293 to +109 (comprising the entire regulatory region of *argO*) was PCR-amplified and shown to possess all the *cis* elements required for *argO* transcriptional regulation in vivo.

Electrophoretic mobility shift assay (EMSA) experiments using the *argO* fragment above revealed that ArgP binds *argO* regulatory region in absence of co-effectors and

that the presence of Lys, but not Arg, leads to an increased affinity of ArgP binding to *argO* DNA (K_ds under three conditions being 6.5 nM, 6.3 nM and 2.4 nM respectively. Deletion experiments suggested that a 26-bp near-palindromic sequence centered at −77 relative to start site of *argO* is not required for ArgP binding. DNase I footprinting showed that ArgP protection extends from −85 to −20 on *argO* DNA which was again verified by an EMSA experiment using the corresponding fragment.

ArgP- and Arg- or Lys-mediated regulation of *argO* transcription was successfully reconstituted in vitro using σ^{70}-bearing RNA polymerase (RNAP) holoenzyme and the PCR-amplified *argO* fragment above, indicating that ArgP acts directly to regulate *argO* expression. To obtain further mechanistic insights into this process, the four steps of transcription initiation (formation of closed complex between *argO* and RNAP, isomerisation to open complex, transition to initial transcribing complex, and finally promoter clearance) were investigated. It was found from EMSA experiments that RNAP alone forms a very unstable binary complex with *argO* promoter and that, in absence of any of the co-effectors, ArgP and RNAP compete for the formation of corresponding binary complexes with *argO* with no evidence of ternary complex formation. On the other hand, each of the three co-effectors (Arg, CAN or Lys) promote both the formation and the stability of the closed ternary complex of *argO* with RNAP and ArgP. This explains the ArgP-mediated activation of *argO* in presence of Arg or CAN and indicates that it is by the recruitment and stabilization of RNAP binding to *argO*. However, these data also indicate that Lys-mediated inhibition is not merely by inhibiting the RNAP recruitment at *argO* promoter. The ternary complexes of *argO*-ArgP-RNAP assembled in presence of co-effectors were found to be sensitive to heparin in absence of NTPs and become heparin resistant upon addition of NTPs. In

vivo $KMnO_4$ footprinting experiments also revealed that 1 mM Lys addition to the cultures does not impede open complex formation at *argO*, despite the complete inhibition of *argO* transcription under these conditions.

Most interestingly, the inhibition of *argO* transcription in presence of Lys was also not associated with synthesis of abortive transcription products. The possibility that the –10 promoter motif like sequence in the *argO* initial transcribed sequence (ITS) contribute to stalling of RNAP in the ITS in presence of ArgP and Lys was also excluded by site directed mutagenesis experiments, thereby suggesting a novel paradigm of regulation in this case. When the position of RNAP on the *argO* promoter in presence of ArgP and co-effectors was examined by the exonuclease III protection assay, the downstream edge of RNAP protection at *argO* promoter was found to be identical both in the case of ArgP with Arg (without NTPs) and in the case of ArgP with Lys (with or without NTPs), at position +25 in *argO*. These data suggested that in the ternary complex which is inactive for both abortive and productive transcription in presence of ArgP and Lys, RNAP is trapped at the step of the open complex itself or immediately thereafter. It was finally shown by a variety of Arg-chase experiments that this inactive complex can be rendered productive by displacement of Lys with Arg, suggesting that the complex is in a reversible state.

Accordingly, the results presented in this thesis support a novel mechanism of *argO* regulation in which Lys-bound ArgP reversibly restrains RNA polymerase at the promoter, at a step (following open complex formation) that precedes, and is common to, both abortive and productive transcription. Therefore, it is proposed that, under this condition, RNAP exists in a dormant "Rip Van Winkle" ensemble after melting of the promoter duplex at the –10 region, which can be chased into the productive state by

competitive replacement of the Lys ligand which is bound to ArgP by Arg. This represents the first example of an environmental signal regulating the final step of promoter clearance by RNA polymerase in bacterial transcription. It can also be proposed that the *argO*-ArgP-RNAP ternary complex remains assembled and poised in vivo at all times to respond, positively or negatively, to instantaneous changes in the ratio of intracellular Arg to Lys concentrations.

Furthermore, it was demonstrated in this study that the *argP*$^{-}$ mutant alleles that render the strain CAN^{R} are divisible into different classes depending upon whether the *argO* expression in vivo is modulated by Lys or Arg addition. Using one representative CAN^{R} mutant (ArgP^{d}-S94L), transcription of *argO* in vitro was studied in detail. Preliminary evidence was obtained to suggest that Arg or Lys may not interact with the mutant ArgP^{d} protein. Besides, an unexpected observation was that ArgP^{d} mutant protein by itself does not form a stable complex with the *argO* DNA, however, it could bind in presence of RNAP to form a stable ternary complex and activate the transcription.

Finally, comparison of the binding affinities of ArgP revealed that *argO* has 10- to 100-fold higher affinity to ArgP than the other putative target DNAs like *dnaA, nrdA, oriC, gdhA,*or *yqfE*, etc previously reported to bind the protein. Hence, *argO* may be considered as the sole bonafide target of ArgP so far identified.

CHAPTER 1

General Introduction

1.1 Introduction to cell envelope:

1.1.1 Cell envelope : Structure and Function:

The cell envelope in gram negative bacteria is comprised of two cell membranes, an inner membrane (IM) or cytoplsmic membrane which surrounds the cytoplasm and and outer membrane (OM) which lies outside the IM and is separated from it by periplasmic space. The rigidity to the cell envelope is provided by the peptidoglycan layer which lies outside the IM in the periplasmic space. This arrangement is different from that in Gram-positive bacteria, in which there is no OM and the peptidoglycan layer is much thicker (reviewed in Ruiz et al. 2006). A schematic diagram of the *Escherichia coli* cell envelope is shown in Figure 1.1.

1.1.1.1 Outer membrane (OM):

The lipoprotein-rich outer membrane OM is an atypical membrane in comparison with the inner membrane, and has higher buoyant density (1.22) (Schnaitman 1970; Osborn et al. 1972). The components of OM can be divided into two, lipids and characteristic proteins. Besides, a unique polysachharide, enterobacterial common antigen (ECA) is also present in the OM. ECA is an acidic polysachharide containing N-acetyl-D-glucosamine, N-acetyl-D-mannosaminuronic acid, and 4-acetamido-4,6 di-dioxy galactose (Lugowski et al. 1983). It is anchored to the OM through a phospholipid moiety (Kuhn et al. 1983). Besides many lines of evidence suggests that the lipid bilayer forms the basic continuum of OM. However the distribution of lipid is asymmetrical and most of the lipopolysachharides (LPS) exist in the outer leaflet of the bilayer (Muhlradt and Golecki 1975).

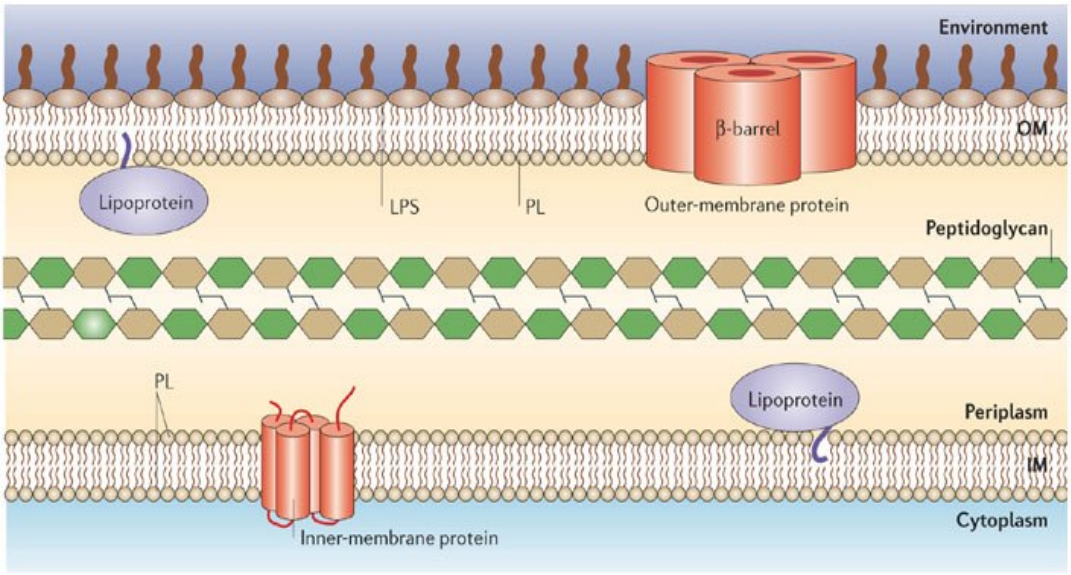


Fig. 1.1 Schemetic representation of *E. coli* cell envelope (adapted from Ruiz et al. 2006). Various membranes, inner and outer membrane proteins, lipoproteins, peptidoglycan layer etc. are indicated. OM, outer membrane; IM, Inner membrane; PL, phospholipids, LPS, lipopolysachharides.

The major lipid components of OM are LPS and phospholipids (PL). The phospholipid components of OM are similar to that of inner membrane with a slight enrichment in phosphatidylethanolamine (Osborn et al. 1972). LPS is a unique constituent of the bacterial outer membrane (reviewed in Raetz and Whitfield 2002; Nikaido 2003). LPS is composed of three parts: the proximal, hydrophobic lipid A region; the distal hydrophilic O-antigen polysaccharide region; and the core oligosaccharide region that connects the two. The lipid A region is presumably essential for the assembly of the outer membrane whereas the more proximal hydrophobic region is responsible for maintaining the barrier property of the OM. The O-antigen is essential for escaping phagocytosis and virulence. *E. coli* K12 completely lacks the O-antigen (Takayama and Quareshi 1992; Vaara 1993; Reevs 1994).

The major proteins of bacterial OM include the following:

(i) Murein lipoproteins: These function in stabilisation of OM peptidoglycan complex. About one third of the murein lipoproteins molecules are bound covalently to the peptidoglycan layer through the ε-amino group of the C-terminal Lys thus fixing OM to the underlying peptidoglycan. Mutants through the deletion of murine lipoprotein gene (*lpp*) produces unstable OMs resulting in the release of OM vesicle and periplasmic enzymes into the medium (Hirota et al 1977).

(ii) The porin proteins: These include protein such as OmpF, OmpC, PhoE, and LamB (Nikaido and Vaara 1987). The porin proteins produce relatively non-specific pores or channels that allow passage of small hydrophilic molecules (of molecular mass less than 600 Daltons) across the OM. The latter two proteins, PhoE and LamB are produced under specialised conditions of growth i.e. upon phosphate starvation and upon maltose inductions, respectively, to facilitate the transport of polyphosphates and maltodextrins. OmpF and OmpC are the major porins present under most culture condition. The relative abundance of these two proteins is regulated by a number of environmental parameters such as osmolarity of the medium, temperature, oxygen availability, pH and carbon limitation (Lugtenberg et al. 1976; Bassford et al. 1977; Alphen and Lutenberg 1977; Graem-Cook et al. 1989).

1.1.1.2 The peptidoglycan layer:

The peptidoglycan matrix is the rigid shape-determining structure in the bacterial cell envelope. This layer is composed of highly crosslinked peptidoglycan which is sensitive to lysozyme. The peptidoglycan layer is located in the periplasm between OM and IM. In *E. coli* it is a seen as a dense layer 2-3 nm thick associated with OM in electron microscopy (Hobot et al. 1984). This central line of peptidoglycan divides the periplasmic space into inner and outer periplasmic regions. It has been proposed that

relatively uncrosslinked peptidoglycan polymers are found in the inner periplasmic space forming a periplasmic gel (Hobot et al. 1984).

The peptidoglycan layer contains roughly equal amounts of polysaccharides and peptides and is composed of glycan chains that are crosslinked by oligopeptides, but there are also proteins associated with the peptidoglycan layer (Vollmer and Holtje 2004). The peptidoglycan is a complex polymer whose subunits consists of two amino sugars and at least four amino acids. The chemical structure of peptidoglycan is composed of N-acetyl glucosamine (GlcNAc), N-acetylmuramic acid (MurNAc), D-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP) and D-alanine all in equimolar amounts except for D-alanine. These components form the repeating unit of the peptidoglycan polymer chain. The sugars form linear chains of alternating units of GlcNAc and MurNAc linked in β 1→4 bonding. This structure is shared by all gram negative bacteria (Schleifer and Kandler 1972). The average glycan strand is about 30 muropeptides in length (Holtje et al. 1975). A molecule of lipoprotein is attached to about every tenth glycan-peptide (Braun and Rehn 1969).

The peptidydoglycan is firmly attached to the OM by covalently linked liproprotein molecule that form part of the OM (Bruan 1975). In addition, OmpF and OmpC also appear to contribute to the high degree of association between peptidoglycan and the OM, since these proteins do not separate from murein peptidoglycan layer even after heating to 60ºC in the presence of sodium dodecyl sulphate (Nikaido and Vaara 1987).

1.1.1.3 Inner membrane (IM):

The IM or cytoplasmic membrane is the boundary between the cytoplasm and the environment and is primarily responsible for regulating the flow of nutrients and the metabolic products in and out of the cell. It is involved in almost every aspect of

bacterial growth and metabolism. The IM is believed to lack mechanical rigidity and therefore cannot support an osmotic gradient.

The IM is made up of phospholipid bilayer and composed of roughly equal amounts of proteins and phospholipids. It contains 65 to 75% of the total cellular phospholipids and 6 to 9 % of cellular protein (about 60 % of the cell envelope). Three major phospholipid species present are phosphatidyl-ethanolamine (PE), phosphatidyl-glycerol (PG) and cardiolipin (CL) or diphospahatidyl glycerol. There are small amounts of phosphatidic acid and phospahtidyl serine (PS), lysophospholipids, and diacylglycerol. The predominant fatty acids present in the cytoplasmic membrane lipids are saturated palmitic acid and the unsaturated species palmitoleic acid and cis-vaccenic acid.

Some proteins are tightly bound to the IM and they are called as integral membrane proteins. On the other hand, those which are loosely bound without spanning the membrane are called as peripheral membrane proteins. Integral membrane proteins span the membrane with α-helical transmembrane domains, and lipoproteins are anchored to the outer leaflet of the membrane by lipid modifications (Tokuda and Matsuyama 2004). Two dimensional electrophoresis reveals around 100 varieties of protein associated with the IM (Sato et al. 1977). The presence of specific transport proteins in the membrane renders it selectively permeable. Proteins in IM are involved in bioenergetic transport functions.

The fluid mosaic model of the bilayer IM portrays that the membrane proteins are floating freely in the lipid bilayer where they have restricted vertical (flip-flop) movements but are free to diffuse within the plane of the membrane (horizontal movement). The lipid bilayer of the membrane forms a hydrophobic barrier that

prevents the uncontrolled movements of polar molecules and allows the accumulation and retention of metabolites and proteins. They also provide a proper matrix for the protein complexes involved in bioenergetic and biosynthetic functions. The lipid composition determines the dynamics and the interactions of lipid molecules to determine the permeability properties of the membrane and also influence membrane topology, interaction and function (Nichole et al. 1980; Cronan et al. 1987; Bloom et al. 1991; Hoch 1992).

1.1.1.4 Periplasm:

The periplasmic space lies between the inner and the outer membranes. It is nearly 10% of the cell volume and is a highly viscous compartment (Van Wielink et al. 1990). Based upon a method for preparation of cells for electron microscopy, Hobot et al. (1984) conceptualised that the periplasm is filled with a gel matrix composed of highly hydrated un-crosslinked strands of peptidoglycan that maintain the distance between outer peptidoglycan cell wall and the inner cytoplasmic membrane. Periplasmic proteins would be freely diffusible within this gel.

The proteins in the periplasmic compartment can be divided into several groups. These include i) substrate-binding proteins that function both in active transport of small molecules like sugars, amino acids, peptides, vitamins and ions and in chemotaxis, ii) scavenging enzymes which break down complex molecules into simpler precursors for transport across the membrane, iii) detoxifying enzymes that inactivate toxic molecules, and iv) enzymes or proteins that promote the biogenesis of the major envelope proteins or proteinaceoius appendages, peptidoglycans, LPS capsules or membrane derived oligosaccharides (MDOs) (Stock et al. 1977; Oliver 1987). Besides, periplasmic proteins are also responsible for the biogenesis of molecules entering the compartment

and facilitates incorporation to destined peptidoglycan, OM, IM or capsular layers. The periplasmic content of small molecules also serves to buffer the cell from changes that occur in local environment. Some protein folding and trafficking factors that build and maintain the cell envelope are also located in the periplasm (Duguay and Shilhavy 2004; Mogensen and Otzen 2005).

The major solutes identified in large amounts specifically in the periplasm are a class of compounds called membrane derived oligosaccharides (MDO) of molecular weight >2500 Da. MDO are highly anionic (average charge −5) oligosaccharide consisting of 8-10 glucose units joined by β-1→2 and β-1→6 linkages and variously substituted with phosphoglycerol and phosphoethanolamine derived from membrane phospholipids. Some contain O-succinylester residues. MDO have been postulated to play a role in osmotic regulation in the periplasm. Accumualtion of MDO has been shown to attract high concentrations of Na^+ and K^+ ion and thus cause an unequal distribution of these cations between the periplasm and extracellular medium. This results in generation of an electric potential called the Donnan potential across the OM. The Donnan potential has been measured by determining the distribution of Na^+ and Cl^- between the periplasm and cell exterior. The potential varied with ionic strength of the medium and was shown to be 30 mV, negative inside (Stock et al. 1977).

1.2 Introduction to amino acid transport in *Escherichia coli*:

All metabolites, nutrients and solutes constitute a pool inside the cell of a bacterium. A pool can be simply defined as the total quantity of low molecular weight compounds that may be extracted from the cells under the condition such that the macromolecules are not degraded into low molecular weight subunits. Pool formation and maintenance is an expression of the ability of a cell to contain nutrients, which are present at very

low concentrations in the environment, for supply to the cellular machinery at high concentration.

Like other cellular metabolites, amino acids are also a part of the cellular pool. A cell maintains a very high pool size of amino acids against the concentration of that amino acid in the surrounding environment. All amino acids in the pool are mainly utilised for protein synthesis. The cellular pool of amino acid is maintained in two ways, namely by biosynthesis and by active uptake from the outside environment (reviewed in Britten and McClure 1962). *E. coli* cells accumulate amino acids by systems some of which are specific for individual substrates and others which are general for groups of related amino acids (reviewed in Piperno and Oxender 1968; Oxender 1972; Halpern 1974).

1.2.1 Amino acid uptake systems in *E. coli:*

All molecules passing across the cell envelope are transported through porins in the OM. However, the specificity and the flow are regulated in the IM by the existence of a large number of transport proteins. The active uptake of nutrients across the *E. coli* inner membrane is mediated by three different classes of transporter systems, two of which are involved in the uptake of different amino acids (Neu and Heppel 1965; Wilson 1978; Ames 1986). (1) The binding protein-dependent transport systems (or osmotic shock-sensitive system) are multi-component system each of which includes a specific periplasmic binding protein which can be released by mild osmotic treatment [examples include leucine (Leu), arginine (Arg), histidine (His), glutamine (Gln) etc.]. (2) The membrane-bound systems are shock-resistant, and their transport elements are integral components of cytoplasmic membrane [examples include the transporters of proline (Pro permease), phenylalanine (Phe), alanine (Ala), aspartate (Asp), glycine (Gly), serine (Ser), tyrosine (Tyr) etc.]. (3) The third class of active transporters are the

group translocation systems which are energised by substrate level phosphorylation and include several sugar transporters but none of them are involved in amino acid uptake. Whereas the binding protein-dependent system has obligatory requirement of phosphate bond hydrolysis provided by ATP or related molecules for energy, the membrane-bound system uses the chemi-osmotic proton motive force (PMF), either directly or indirectly, to drive the active transport.

In a functional catagorisation, amino acid influx systems can be divided into the following classes: 1. Neutral amino acid uptake systems [Ala, Gly, Ser, threonine (Thr)], 2. aromatic amino acid transport systems [Phe, Tyr, tryptophan (Trp)], 3. basic amino acid transport system [Arg, Lys, ornithin (Orn), His], 4. branched-chain amino acid transport permeases [Leu, isoleucin (Ile), valine (Val)], 5. dicarboxylic amino acid transporters [Asp and Glu], 5. asparagine (Asn) and glutamine (Gln) transport and, 6. other amino acids Pro, cystein (Cys), methionine (Met) etc. Since this study was concern with the basic amino acids Arg and Lys, the current knowledge on their uptake system is described below.

1.2.2 Basic amino acid transport systems (Arg, Lys, Orn and His):

Gram negative bacteria like *E. coli* possess a number of systems for active uptake of basic amino acids. Of the basic amino acids, His transport is mediated by the His permease coded by the *hisPMQJ* operon (Liu and Ames 1997, 1998). HisPMQJ is an ATP-dependent His uptake system that is a member of ATP binding cassette (ABC) superfamily of the transporters. It is comprised of the following components: the receptor which is the periplasmic His-binding protein, HisJ; integral membrane-bound complex (HisQ/M) containing two hydrophobic subunits HisQ and HisM; and the two subunits of inner-membrane associated ATP-binding component HisP. A model of

transport was proposed in which liganded HisJ interacts with HisQ/M/P, thus initiating ATP hydrolysis and consequent substrate translocation leading to the transport of the ligand.

In *E. coli*, a common system shared by Arg, Lys and Orn is called as the LAO system (Rosen 1971; Celis 1981; Ames 1986). This system represents one of the prototypes of the binding protein dependent uptake systems, also called ABC-transporters (Ames 1986; Higgins 1992). The LAO transporter is a high-affinity system with the affinities of LAO binding proteins in the following order for its three substrates K_d (Arg) > K_d (Lys) > K_d (Orn). This system is unusual in that it has a distinct periplasmic binding protein (LAO) for Lys, Arg and Orn but the other components (that is, the integral membrane proteins HisQ and HisM and ATP-hydrolysing protein HisP) are shared with that of the His uptake system (which has its own binding protein HisJ). In other words, the two periplasmic proteins bind different substrate amino acids but then interact with common membrane proteins uptake of these amino acids. The corresponding genes are organised into two adjacent transcriptional units i.e., *argT* (encoding LAO) and *hisJQMP* (encoding HisJQMP proteins). In *E. coli*, the corresponding genes of this system are located at 50 min on the chromosome; however it is less well characterised as compared to that in *Salmonella enterica Serovar typhimurium* (previously *S. typhimurium*) (Nonet et al. 1987; Kraft and Leinwand 1987). It was reported that the expression of proteins constituting the LAO system is repressible only by Lys (Celis et al. 1973). It is the only Arg transport system (*argT*) which expression is σ^{54}-dependent (Reitzer and Schneider 2001). However, the P_{hisJ} (for *hisJQMP*) is σ^{70}-controlled promoter repressible by ArgR protein (Caldara et al. 2007).

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 subsequently described by Celis (1977a,b, 1981). The Arg-Orn system includes a specific periplasmic binding protein, the AO-binding protein which binds Arg and Orn with lower affinity than LAO-system. It was reported to share with LAO-system, a common inner membrane transport protein which is able to hydrolyse ATP and also phosphorylate the two periplasmic binding proteins, the enzyme proposed to be ArgK (Celis 1990; Celis et al. 1998). The gene locus for the structural gene of Arg-Orn binding protein (*abpS*) was mapped roughly at 63.5 min of the *E. coli* chromosome (Celis 1981, 1982). Its expression is σ^{54}-independent (Reitzer and Schneider 2001). 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).

It appears now that the AO uptake system of *E. coli* is also encoded by the *artPIQM-artJ* locus (*art* for Arg transport) of the Arg-specific system. Recently *artI* was proposed to be the gene encoding the periplasmic AO binding protein (ArgBP-II) instead of *abpS*, as proposed previously (Caldara et al. 2007). A computer analysis also showed that ArgI is the closest *E. coli* protein to the AO protein, and that no protein near the proposed site of *abpS* had anything resembling this amino acid composition (Reitzer and Schneider 2001).

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 Arg-BP I) of lower content and affinity. The third uptake system was analysed and characterised at gene and protein levels by Wissenbach et al. (1995). This third system is encoded by five adjacent genes, *artPIQMJ* (*art* standing for Arg transport), which was organised 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 and ArtM are similar to the trans-membrane proteins, and ArtP to 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 was not known, and the isolated protein did not bind common amino acids, various basic uncommon amino acids such as Orn or citrulline, or amines, such as putrescine (Wissenbach et al. 1993, 1995).

1.2.3 Amino acid export systems in *Escherichia coli*:

In contrast to uptake systems, far fewer nutrient efflux systems or exporters in bacteria have been identified and characterised (Lolkema et al. 1998; Aleshin et al 1999; Burkovski and Kramer 2002). Traditionally, bacterial export carriers were thought to confer resistance to antibiotics and metals or to remove waste products. It was believed that excretion of nutrient solutes occurs as a result of changed (leaky) membrane (Britten and McClure 1962).

However, it has now become clear that carriers also export primary metabolites such as sugars and amino acids but in many situations the primary functions of the nutrient export carriers are not known. The paradox is why a cell would possess systems that mediate the loss of essential nutrients. Recent studies have suggested that the function of amino acid exporters is at least to serve as safety valves to prevent the accumulation to toxic levels of the nutrients in question, as first shown from studies of the basic amino acid exporter LysE of *Corynebacterium glutamicum* (Broër and Krämer 1991a,b; Eggeling and Sahm 2003). The safety-valve hypothesis has since been validated by genetic studies in a number of other instances (Vrljić et al. 1996; Simic et al. 2001; Kennerknecht et al. 2002; Nandineni and Gowrishankar 2004; Kutukova et al.

2005; Bucarey et al. 2006). One would also expect that the exporters are tightly regulated (Bellmann et al. 2001; Nandineni and Gowrishankar 2004; Trötschel et al. 2005), in order to minimise futile cycling of substrate flux across the cell membrane.

The amino acid exporters so far identified for *E. coli* include RhtB and RhtC for Thr and homoserine, and YdeD and YfiK for Cys and O-acetylserine, LeuE for Leu export and very recently YgaZH for Val export. Most of these export systems have been identified based on their ability to confer increased production that is, excretion of the cognate amino acid upon their overexpression and are further described in the following section, as also the LysE system for Arg export in *C. glutamicum*.

1.2.3.1 RhtB and RhtC for Thr and homoserine export:

RhtB was identified by Zakataeva et al. (1999) as the efflux system of homoserine and Thr. In other studies, three different open reading frames encoded by *rhtA, rhtB* and *rhtC* involved in the resistance of *E. coli* to high concentrations of Thr and homoserine were reported as being responsible for Thr export into the culture medium from L-Thr overproducing strain (Kruse et al. 2002; Burkovski and Krämer 2002; Livshits et al 2003). While single or double deletions of *rhtB* and *rhtC* conferred no difference in the production of L-Thr, over-expression of either *rhtB* or *rhtC* improved the specific production of L-Thr overproducer strains to some extent (Kruse et al. 2002).

The proteins RhtB and RhtC belong to a new RhtB family of membrane proteins which also includes other exporters LysE and LeuE. RhtB is highly hydrophobic and contains six predicted trans-membrane segments. RhtB carries out excretion of not only Thr and homoserine but also homoserine lactones and probably some other amino acids as well. RhtC is also a homologue of RhtB. The expression of these genes *rhtB* and *rhtC* are regulated by the global regulator Lrp, which is a Leu binding protein.

The *rhtA* gene was also identified as involved in the Thr and homoserine efflux (Livshits et al. 2003). This gene also codes for a highly hydrophobic membrane protein with 10 predicted trans-membrane segments. In addition, the *E. coli* genome encodes at least 10 paralogues of RhtA. The over-expression of *rhtA* gene results in resistance to inhibitory concentrations of homoserine and decreased accumulation of homoserine and Thr in the cell.

1.2.3.2 YdeD and YfiK for Cys and O-acetylserine (OAS) export:

YdeD and YfiK are the two efflux proteins for Cys and OAS which are coded by genes *ydeD* and *yfiK* respectively (Daßler et al. 2002; Franke et al. 2003). *ydeD* was detected as a gene augmenting the yield of Cys overproducing strain. Mutation of *ydeD* did not cause any distinct phenotype although the overexpression of this gene causes the cell to excrete considerable amounts of Cys and OAS in presence of thiosulphate. YdeD belongs to PecM family of transporters and has 9 to 10 putative trans-membrane helices (Daßler et al. 2002).

yfiK was identified during the gene bank screening for Cys overproducers. It was also discovered as a gene augmenting Cys production when it was over-expressed in an industrial *E. coli* production strain. The gene product is an integral membrane protein with about 6 predicted trans-membrane helices. It belongs to RhtB family of export proteins. YfiK overproduction from a plasmid leads to drastic and parallel secretion of Cys and OAS into the medium (Franke et al. 2003).

1.2.3.3 LeuE for Leu export:

The *yeaS* locus was identified by Kutukova et al. (2005) to code for a Leu exporter protein and it was renamed as *leuE* for Leu export. LeuE belongs to RhtB transporter

family of proteins and it serves as efflux system for Leu. The over-expression of *leuE* conferred upon cells resistance to the dipeptide glycyl-L-Leu, Leu analogues (such as L-α-amino-N-butyric acid, 4-Aza-DL-Leu), some structurally unrelated amino acids (such as His, Met, Lys etc.) and their analogues. The global regulator Lrp is involved in the repression of *leuE* gene in *E. coli*, and *leuE* expression is induced by Leu, its natural analogue L-α-amino-N-butyric acid and a number of other amino acids (Kukutova et al. 2005).

1.2.3.4 Valine (Val) exporter:

The exporter of Val (coded by *ygaZH*) was described very recently by Park et al. (2007) in the process of construction of a Val overproducing *E. coli* strain by rational metabolic engineering. It was reported that the amplification of *lrp*, *ygaZH*, and *lrp-ygaZH* genes led to the enhanced production of L-Val. Lrp acts as the regulator of Val exporter which is coded by *ygaZH*. The proteins coded by *ygaZH* genes show significant homology to the known *C. glutamicum* L-Val exporter. It was proved by knocking-out and amplification studies that it is truly involved in the export of L-Val in *E. coli* as well.

1.2.4 Arg exporter in bacteria:

The first bacterial Arg exporter to be identified and characterised was LysE of *C. glutamicum*. Although Broër and Krämer (1991a,b) showed the presence of a specific Lys excretion system, it was identified and isolated as LysE by Vrljic et al. (1996) and cloned the *lysE* gene and further characterise the protein (Vrljić et al. 1996, 1999). Bellmann et al. (2001) reported that in addition to Lys, LysE also exports Arg. However, this transporter is not involved in the export of other basic amino acids like His or citrulline. The export of Arg or Lys by LysE is driven by proton motive force.

LysE functions as a dimer and represents a novel type of structure containing five transmembrane-spanning helices and a sixth hydrophobic segment that may dip into the membrane or surface localised (Vrljić et al. 1999; Eggeling and Sahm 2003; Burkovski and Kramer 2002). This exporter is a prototype of the LysE superfamily of transporters, the members of which are present both in eubacteria and archaebacteria (Aleshin et al. 1999; Vrljic et al. 1999).

The mutant of *lysE* with a modified efflux of Lys was isolated by Vrljic et al. (1995) and in absence of this carrier, L-Lys could reach a concentration of more than 1100 mM, which prevents the cell growth. Therefore, it was implied that LysE serves as a valve for exporting excess Lys that might be harmful to the cell (Vrljic et al. 1996; Eggeling and Sahm 2003; Burkovski and Kramer 2002).

It was also shown that *lysE* expresson is positively regulated by a LysR type of transcriptional regulator, LysG. Lys and Arg induces the *lysE* expression via LysG. Although, His and Citrulline are not the substrate of LysE, they also induce the *lysE* expression (Bellmann et al. 2001; Eggeling and Sahm 2003).

1.2.4.1 ArgO as an Arg exporter in *E. coli*:

Nandineni and Gowrishankar (2004) reported the identification of an Arg exporter protein in *E. coli* that was encoded by an anonymous open reading frame, *yggA* which was then re-designated as *argO* (for Arg outward transport). Null insertions in *argO* confer a CAN^{SS} (canvanine supersensitive) phenotype. (CAN is plant-derived naturally occurring antibacterial metabolite; it is an Arg analogue and it is also transported by Arg transporters.) The CAN^{SS} phenotype of the *argO* null mutants has been explained to be consequence of the inactivation of CAN export through the ArgO exporter.

ArgO shows high similarity with LysE of *C. glutamicum.* Based on the homology between ArgO and other members of the membrane-associated efflux systems, it was suggested that *argO* encodes a membrane protein with six trans-membrane helices involved in efflux of Arg. Nevertheless, despite the primary sequence similarity of ArgO to LysE, which is an exporter of both Arg and Lys in *C. glutamicum* (Vrljić et al. 1996; Bellmann et al. 2001), there is no evidence of Lys export by ArgO. Just as LysE in *C. glutamicum* is under the transcriptional control of LysG, *argO* is under the transcriptional control of a LysR-type transcriptional regulator ArgP which is an analogue of LysG.

1.3 Introduction to ArgP (IciA) Protein:

As mentioned above, it was recently identified (Nandineni and Gowrishankar 2004) that the ArgO exporter is under the transcriptional control of ArgP, which is homologous to LysG of *C. glutamicum*. ArgP protein is 297 amino acids long (with a molecular mass of 33,471 Da) and is encoded by the *argP* gene which lies at 62.8 min on the chromosomal map of *E. coli.* ArgP was shown to be an elongated dimer with a helix turn helix DNA binding motif at the N-terminus (Thony et al. 1991; Hwang et al. 1992). ArgP belongs to a family of LysR type of transcriptional regulators (Schell 1993) and recognises the AT-rich DNA sequences (Celis 1999).

The ArgP protein was originally identified and purified by Hwang and Kornberg (1990) as an inhibitor of chromosome replication initiation at *oriC* in vitro, and was designated as IciA. In a large number of publications thereafter, this protein has been worked upon as IciA. Besides the role in DNA replication, IciA was also implicated to be involved in the regulation of some target genes such as *dnaA* and *nrdA* which are required respectively in the synthesis of DnaA protein and the deoxyribonucleotides.

Nevertheless, *iciA* (that is, *argP*) knock out mutants have shown no replication related or DNA-synthesis related phenotypes and hence the physiological role of IciA in such function is unclear.

ArgP was also reported to be a member of Pho regulon and is activated by PhoB protein (Han et al. 1998). Besides, in one of the study, ArgP was suggested to be one of the DNA binding nucleoid proteins (Azam and Ishihama 1999). It has similarity to an *Edwardsiella ictaluri* protein that provokes an immune response in catfish (Moore et al. 2002). Yoo et al. (1993) showed that ArgP is specifically cleaved by protease "Do" but not with other soluble proteases in *E. coli*.

The in vivo role of ArgP was not known except for the report that the over-expression of *argP* on high copy number plasmid exhibits a pronounced lag upon transfer to a fresh medium (Thony et al. 1991). Around 100 molecules of ArgP (IciA) is present in the fast growing bacterial cells in log phage of growth which is increased fourfold (around 400 dimers per cell) as cells approach stationary phase that is from around, 0.4 μM to 1.6 μM of ArgP monomers in the cytoplasm (Hwang et al. 1992). A recent study from this laboratory suggested in vivo role of ArgP in Arg (and toxic analogue CAN) export from the cell (Nandineni and Gowrishankar 2004).

1.3.1 IciA and DNA replication at *oriC*:

As mentioned above, the first identification of IciA was by Arthur Kornberg and coworkers more than fifteen years ago as a protein that binds to *oriC* to *i*nhibit *c*hromosomal replication *i*nitiation in vitro (Hwang and Kornberg 1990, 1992; Thony et al. 1991; Hwang et al. 1992). *E. coli* chromosomal replication is initiated by binding of DnaA proteins to the DnaA boxes (9-mers) within the *oriC* sequence. After being bound to the 9-mers, DnaA protein along with nucleoid proteins such as HU or IHF

opens the AT-rich regions containing the three 13-mers (which are very conserve sites at *oriC*) to admit DnaB helicase and other replicative proteins that enlarge the opening and set the stage for priming of bidirectional replication for initiation of DNA synthesis. The cell cycle in *E. coli* is regulated at the stage of initiation, and it is at this point that one might expect positive and the negative effectors to exert their action (Von Meyenberg and Hansen 1987; Kornberg and Baker 1992). ArgP protein binds the entire 13-mer regions with specificity and prevent opening of this region to start replication thereby blocking the initiation of replication. Once 13-mers are opened ArgP has no effect on the subsequent events in the replication of *oriC* (Hwang and Kornberg 1990, 1992; Thony et al. 1991). However, *argP* (*iciA*) mutants are viable and exhibit no replication-associated phenotype (Thony et al. 1991; Lee et al. 1996; Celis 1999; Nandineni and Gowrishankar 2004). Besides, it was also reported that ArgP binds AT-rich regions of origin of replications of plasmids F (*oriS*) and R1 (*oriR*) (Wei and Bernander 1996).

1.3.2 IciA (ArgP) in *dnaA* regulation:

dnaA gene codes for DnaA protein which binds to DnaA boxes (or 9-mers) within the *E. coli oriC* to initiate the chromosomal DNA replication (Bramhill and Kornberg 1988; Kornberg and Baker 1992; Hwang and Kornberg 1992). This gene has two promoters 1P and 2P. The binding of DnaA protein to the consensus DnaA box in the promoter region followed by subsequent oligomerisation over the two promoters results in the repression of the transcription from these two promoters presumably by occluding the binding of RNAP (Lee and Hwang 1997; Braun et al. 1985). This autoregulation of *dnaA* has been suggested to contribute to the regulation of initiation of chromosomal DNA replication. The *dnaA* regulatory region has been reported to contain two binding sites for IciA protein, IciA-I and IciA-II, located upstream of the

promoter 1P and downstream of the promoter 2P, respectively (Lee et al. 1996). Of the two *dnaA* promoters, transcription from the promoter 1P was specifically enhanced by IciA both in vitro and in vivo (by overproduction of IciA protein) (Lee et al. 1997). ArgP function is dominant over the DnaA protein and the mechanism may involve the ability of ArgP protein to counteract the DnaA dependent occlusion of RNAP from this promoter. In vitro transcription assays carrying various combinations of ArgP binding sites on *dnaA* gene revealed that IciA-I which is located upstream of the promoter is responsible for the transcriptional activation (Lee et al. 1996, 1997). The authors postulated that the two dimers of IciA bind cooperatively on *dnaA* promoter 1P, enhancing the binding of RNAP and thus resulting in the activation of transcription from *dnaA* promoter 1P (Lee et al 1996, 1997).

1.3.3 IciA and *nrdA* expression:

The essential genes *nrdA and nrdB* in the *E. coli nrd* operon encode two non-identical subunits (B1 and B2) of ribonucleoside diphosphate reductase which is required for deoxyribonucleotide synthesis (Brown et al. 1969; Han et al. 1998). The *nrd* promoter contains binding sites for DnaA and Fis proteins which have been reported to be transcriptional activators of *nrd* gene (Augustin et al. 1994). Previous studies have shown that IciA protein binds specifically to the AT-rich upstream region of *nrd* promoter. Three binding sites of ArgP on the *nrd* promoter were reported by DNase I footprinting. In vivo over-expression of IciA led to increase in the expression of *nrd* genes by four- to five-fold, suggesting that ArgP acts as transcriptional activator of *nrd* genes (Han et al. 1998). However purified ArgP did not affect the in vitro transcription of *nrd* promoter from supercoiled or linear template.

1.3.4 ArgP's role in Arg metabolism and transport:

The *argP* genetic locus was first described independently by Celis et al. (1973) and Rosen (1973b) by mutations conferring canavanine resistance (CAN^R) and it was postulated to be a regulator of Arg uptake. Subsequenly, molecular characterization of the locus showed that the protein is identical to IciA. The *argP* mutant showed a reduction of 90% in Arg transport and was also affected in the transport of Lys and Orn

Genetic studies of Arg metabolism and transport have often employed the Arg analogue canavanine (CAN). CAN is a plant derived naturally occurring bacterial anti-metabolite, that is also transported into the cell by Arg 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.

It was hypothesised that reduced uptake of CAN in the *argP* mutant was responsible for its CAN^R phenotype. The CAN^R mutation in *argP* was shown to be a missense allele that led to the substitution of Pro to Ser at position 274 (P274S) in the 297-amino acid long protein. ArgP was suggested to activate the transcription of a second gene *argK* which was postulated to be an ATPase that is necessary for ATP hydrolysis coupled to the transport of amino acids. Thus, the *argP* locus was proposed to encode a protein that regulates Arg transport in *E. coli* in by regulating the expression of the *argK* gene (Celis, 1999).

Contrary to these conclusions, however, studies from this laboratory (Nandineni 2003; Nandineni and Gowrishankar 2004) reported that the null mutants of *argP* (*argP::Kan*), exhibit more sensitivity to CAN [CAN supersensitive (CAN^{SS})] than the wild type ($argP^{+}$) strain. This was unexpected since, according to the model of Celis (1999), null insertions in genes involved in Arg uptake are expected to give a CAN^{R} phenotype. Besides, from the in vivo genetic evidence, it was argued that ArgP neither regulates ArgK nor any Arg importer, rather that it transcriptionally regulates the Arg exporter ArgO. The failure to export CAN from the cell in an *argP* mutant was suggested be the cause for CAN^{SS} phenotype in the *argP* null strain.

1.3.5 ArgP regulation of *argO* expression:

As mentioned in the previous Sections, ArgP protein was reported to control the transcription of an Arg exporter gene called *argO*. In vivo regulation studies had shown that Arg addition activates the transcription of *argO* promoter and Lys causes the *argO* expression to be shut off. Arg analogue, CAN was also reported to induce *argO* expression mediated through ArgP (Nandineni and Gowrishankar 2004).

Nandineni and Gowrishankar (2004), also isolated seven gain of function *argP* missense mutations which render the strain CAN resistant (CAN^{R}) all but one were dominant ($argP^{d}$) over wild-type $argP^{+}$. The dominant CAN^{R}-$argP^{d}$ mutant rendered the *argO* expression constitutive and independent of co-effectors Arg, CAN or Lys. It is therefore likely that the CAN^{R} missense mutation in *argP* originally identified by Celis (1999) also represents a gain of function $argP^{d}$ mutation rather than a negative dominant (i.e., loss of function) mutation in the gene as proposed by him.

1.3.6 ArgP protein in *argP* expression (auto-regulation of *argP*):

Based on the data from in vitro experiments, it was suggested by Celis (1999) that ArgP negatively auto-regulates its expression, but only so in presence of Arg. It was postulated by Celis (1999) that the ArgP protein might bind to the T-N_{11}-A consensus sequence located at the position –40 with respect to the start codon of *argP* structural gene and that the inhibition of initiation of transcription might result from hindrance to the binding of RNAP or another essential transcription factor to the promoter by ArgP.

Interestingly, there is one gene *yqfE* of unknown function adjacent to and upstream of *argP* which is transcribed divergently from *argP*. *argP* and *yqfE* hence share a common and overlapping regulatory region. In many instances, the target gene for a LysR-type transcription regulator lies immediately upstream of and is transcribed divergently from the gene encoding the regulator itself. Since ArgP is a member of the family of LysR-type transcription regulators, it is conceivable that it actually recognise this region and controls either *yqfE* or itself.

However the claim that *argP* transcription is autoregulated was contradicted by findings from this laboratory (Nandineni and Gowrishankar 2004), which purported that ArgP transcription is constitutive. In their study, β-galactosidase expression from a mutant *argP*202 null insertion allele (that generates a *lac* operon fusion in correct orientation with the chromosomal *argP* gene) was not affected by $argP^+$ or $argP^d$ (that is, the transdominant CAN^R *argP* allele S94L, which render *argO* expression constitutive) nor by the co-effector Arg or Lys.

1.3.7 Role of ArgP in osmosensitivity (ArgP in *gdhA* expression):

In *E. coli*, the process of NH_4^+ assimilation and Glu synthesis are intimately coupled in that all NH_4^+ assimilation is mediated through Glu biosynthesis. It is also known that

growth in high-osmolar media is associated with intracellular accumulation of Glu (as a counterion for K^+ which also accumulates under these conditions) mediated by its increased biosynthesis. There are two pathways for Glu synthesis and NH_4^+ assimilation. They are: (1) GS-GOGAT pathway in which one molecule of Glu reacts first with NH_4^+ to be converted into one molecule of Gln with the aid of enzyme Gln synthetase (GS), and in the second step, Gln reacts with 2-oxoglutarate to form two molecules of Glu in the presence of Gln-2-oxoglutarate aminotransferase (GOGAT), with a result of net gain of 1 Glu molecule synthesised; and (2) the glutamate dehydrogenase (GDH) pathway which is catalised by Glu dehydrogenase wherein NH_4^+ is condensed with 2-oxoglutarate to give Glu.

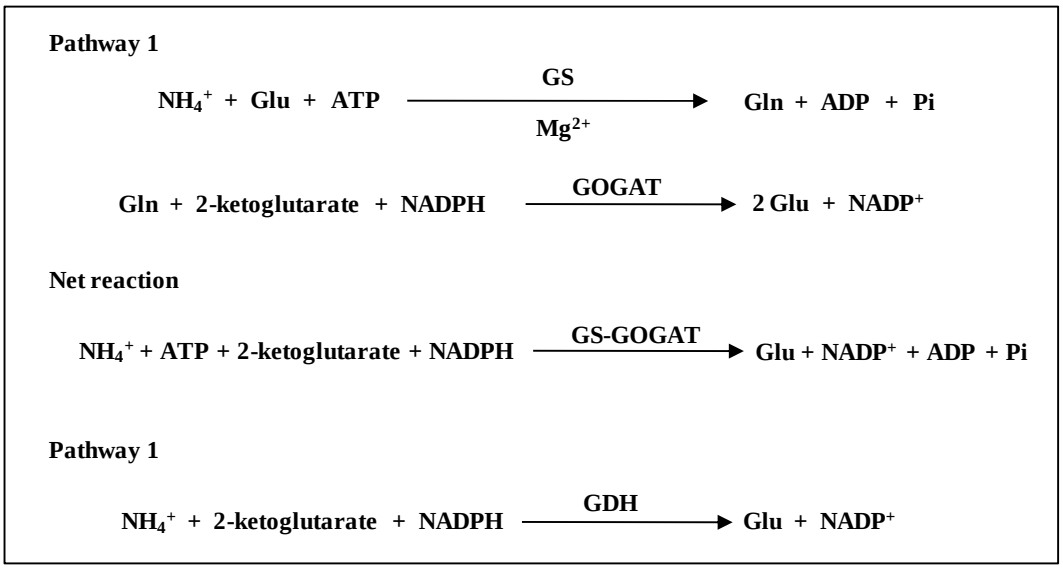


Figure 1.2 Ammonium assimilation and Glu biosynthesis in *E. coli*

Whereas both pathways are associated with oxidation of NADPH to $NADP^+$, an important difference between the pathways is that the former does not consume ATP, while the latter does. The K_m of GDH for NH_4^+ is ~20-fold higher than that of GS for NH_4^+. The GDH pathway is therefore functional during only during growth in high

[NH_4^+] when it is the preferred pathway, while the GS-GOGAT pathway is functional with low [NH_4^+] or during growth with a nitrogen source other than NH_4^+.

The GOGAT enzyme is encoded by *gltBD* gene, and hence in a *gltBD* mutant strain the GS-GOGAT pathway is rendered non-functional. Nevertheless, the *gltBD* mutants are not defective for growth or for osmoregulation in media with high NH_4^+, where the pathway mediated by GDH is functional. Therefore, the finding by Nandineni (2003) that in *gltBD* mutant strain, mutations in *argP* conferred osmosensitivity, led to the suggestion for a possible role of ArgP in the expression *gdhA*, the structural gene for GDH. According to this model *argP gltBD* mutants are osmosensitive in high NH_4^+ media because they are compromised in both the pathways of biosynthesis and fail to accumulate Glu under these conditions.

1.3.8 LysR family of transcriptional regulators:

This family of proteins is composed of more than 50 similar sized transcriptional regulators (LTTRs) those regulate their own transcriptions (reviewed in Schell 1993). They are apparently evolved from a distant ancestor into sub-families found in diverse prokaryotic genera. This family of transcriptional regulators was first reported by Henikoff et al. (1988). It is one of the most common types of positive regulators in prokaryotes.

The most important characteristic feature of LysR family of proteins include mass of 30-36 KDa and function as transcriptional regulators with a helix turn helix DNA binding motif. Most of the family members share four other characteristics (a) they encode co-inducer responsive transcriptional activator proteins, (b) independent of the presence of co-inducer, they bind at regulated target DNA sequences that have similar position and structural motif, (c) each is diversely transcribed from a promoter that is

very close to and often overlap a promoter of the regulated target gene, (d) because an overlapping divergent promoter allows simultaneous bidirectional control of transcription (Beck and Warren 1988), most LTTRs repress their own transcription by 3- to 10- fold (negative autoregulation) possibly to maintain constant levels. The DNA binding domain, helix-turn-helx motif extends from residues 1 to 65 and the co-inducer recognition or response domain extends from residues 100 to 173 and 196 to 203 that form a ligand binding pocket specially the residues 149 to 154 in the protein structure.

1.4 Introduction to transcription regulation in *E. coli*:

1.4.1 Transcription:

Transcription is the process through which the sequence of one strand of DNA serves as template for enzymatic action by a DNA dependent RNA polymerase (RNAP) to produce a complementary strand of RNA (transcript). As in DNA replication, RNA synthesis, also proceeds in a 5'→3' direction. The strand of DNA that is used as template for RNA synthesis is called as the template (non-coding) strand, and the complementary DNA is called as non-template (coding) strand (whose DNA sequence is the same as that of the RNA transcript). There are three steps in the process of transcription. They are 1. Initiation 2. Elongation and 3. Termination. The process of initiation is described in further detail below.

1.4.1.1 Transcription initiation:

The principal events in transcription initiation involve the interaction of RNAP with a special *cis*-acting element in DNA called the promoter. The detail feature of promoters are described in a later Section (Section 1.3.2.2). Initiation of transcription in *E. coli* may be divided into four steps (reviewed in McClure 1985; Reznikoff et al. 1985; Young et al. 2002; Browning and Busby 2004; Record et al. 1996): 1, binding of RNAP

to the promoter to form a reversible closed complex; 2, isomerisation of the closed complex into an irreversible open complex – in this step, the DNA is unwound and becomes single-stranded (open) in the vicinity of the initiation site (defined as +1) to form a bubble of around 12 to 18 nucleotides to facilitate the initiation of RNA synthesis; 3, formation of the initial transcribing complex (i.e., initial synthesis of RNA where RNAP is engaged in abortive initiation. The RNAP transcribes the DNA without any change in its position, but produces abortive (short, non-productive) transcripts; and 4, promoter escape, i.e. the release of RNAP from the promoter to enter into the elongation mode of transcription. RNAP covers around 80-bp extending from −55 to +20 at a particular promoter. During initiation phase RNA does not change its footprint on promoter DNA.

In prokaryotes, in more than 90% of the cases, the first incorporated ribonucleotide is a purine (either A or G) and it is incorporated as a triphosphate. The subsequent additions of nucleotides involve phosphodiester bond formation by a reaction between 3'-OH group of the last nucleotide and 5'-triphosphate of the incoming nucleotide with the release of a pyrophosphate in each cycle of nucleotide addition.

1.4.2 Components of Transcription Initiation Machinery:

The transcription initiation machinery includes DNA, RNAP and required accessory factors and co-effectors.

1.4.2.1 RNA polymerase (RNAP):

The *E. coli* RNAP is a complex enzyme with multiple subunits and is responsible for most if not all transcriptions (Darst 2001). (DnaG also synthesises short transcript that serve as primers for DNA replication.) Highly purified RNAP holoenzyme consists of

following subunit polypeptides: α, β, β', σ and ω (reviewed in Burgess 1969a,b; Losick 1972; McClure 1985; Ishihama 2000; Darst 2001). These subunits have molecular weights of 40,000, 155,000, 160,000, 90,000, and 10,000 dalton (Da) respectively in the stoichiometry of α_2, β, β', σ, $\omega_{(0.5\text{-}2)}$. RNAP holoenzyme is reversibly dissociated into core enzyme (α_2, β, β', ω) and sigma (σ) subunit. The ω chain is not required for RNA synthesis since in some active preparations of core enzyme, this polypeptide was missing (Burgess 1969); furthermore, knockout mutants of ω are viable and cells deleted for the gene encoding ω (*rpoZ*) have no discernible mutant phenotype (Gentry and Burgess 1989; Gentry et al. 1991) whereas other RNAP subunits are essential for bacterial growth. The core subunit α_2, β, β' are apparently the only polypeptides needed for RNA synthesis (Heil and Zillig 1970), indicating that σ is not required for the basic catalytic activity of the enzyme. σ is responsible for specific promoter recognition by RNAP and it also decreases the affinity of RNAP binding for non-specific DNA.

At least *E. coli* has seven alternative sigma factors that are able to bind the core RNAP and catalyse transcription initiation at different promoters (reviewed in Ishihama 2000; Gruber and Gross 2003). This include 1. σ^{70} encoded by *rpoD* which is the principal housekeeping sigma factor; 2. σ^{54} encoded by *rpoN* involved in nitrogen regulated gene expression; 3. σ^{32} encoded by *rpoH* involved in heat shock response; 4. σ^{S} encoded by *rpoS* for gene expression in the stationary phase cells; 5. σ^{F} encoded by *rpoF* for flagellar operon; 6. σ^{FecI} encoded by *fecI* for regulation of the *fec* genes for iron dicitrate transport and 7. σ^{E} (*rpoE*) for envelope stress response. There are also anti-sigma factors that inhibit the function of sigma factors. In the phase of transcription elongation, the core RNAP will continue to catalyse RNA synthesis without σ factor, and upon transcription termination the released core complex can again bind a sigma

subunit before starting a new cycle of RNA synthesis (Travers and Burgess 1969; Burgess 1969a).

The principal sigma factor (σ^{70}) comprises of three flexibly linked domains σ^2, σ^3 and σ^4 which are anchored on the surface of the enzyme along the active cleft and make different contacts on the DNA: σ^2 with –10 region, σ^3 with base at –14/–15, and σ^4 with –35 region. σ-subunit was not crystallisable alone but studies were with co-crystallisation as holoenzyme (Campbell et al. 2002; Murakami et al. 2002a, 2002b; Hsu 2002b)

The current understanding of the transcription process has been greatly enhanced by the availability of crystal structure data of bacterial RNAP holoenzyme alone and complex with promoter DNA, as also of structural data on elongating RNAP. These studies have also revealed the substantial similarity of the basic transcription process in the prokaryotes and eukaryotes (Ebright 2000). Briefly, the structural data have revealed that the core enzyme (which lacks sigma) resembles a crab claw with the β and β' subunits each constituting the pincers (the holoenzyme also maintains the similar overall structure.). The two pincers surround an open channel (or cleft) with active site Mg^{2+} centrally located on the channel floor. This channel is 27Å in diameter and has many positively charged residues to accommodate negatively charged duplex DNA (Darst et al. 1998; Darst 2001; Zhang et al. 1999; Campbell et al. 2002; Murakami et al. 2002a; Vassylyev 2002). This active site is defined by the three invariant Asp residues of the β' subunit (Asp739, Asp741, and Asp743), located within a highly conserved sequence motif. Two Mg^{2+} ions are chelated to Asp residue along with water molecules in a configuration suitable for catalysis (Vassylyev 2002; Hsu 2002b; Campbell et al. 2002).

Studies on structures of RNAP with promoter DNA suggested that DNA lies across the holoenzyme face completely outside the RNAP active site channel. DNA enters and exits the polymerase as duplex but is melted within the enzyme with the two single strands of the transcription bubble taking different paths: the template strand goes near the active site whereas the non template strand is lodged elsewhere. The holoenzyme undergoes a large conformational change and produces a series of kinks in the upstream DNA, bending the DNA around the RNAP. Besides the overall trajectory DNA takes sharp 90º bends at the double/single strand junction. Presumably the large bending and untwisting of DNA with accompanying conformational change in the holoenzyme activates the polymerase-DNA complex (Korzheva et al. 2000; Murakami et al. 2002a and b; Vassylyev 2002; Hsu 2002b).

1.4.2.2 *E. coli* promoter:

The promoter is a sequence of DNA upstream of the structural gene that is recognised by RNAP holoenzyme to initiate transcription. Besides the transcription start site, an *E. coli* promoter consists of five important sequence elements: two hexamers centered at or near –10 and –35 upstream of the transcription start site, the spacer DNA separating them, a region between –40 to –60 (UP element) and extended –10 elements (reviewed in Rosenberg and Court 1979; McClure 1985; Reznikoff et al. 1985; deHaseth et al. 1998; Browning and Busby 2004).

1. The sequence located at 10-bp upstream from the transcription start site is called the –10 element (hexamer) or the Pribnow box, and usually consists of the six-nucleotide consensus TATAAT (all sequences are for the non-template DNA strand). The Pribnow box is absolutely essential to start transcription in prokaryotes. Promoter –10 elements are recognised by domain 2 of RNAP sigma subunit (specifically the region 2.4)

(deHaseth et al. 1998; Campbell et al. 2002; Murakami et al. 2002b; Browning and Busby 2004).

2. The other sequence at –35 (the –35 element), located 35 bp upstream of transcription start site, consists of the consensus six-nucleotide sequence, TTGACA. The promoter –35 region is recognised by domain 4 of RNAP sigma factor (specifically region 4.2) (deHaseth et al. 1998; Campbell et al. 2002; Murakami et al. 2002b; Browning and Busby 2004).

3. The distance between the two conserved elements (–10 and –35) is also important for the interaction with RNAP and in most of the cases it is 17±1 bp as given below (Mulligan et al. 1985; Ayers et al. 1989; deHaseath et al. 1998; Browning and Busby 2004):

TTGACA ---- 17±1 ---- TATAAT

4. The extended –10 element is a 3- to 4-bp motif located immediately upstream of the –10 hexamer that is recognised by the domain 3 of RNAP sigma factor, the consensus sequence being (along with the –10 element itself) TGNTATAAT. This is present in a subclass of *E. coli* promoters which are able to function quite well without a recognisable –35 region or without any activating proteins (Minchin and Busby 1993; Voskuil et al. 1995; Dombroski 1997; Brown et al. 1997; Murakami et al. 2002b; Sanderson et al. 2003).

5. UP element is a ~20-bp sequence located upstream to –35 hexamer that is recognised by C-terminal domain of RNAP α-subunit. This DNA sequence is AT-rich and located between –40 and –60 and increases the promoter activity by providing a point of contact for RNAP α-subunit C-terminal domain (Ross et al. 1993, 2001; Blatter et al.

1994; Rao et al. 1994; Landini and Volkert 1995; Gaal et al. 1996; Browning and Busby 2004).

Over the years, a number of authors have compiled lists of *E. coli* promoters and analysed their sequences. It was from the compilations that the consensus sequence elements were determined. However, as more and more promoter sequences were identified, it has also become clear that not all consensus residues are well conserved. Harley and Reynolds (1987) described an analysis of 263 phage, plasmid and bacterial promoters, and the degree of conservation (%) of each residue in their compilation of the consensus hexamers was (where the subscript indicates the % conservation):

$$\mathbf{T_{78}T_{82}G_{68}A_{58}C_{52}A_{54}} \text{ ---------- } \mathbf{16_{21}17_{52}18_{19}} \text{ ---------- } \mathbf{T_{82}A_{89}T_{52}A_{59}A_{49}T_{89}}$$

–35 **spacer** **–10**

Later, Lisser and Margalit (1993) looked exclusively at *E. coli* promoters. Their results showed some changes in the degree of conservation of some bases, but overall the same pattern was obtained:

$$\mathbf{T_{69}T_{79}G_{61}A_{56}C_{54}A_{54}} \text{ ---------- } \mathbf{16_{17}17_{43}18_{17}} \text{ ---------- } \mathbf{T_{77}A_{76}T_{60}A_{61}A_{56}T_{82}}$$

-35 **Spacer** **-10**

With few exceptions, the general rule holds that the greater the similarity of the –10 and –35 regions to the consensus sequence, the better the promoter functions in vitro as well as in vivo (Hawley and McClure 1983; Kammerer et al. 1986; Harley and Reynolds 1987; Ellinger et al. 1994).

1.4.2.3 Transcription factors, small ligands and co-effectors:

The *E. coli* genome contains more than 300 genes that encode proteins which are predicted to bind the DNA and to either up-regulate or down-regulate transcription (Pérez-Rueda and Collado-Vides 2000; Babu and Teichmann 2003). Most of these proteins (predicted or established transcription factors) are sequence-specific DNA-

binding proteins. Some of these proteins control large numbers of genes, whereas others control just one or two genes. So far, a dozen families of *E. coli* transcription factors have been identified (Pérez-Rueda and Collado-Vides 2000), the best characterised of these being the LacI, AraC, LysR, CRP and OmpR families. Some transcription factors function solely as activators or repressors, whereas others can function as either according to the target promoter (Pérez-Rueda and Collado-Vides 2000).

The activity of the transcription factors can in turn be modulated in a variety of ways. First, the DNA-binding affinity of transcription factors can be modulated by co-effector molecules. The best example of this is the alteration in the DNA-binding affinity of the Lac repressor by the small molecule, allolactose, which signals the presence of lactose in the growth medium (Müller-Hill 1996). Second, the effect on transcription initiation can also be altered by co-effectors. The best example is TyrR protein which binds the *tyrP* operator in absence or presence of co-effector Phe but activates it only in presence of Phe (Yang et al. 2004). Besides, the activity of some transcription factors is modulated by covalent modification as typified by the two component regulator systems. In each of these systems, there is a sensor kinase that is regulated by extracellular signals, which then phosphorylates its cognate response regulator, and it is only the phosphorylated protein that can bind target DNA and activate transcription. For example, the response regulator such as NarL bind to their target DNA only when phosphorylated by their cognate kinase (Stock et al. 2000). Fourth, the concentration of some transcription factors in the cell controls their activity. For example, one cellular response to oxidative stress is controlled by the concentration of the SoxS protein. The transcription of the gene encoding SoxS is controlled by SoxR, which in turn is regulated directly by interactions with oxidizing ligands (Demple 1996). Fifth, activity

can be controlled by protein fold switch (which also includes oligomerisation) exemplified by OxyR, where there is a disulphide bond formation leading to a functional oligomer upon oxidative stress to become transcription activator (Choi et al. 2001). Finally, a less common mechanism for regulating the effective concentration of a transcription factor is sequestration by a regulatory protein to which it binds; for example the PtsG in its dephosphorylated form sequesters Mlc protein resulting in the de-repression of the Mlc repressed genes (Plumbridge 2002).

1.4.3 Gene regulation in *E. coli:*

Gene regulation refers to the cellular control of the amount of the functional product of a gene. Any step of the expression of a gene may be modulated, from transcription to the post-translational modification of a protein (Grieve and von Hippel 2005). Gene regulation is executed mostly at the level of transcription. Numerous factors regulate transcription by influencing the ability of RNAP to access, bind and transcribed specific genes in response to various signals. The transcription cycle (which includes the three main phases viz. initiation, elongation and termination) is subject to regulation by a host of transcription factors that act on RNA, DNA or RNAP during all stages of the cycle. These factors include proteins, small peptides, non-coding RNAs (for example, *E. coli* 6S RNA), polyphosphates, amino acids, vitamins and other molecules (Storz 2002; Grundy and Henkin 2004).

The control of the rate of transcription initiation is the predominant mode for control of gene expression in bacteria. Among the protein factors known to affect transcription, a vast majority of them are DNA-binding proteins that act during transcription initiation by either increasing or decreasing the occupancy of promoter by RNAP holoenzyme (Ishihama 2000). The activity of RNAP at a given promoter is regulated by interaction

with accessory proteins either positively (activators) or negatively (repressors). Many activators recruit RNAP to specific promoter sites by direct protein-protein interactions, whereas repressors occlude promoters from RNAP or render the RNAP-promoter complex functionally inactive. Some anti-σ-factors can regulate transcription initiation by sequestering σ from association with core, causing selective inhibition/activation of different σ-specific promoters (see Dove et al. 2003).

On the other hand, the DNA sequence in the initial transcribed region (ITS) may also contribute to the regulation (delay) of promoter escape (the last step of initiation) by mediating the binding of σ^{70} to the –10 like elements. It was first reported from the lambdoid phage where the $\sigma^{70}$of RNAP holoenzyme induce a paused transcription complex after RNA is synthesised around +16 to +17 at critical bases of −10 like sequence motif at the initial transcribed region where the antiterminator Q protein modifies RNAP to relieve from this extended pause in late gene promoter transcript (Roberts et al. 1998; Ko et al. 1998; Marr et al. 2001). Similar pausing was also reported for *lacUV5* promoter and additional promoters in *E. coli* (Nickels et al. 2004; Brodolin et al. 2004). Besides, Shimamoto and co-workers have described the existence of a moribund complex of RNAP with promoter DNA in which RNAP is unable transit from abortive to productive transcription (Kubori and Shimamoto 1996; Sen et al. 2001; Susa et al. 2006). It was reported for the transcription from promoters *lacUV5* and further demonstrated to occur at least in the following promoters *atpC*, *cspA* and *rpsA* (Susa et al. 2006).

Like transcription initiation, the elongation and termination stages of the transcription cycle also serve as important targets for regulatory factors in prokaryotes. A number of proteins are reported to act during elongation and termination stages of transcription by

direct modification of RNAP properties. In *E. coli*, they include: Nus factors [NusA, NusB, NusG and NusE (S10)], RfaH, ribosomal protein S4, Gre-factors (GreA, GreB), Mfd, RapA (HepA), ρ (Rho) and phage encoded proteins such as N and Q of lamda phage (Bailey et al. 2000; Squires and Zaporojets 2000; Sukhodolets et al. 2001; Fish and Kane 2002; Nudler and Gottesman 2002; Roberts and Park 2004). These factors affect RNAP processivity by modulating transcription pausing (temporary interruption of transcription), arrest (permanent stalling of elongation complex without dissociation), termination (release of RNA transcript and complex dissociation) or anti-termination (prevention of termination).

A different way of gene regulation, the control by modulating RNA structure also known as transcription attenuation, was discovered in the *trp* operon of *E. coli* (Yanofsky 1981), and it regulates the expression of some genes by controlling the ability of RNAP to continue elongation past specific sites. In this system, regulation is accomplished by alternative folding of an mRNA in the leader region upstream of the coding sequence, resulting in either premature termination of transcription or read-through.

Besides, several cases of gene regulation in bacteria have indicated the existence of another regulatory mechanism. In 2002, two studies reported the first experimental evidence for gene regulation through direct binding of small metabolites to structured 5′-leader regions of mRNA termed ‘riboswitches’ (Mironov et al. 2002; Winkler et al. 2002). One of these riboswitches supported a transcription attenuation mechanism in which alternative RNA folding is induced by binding of flavin mononucleotide (FMN) to the leader region of nascent riboflavin mRNA in *B. subtilis* (Mironov et al. 2002). In contrast to the traditional attenuation where translation of a leader region leads to

premature termination of transcription, in the riboswitch mechanism it is the binding of a small metabolite that induces an alternative RNA fold that leads to premature transcription termination (Brantle 2004, 2006).

1.4.4 Control of regulation at transcription initiation:

As mentioned above, the regulation of bacterial gene expression is commonly mediated at the step of transcription initiation. Any of the steps of transcription initiation (viz. closed complex formation, isomerisation to open complex, formation of initial transcribing complex and promoter escape) are subject to regulation at different promoters by accessory protein factors in *E. coli*. The activity of RNAP at the promoter is either assisted or hindered by regulatory protein factors. Repressors commonly act by steric hindrance of RNAP binding to promoters while activators do so by "recruiting" RNAP to promoters by direct protein-protein interactions and/or via co-effector molecules. Several regulators can act as both activator and repressor at different promoters (stimulates at one promoter and inhibits at the other) and sometimes at the same promoter under different conditions. The regulation at the step of transcription initiation can be studied under two heads: activation and repression.

1.4.4.1 Activation (positive regulation) of transcription initiation:

The majority of the instances of activation rendered by accessory protein factors at the initiation of transcription involve the recruitment of RNAP at the promoter. Besides, reports are available for the activation at the post binding steps i.e. at the promoter opening and clearance (reviewed in McClure 1985; Reznikoff et al. 1985; Adhya and Garges 1990; Adhya et al. 1993; Ptashne and Gann 1997; Browning and Busby 2004). Three general mechanisms have been described for simple activation by recruitment of

RNAP at the bacterial promoter: the class I activation in which the activator binds to a target located upstream of the promoter −35 element and recruits RNAP to the promoter by directly interacting with the C-terminal domain of the alpha subunit (α-CTD) of RNAP. The best example of Class I activation is the action of the cyclic AMP receptor protein, CRP, at the *lac* promoter (Ebright 1993). In Class II activation, the activator binds to a region that overlaps the promoter –35 element of the promoter and recruits RNAP by contacting region 4 of σ-subunit (Dove et al. 2003), example includes the activation at the bacteriophage λ P_{RM} promoter by λcI protein through interaction with σ-subunit of RNAP thereby stabilizing the initial binding of RNAP from established from various genetic and structural studies (Dove et al. 2000; Nickels et al. 2002). [However, earlier reports (as also described in the next para) from the kinetic analysis had established that λcI does not stabilise the initial binding of RNAP to promoter P_{RM}, but rather stimulates the isomerisation from closed to open complex (Hawley and McClure 1982; Li et al. 1997).] At some promoters that are subject to Class II activation, the activator contacts other parts of the RNAP (for example, α-N-terminal domain) but still binds to a target sequence that overlaps with the promoter −35 element (Busby and Ebright 1997). In the third mechanism, the activator protein changes the conformation of the target promoter so that the interaction of RNAP with the promoter –10 and/or –35 elements will be favoured. For this, the activator has to bind very near to the promoter elements, or at a distance causing conformational changes in the promoter DNA (Sheridan et al. 1998, 2001). An example is the genes controlled by Mer-family of activators where the spacing between the –10 and –35 region is not optimal and the binding of the protein twists the DNA to re-orient the –10 and –35 elements so that they can be bound by the RNAP σ-subunit (Ansari et al. 1992, 1995; Brown et al. 2003, Heldwein and Brennan 2001).

Activators could also act to recruit RNAP by influencing the post-binding step, such as isomerization or promoter clearance (McClure 1985; Reznikoff et al. 1985; Choy and Adhya 1993; Tagami and Aiba 1995, 1998; Hsu 2002a). In other words, enhancement of open complex formation is not mutually exclusive as a mechanism of activation from that of RNAP recruitment discussed above, since the irreversible conversion of closed to open complex will be expected to drive the preceding (reversible) step of RNAP binding to the promoter towards the direction of further formation of the closed complex. One example of activation by enhancing open complex formation is that of CRP in *malT*, *lacP1* and *galP1* promoters (Herbert et al. 1986; Straney et al. 1989; Lavigne et al. 1992; Tagami and Aiba 1995, 1998). Another example is λP_{RM} promoter, where it was reported that λ-CI activates transcription from λP_{RM} promoter by facilitating the rate-limiting conformational change (isomerization) in open complex formation, a step that occurs after polymerase binds (Hawley and McClure 1982; Shih and Gussin 1983; Li et al. 1997). (A recent finding, as also mentioned in previous para, postulated that this protein stabilises the initial RNAP binding at λP_{RM}, however, did not account for this kinetic observation.)

Various other instances exist for the similar activation at the isomerisation step from closed to open complex: Class II FNR-dependent promoters (Wing et al. 2000) where Ar1 region of FNR protein interacts with RNAP and promotes the isomerisation step; the activation by NarL/NarP and FNR from *nrf* promoter (Browning et al. 2005, 2006); activation of *tyrP* by TyrR protein in presence of phenylalanine (Yang et al. 2004); the requirement of UphA and CRP protein for the formation of open complex at the *uphT* promoter (Olekhnovich et al. 1999); and the dependence on MelR for the stable open complex formation of *melAB* promoter (Belyaeva et al. 2000) are some of the examples in this regard.

Evidence exists for some activators acting at the step of promoter clearance of transcription initiation (reviewed in Hsu 2002a), and in all this cases an increase in productive transcription and concomitant decrease in abortive transcription has been observed. A simple example is that of global regulator CRP at the *malT* promoter for which it was first reported that abortive products were obtained in absence of CRP but not after incubation with CRP (Menendez et al. 1987), and subsequently, (using UV-laser footprinting as a tool for studying transcription complexes) that CRP helps in the promoter escape from *malT* promoter (Eichenberger et al. 1997). [Although, a study from different group, claimed that CRP is dispensable after open complex formation (Tagami and Aiba 1995, 1998), this finding was not explained by their study.] In another example, the dual regulator Arc protein has been shown to accelerate the promoter clearance at the P_{ant} promoter of bacteriophage P22 (Smith and Sauer 1996). DnaA protein, apart from its functions as initiator of DNA replication, was also established to activate transcription from bacteriophage P_R promoter by stimulating promoter escape (Glinkowska et al. 2003). In yet another example, the transactivator protein-C was shown to enhance the promoter clearance at the promoter (P_{mom}) of *mom* gene in bacteriophage Mu (Chakraborty and Nagaraja 2006). Besides, *galP2* promoter (galactose utilization) and *pyrB1* promoter (pyrimidine biosynthesis) were shown to be subject to promoter clearance control in response to changes in UTP concentration (McClure 1985; Jin 1994).

1.4.4.2 Repression (negative regulation) of transcription initiation:

The negative regulation by repressors can also be effected by impeding or hindering any step of transcription initiation (reviewed in McClure 1985; Reznikoff et al. 1985;

Lloyd et al 2001; Browning and Busby 2004). Various mechanisms of inhibition at the step of transcription initiation can be discussed as follows:

A. By impeding the recruitment of RNAP to form closed complex of RNAP with promoter DNA: Repressors can act by preventing the RNAP binding to promoter through steric hindrance, and this is one of the most common ways of inhibition of transcription initiation (Beckwith 1987; Choy and Adhya 1996; Liu et al. 2004). Steric hindrance of RNAP binding to promoter DNA is probably the simplest mechanism of repression. In these instances, the repressor binding site is located in, or close to, the core promoter elements - for example, the Lac repressor binding site at the *lac* promoter (Müller-Hill 1996). Some examples of inhibition at the step of recruitment of RNAP are those involving cAMP-CRP regulated promoters: the *cya* gene which is inhibited by cAMP-CRP binding to and occluding RNAP from *cya* promoter (Aiba 1985; Mori and Aiba 1985); the inhibition of *lac P2* and *P3* promoters and of *galP2* promoter by CRP (Musso et al. 1977; Eschenlauer and Reznikoff 1991; Xiong et al. 1991). It is also possible that repressor binding can prevent the interaction of an activator with RNAP and/or DNA (Bolker and Kahmann 1989).

B. By hindering the isomerisation of the closed complex to a stable open complex: Some repressors act by interfering with post-recruitment steps in transcription initiation (Choy and Adhya 1996; Müller-Hill 1998, Browning and Busby 2004). The Arc repressor involved in bacteriophage P22 lysogeny is believed to repress the P_{ant} promoter by binding at or near the –10 region and distorting the DNA to prevent the formation of stable open complex (Vershon et al. 1987). Salient other examples include the blocking of *lacUV5* promoter by LacI repressor protein (Straney and Crothers 1987); the inhibition at the *aroP P*2 and *tyrP* promoters by the TyrR protein in presence of Tyr (Yang et al. 1999, 2004); the *bgl* operon whose transcription is inhibited by the

Fis protein (Caramel and Schnetz 2000); and FNR-mediated repression from *ndh* and *yfiD* promoters where the configurations of two FNR dimers at these promoters effectively jam RNAP in one of the intermediate states between closed and open complex (Green and Guest 1994; Green and Marshall 1999).

C. At the step of initial transcribing complex formation: This is the step at which phospho-diester bond synthesis starts and is typically associated with the engagement of RNAP in abortive cycles of synthesis and release of short RNA products. Most of the repression at this step is mediated at the synthesis of first phosphodiester bond and hence of both abortive and productive transcripts. An example of repression at this step is that represented by the inhibition at *gal* promoter by the interaction of two GalR molecule bound to different operator regions which generates DNA looping thereby effecting repression at a step prior to formation of the first phosphodiester bond resulting in inhibition of the synthesis of complete as well as aborted transcripts (Choy and Adhya 1992).

D. At the step of promoter clearance: The mechanism of repression is characterised by RNAP trapping within the cycle of abortive transcript synthesis which is often correlated with increased affinity between promoter and RNAP (reviewed in Hsu 2002a). The repressor could make contacts with RNAP and thus block promoter clearance. Lee and Goldfarb (1991) have suggested that the mechanism of *lac* repressor action at the *lacUV5* promoter is by the repressor acting to modify the initiation complex so as to result in continued abortive synthesis of RNA oligomers. Other examples are the regulatory protein P4 from *Bacillus subtilis* phage φ-29 which represses the strong viral A2c promoter (*P*A2c) by preventing promoter clearance (Monsalve et al. 1996a, 1996b), and also, the CRP protein that blocks transcription

from extended −10 *lacP1* promoter by hindering promoter clearance (Liu et al. 2004). In another example, H-NS protein mediates RNAP trapping following open complex formation at promoters such as *rrnB* P1 (Schroder and Wagner 2000; Dame et al. 2002) or *hdeAB* (Shin et al. 2005),

Complex cases have been reported where the repressor functions as an anti-activator. The best examples are at CytR-repressed promoters, which are dependent on activation by CRP. Repression by CytR is through direct interactions between CytR (the repressor) and CRP (the activator) that prevent CRP-dependent activation (Shin et al. 2001).

1.4.4.3 Regulators acting as both activators and repressors:

Several regulators can act as both activator and repressor at different promoters (stimulates at one promoter and inhibits at the other) and sometimes at same promoter under different conditions (Roy et al. 1998). A good example is that of Gal repressor on *gal* operon where, the binding of this repressor in the absence of DNA looping, represses the P1 promoter located on the same face and activates the P2 promoter situated on the opposite face of the DNA helix (Choy et al. 1995). Another example is phage psi-29 protein P4, which activates the late A3 promoter and represses the early A2c promoter both by interacting with the C-terminal domain of the RNAP α-subunit (Monsalve et al. 1997). Yet another example is Arc protein, which acts both as repressor and activator at the variants of P_{ant} promoter (Smith and Sauer 1996). Besides, reports are available where a regulator acts as both activator and repressor at the same promoter, in the presence of different co-effectors. The example is TyrR, which activates *aroP* and *tyrP* genes in presence of Phe and represses them in presence of Tyr (Yang et al. 1993, 1999, 2002).

1.5 Objectives of the present study:

It was reported earlier from this laboratory (Nandineni 2003; Nandineni and Gowrishankar 2004) that *E. coli* strains with either *argP* or *argO* mutations are supersensitive to CAN. In these studies, ArgO (previously YggA) was also characterised genetically to be an Arg exporter protein, and in vivo *argO::lac* expression study established that *argO* is under the strict transcriptional control of a LysR type transcription factor ArgP, with intracellular Arg inducing *argO* expression through ArgP and intracellular Lys mediating a reduction in *argO* expression by abolishing the activating role of ArgP. Besides, seven gain-of-function *argP* mutants which are CAN resistant and exhibit constitutive *argO* expression were also reported. The sensitivity to CAN of the *argP* and *argO* mutants was suggested to be because of the failure to export CAN from the cytoplasm in the mutants.

In this study it was proposed to examine the detailed mechanism of *argO* regulation by ArgP in vitro. The specific objectives of the present work can be summarised as follows:

1. To study the mechanism of *argO* regulation by ArgP and the opposite effects by different environmental cues (co-effectors).
2. To investigate the detailed biochemistry of ArgP and co-effector interaction.
3. To characterise the CAN-resistant gain-of-function *argP* mutants and study the mechanism of their action on *argO* expression.
4. To study other target genes of ArgP (including the gene via which ArgP is involved in osmoregulation) and to compare them with that of *argO* regulation by ArgP.

CHAPTER 2

Materials and methods

2.1 Materials:

2.1.1 Strains and bacteriophages:

The *Escherichia coli* strains used in this study with their genotypes are shown in Table 2.1. All strains other than BL21 (DE3) and BL21-SI employed in protein over expression experiments are derivatives of *E. coli* K12. Bacterial strains were routinely stored on solid agar plates at 4ºC and also as thick suspensions in 20% glycerol either at –20ºC or at –70ºC. Plasmid harbouring strains were freshly prepared by transformation of the required plasmid. The bacteriophage P1*kc* was used for routine transduction to move a locus from one strain to another.

Table 2.1: *E. coli* Strains used in this study

Strain	**Genotype**[a]
BL21(DE3)[b]	F^- *ompTgal* [*dcm*] [*lon*] $hsdS_B$ ($r^- m^-$) *gal* λ(DE3[*lacI lacUV5*-T7 RNAP *ind1 Sam7 nin5*])
BL21-SI[b]	F^- *omp*T *Ion* $hsdS_B$ ($r_B^- m_B^-$) *gal dcm endA1 proUp*::T7 RNAP:: *malQ-lacZ*
DH5α	Δ*(argF-lac)U169 supE44 hsdR17 recA1 endA1gyrA96 thi-1 relA1 (ϕ 80lacZΔM15)*
MC4100	Δ*(argF-lac)U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*
MG1655	Wild-type
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^r)]
GJ216	MC4100 Δ*putPA101 proP222* Δ*(pyr-76::*Tn10)*461recA56 srl-300::*Tn*10*
GJ980	MC4100 *gdhA* λp*lac*Mu
GJ4536	MC4100 *argP202::*Kan

GJ4892	MC4100 *argP202::*Kan *lacZ::*Tn*10*dTet
GJ5319	MC4100 *gdhA* λp*lac*Mu
GJ5320	GJ4892 *gdhA* λp*lac*Mu
GJ5331	GJ4892 *recA56 srl-300::*Tn*10*

a – Genotype designations are as in Berlyn (1998).

b - *E. coli* B strains

2.1.2 Plasmids:

The plasmid vectors used in this study were as follows:

1. pBR329: It is a pMB9-based, medium-copy-number plasmid which carries ampicillin (Amp), tetracycline (Tet) and chloramphenicol (Cm) resistance markers (Covarrubias and Bolivar 1982).

2. pBluescriptII SK (pBSK): It is a high-copy-number, ColE1 based cloning vector with Amp resistance marker carrying a multiple cloning site (MCS) in the *lacZα* region (Stratagene).

3. pCL1920: It is a pSC101-based, low-copy-number vector with spectinomycin (Spc) and streptomycin (Strp) resistance marker carrying the MCS in *lacZα* region and hence provides the advantage of screening the insertions using α-complementation (Lerner and Inouye 1990).

4. pMU575: It is a IncW-based, single-copy, trimethoprim (Tp) resistance bearing promoter probe vector. It carries its MCS upstream of a promoterless *galK'-lacZ* fusion. This fusion has the first 58 codons of *galK* fused to the 8th codon of *lacZ*, and the resultant hybrid polypeptide possesses functional β-galactosidase activity (after assembly as a tetramer). Translation of the hybrid gene is controlled by the ribosome binding site of *galK*. There are stop codons in all the three reading frames between

MCS and initiation codon of *galK* so that there is no interference caused by translational read-through from inserts cloned into MCS region. A strong *pheR* terminator located upstream of the MCS prevents read through from any vector-based promoter into the *lacZ* gene (Andrews et al. 1991).

5. pET21b: It is a ColE1-based, high-copy-number expression vector bearing Amp resistance marker. A strong T7 RNAP-recognised promoter and an efficient ribosome-binding site lie upstream of the MCS to ensure the high level expression of any gene cloned in MCS. A stretch of hexa-histidine (His_6)-encoding codons followed by stop codon is incorporated downstream of MCS to give a C-terminally His_6-tagged recombinant protein (EMD Biosciences).

6. pUC19: It is a small high-copy-number cloning vector containing pMB9 origin of replication from pBR322 but lacks the *rop* gene and carries a point mutation in the RNAII transcript. These changes result in the temperature dependent copy-number of about 75 per cell at 37ºC and 200 per cell at 42ºC. The MCS is in framed with *lacZα* gene allowing screening for insertions using α-complementation (Yanisch-Perron et al. 1985).

7. pCU22: It is a derivative of pUC19 used to prepare supercoiled DNA for in vitro transcription where two strong phage *fd* transcription terminators flank MCS. This will ensure that the transcripts originated from vector based promoters will not interfere with the transcription from the cloned promoter and that the transcript originated from the cloned promoter will be terminated after the MCS (Ueguchi and Mizuno 1993).

8. pCR2.1: It is a TA-cloning vector (linearised) which has single 3′-T-residue overhangs which allows PCR products having 5′-A-residue overhangs ligate efficiently with the vector. The MCS where the 3′-T-residues are present is in the *lacZ* region allowing for α-complementation. Besides having a phage f1 origin, it is pUC origin

based and high-copy-number vector with Kan and Amp resistance markers (Invitrogen).

Plasmid DNA preparations were routinely made from *recA* strain, DH5α and were stored in 10 mM Tris-Cl (pH-8.0) with 1 mM EDTA at –20ºC. The plasmid constructs used in this study are given in Table 2.2:

Table 2.2: Plasmid constructs

Plasmids →	**Specifications**
pHYD915 →	1.8 kb *SalI* fragment containing *argP* from λ-471 cloned in pCL1920
pHYD926 →	*argP* (S94L) mis-sense mutation derived from pHYD915
pHYD927 →	*argP* (P108S) mis-sense mutation derived from pHYD915
pHYD928 →	*argP* (V144M) mis-sense mutation derived from pHYD915
pHYD929 →	*argP* (P217L) mis-sense mutation derived from pHYD915
pHYD930 →	*argP* (L294F) mis-sense mutation derived from pHYD915
pHYD931 →	*argP* (R295C) mis-sense mutation derived from pHYD915
pHYD932 →	*argP* (A68V) mis-sense mutation derived from pHYD915
pHYD1703 →	pBSK construct of *argP* cloned (blunt end) in the *EcoRV* site
pHYD1705 →	pET21b construct of *argP*, for the overexpression of ArgP
PHYD1715 →	pET21b construct of *argP* (S94L) missense mutant for overexpression
pHYD1722 →	pCU22 construct of *argO* regulatory region (–293 to +109)
pHYD1723 →	pMU575 construct of *argO* regulatory region (–293 to +109)
pHYD1724 →	Derivative of pHYD1722 where two nucleotides of a putative –10 sequence of *argO* promoter is modified (TATAGT→CCTAGT)
pHYD1725 →	Derivative of pHYD1722 where two nucleotides of a putative –10 sequence of *argO* promoter is modified (TAGTCT→TCGTCC)
pHYD1726 →	Derivative of pHYD1722 where two nucleotides of a putative –10 sequence of *argO* promoter is modified (TAGTCT→CCGTCT)

pHYD1727 → Derivative of pHYD1722 where two nucleotides of a putative –10 sequence of *argO* promoter is modified (TAGTCT→TCGTCC)

pHYD1728 → Derivative of pHYD1722 where a 26-bp region on the *argO* regulatory region (from –102 to –77) is deleted

pHYD1729 → Derivative of pHYD1722 where a 26-bp region on the *argO* regulatory region (–115 to –90) is deleted

pHYD1730 → Derivative of pHYD1722 where a 26-bp region on the *argO* regulatory region (–115 to –90) is deleted.

pHYD1731 → *argO* regulatory region from pHYD1726 sub-cloned into pMU575

pHYD1732 → *argO* regulatory region from pHYD1727 sub-cloned into pMU575

pHYD1733 → *argO* regulatory region from pHYD1724 sub-cloned into pMU575

pHYD1734 → *argO* regulatory region from pHYD1725 sub-cloned into pMU575

pHYD1735 → Derivative of pHYD1722 where one of the –10 like hexamer motif in initial transcribed sequence of *argO* is modified (GATACT→GCTACC)

pHYD1736 → Derivative of pHYD1722 where one of the –10 like hexamer motif in initial transcribed sequence of *argO* is modified (GAATAT→GCATAC)

pHYD1737 → *argO* regulatory region from pHYD1735 sub-cloned into pMU575

pHYD1738 → *argO* regulatory region from pHYD1736 sub-cloned into pMU575

pHYD1739 → *argO* regulatory region from pHYD1728 sub-cloned into pMU575

pHYD1740 → *argO* regulatory region from pHYD1729 sub-cloned into pMU575

pHYD1741 → *argO* regulatory region from pHYD1730 sub-cloned into pMU575

pHYD1742 → *dnaA* regulatory region cloned in plasmid pCR2.1

pHYD1744 → *nrdA* regulatory region cloned in plasmid pCR2.1

pHYD1746 → *dnaA* regulatory region from pHYD1742 sub-cloned into pMU575

pHYD1747 → *nrdA* regulatory region from pHYD1744 sub-cloned into pMU575

Plasmids pHYD915 to pHYD926 have been described earlier (Nandineni and Gowrishankar 2004). Plasmids pHYD1703 to pHYD1747 were constructed for this study.

2.1.3 Primers:

Table 2.3: Oligonucleotide primers:

Oligonucleotides ID		Sequence (5′→3′)
JGARGP1r	→	AGCAGACAACA*CATATG*AAACGCCCGGA
JGARGP3r	→	ATTATTTGAT*CTCGAG*ATCCTGACGAAG
JGARGO4r	→	CTGTAATGTTCTGTAGTCCGGGCGTTTC
JGARGO8f	→	GTGCGCCTGCAGGAACTTGGTG
JGARGO9r	→	GTATGCCCGGATCCATCACAAAA
JGYPROf (1)	→	AATCCCGCGATA*CC*TCTTCTGCATCAGATA
JGYPROr (1)	→	CTGATGCAGAGAC*GG*TATCGCGGGATT
JGYPROf (2)	→	AATCCCGCGATAT*C*GTC*C*CTGCATCAGATAC
JGYPROr (2)	→	GTATCTGATGCAG*G*GAC*G*ATATCGCGGGATT
JGYPROf (3)	→	ACAATCCCGCGA*CC*TAGTCTCTGCATC
JGYPROr (3)	→	GATGCAGAGACTA*GG*TCGCGGGATTGT
JGYPROf (4)	→	ACAATCCCGCGAT*C*TAG*C*CTCTGCATCAGATA
JGYPROr (4)	→	TATCTGATGCAGAG*G*CTA*G*ATCGCGGGATTGT
JGAU2Odelf	→	CACCG*GAATTC*GAGGCCAGATAATACTCT
JGAU2Odelr	→	TCCG*GAATTC*TTTCACCCGCTTAAAGTT
JGAU1Odelf	→	CACCG*GAATTC*TCTCTTATTAGTTTTTCTG
JGAU1Odelr	→	TCCG*GAATTC*AGCGTTGATTACGCAGCT
JGAOdelf	→	CACCG*GAATTC*ATTAATATTATCAATTTCC
JGAOdelr	→	TCCG*GAATTC*GAGGCCAGAGAGTATTAT
JGARGOf-bio	→	GTGCGCCTGCAGGAACTTGGTG
JGARGOr-bio	→	GTATGCCCGGATCCATCACAAAA

JGA10L 1f	→	AGTCTCTGCATCAGCTACCTAATTCGGAATATCC
JGA10L 1r	→	GGATATTCCGAATTAGGTAGCTGATGCAGAGACT
JGA10L 2f	→	ATACTTAATTCGGCATACCCAACGTGTTTTC
JGA10L 2r	→	GAAAACACGTTGGGTATGCCGAATTAAGTAT
JGAY-OF	→	CTGAGGCCAGATAATACTCTCTGGCCTCTC
JGAY-OR	→	GAGAGGCCAGAGAGTATTATCTGGCCTCAG
JGAY1stHF	→	GTAATCAACGCTGAGGCCAGATAAT
JGAY1stHR	→	ATTATCTGGCCTCAGCGTTGATTACG
JGAY2ndHF	→	ACTCTCTGGCCTCTCTCTTATAAGTT
JGAY2ndHR	→	AACTAATAAGAGAGAGGCCAGAGAGT
JGANRDf	→	AAGTCATGAATAATTTTCTTATAA
JGANRDr	→	GTATGTTTGGGATTCACTGCA
JGADNAf	→	GTAAAGCGAAGGATCGTCCT
JGADNAr	→	TCTGTGGCTGGTAACTCATCCT
JGAGDHf	→	ATTTTGATCCTGCAGAACGCAGCACTG
JGAGDHr	→	GTTTGATTCGGATCCCGCTTTTGGACATG
JGAORICf	→	GGATCCGGATAAAACATGGTGA
JGAORICr	→	ATCCTTGTTATCCACAGGGCA
JGJYQFE-Of	→	GGCGCACCAGTCGACCGTTCGCAA
JGJYQFE-Or	→	TAATGCGCTGGTCGACGGCTGATT

2.1.4 Media:

All media and buffers were sterilised by autoclaving at 121ºC for 15 minutes. Media and buffers used in this study are given below:

Glucose Minimal A medium

K_2HPO_4	0.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate, $2H_20$	0.5 g
H_2O to	1000 ml

After autoclaving the following solutions were added

$MgSO_4$ (1M)	1 ml
Glucose (20%)	10 ml
Vitamin B1 (1%)	0.1 ml

Amino acids when required, were added to a final concentration of 40 µg/ml. When growth on other carbon sources was to be tested, glucose was substituted with appropriate sugar at 0.2%.

Minimal A agar:

It contains 1.5% bacto-agar (Difco) in Minimal A medium. The plates were poured after mixing double strength Minimal A with 3% agar.

LB medium:

Tryptone	10.0 gm
Yeast Extract	5.0 gm
NaCl	10.0 gm
H_2O to	1000 ml

pH adjusted to 7.0 to 7.2 with 1 N NaOH

LB agar:

LB medium	1000 ml
Bacto-agar	15 gm

Z broth (for P1 transduction):

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

Z agar (for P1 transduction):

Z broth	100 ml
Bacto-agar	0.75 gm

LBON medium:

Tryptone	10 gm
Yeast Extract	5 gm
H_2O to	1000 ml

pH adjusted to 7.0 to 7.2 with 1N NaOH

LBON Agar:

LBON medium	1000 ml
Bacto-agar	15 gm

MacConkey agar:

MacConkey agar (Difco)	51.5 gm
H_2O to	1000 ml

W-salt medium:

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
H_2O to	1000 ml

After autoclaving following ingredients were added.

$MgSO_4$ (1M)	1 ml
Glucose (20%)	10 ml
Vitamin B1 (1%)	0.1 ml

2.1.5 Buffers and solutions:

Citrate buffer:

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

SM buffer:

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

TE buffer:

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

TBE buffer:

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

This was prepared as 10 X stock solution and used at 1 X concentration.

TAE buffer:

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

This was prepared at 50 X concentrated stock solution. Both TBE and TAE were used as standard electrophoresis buffers.

MOPS buffer:

MOPS		4.16 gm
0.5 M EDTA		1.0 ml
Sodium acetate		0.68 gm
Water (nuclease free)	to	500 ml

It was filter sterilized and stored in an amber colored bottle. This was prepared as 10 X stock solution and used at 1 X concentration

INOUE (PIPES) 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 gets into solution when the pH is greater than 6.7. $MnCl_2$ was dissolved separately and added drop by drop with stirring. The pH was adjusted to 6.7 and filter sterilized and stored at –20ºC.

Z buffer (for β-galactosidase assay):

Na_2HPO_4	16.1 gm
NaH_2PO_4	5.5 gm
KCl	0.75 gm
$MgSO_4.7H_2O$	0.246 gm
β-mercaptoethanol	2.7 ml
H_2O to	1000 ml

pH was adjusted to 7.0 and stored at 4ºC.

SDS running buffer:

Tris-base	30.3 gm
Glycine	144 gm
SDS	10 gm
H_2O to	1000 ml

It was prepared in 10 X concentration and diluted to 1 X for running.

Cell lysis buffer:

Tris-Cl (pH 8.0)	20 mM

NaCl	500 mM
DTT	10 mM

Storage and working buffer for protein:

Tris-Cl (pH 8.0)	20 mM
NaCl	300 mM
DTT	10 mM

Glycerol was added at 20 – 40 % for storage purposes.

Hybridization buffer:

Tris-Cl (pH 8.0)	9 mM
EDTA	0.35 mM

Sample buffer (for SDS-PAGE):

Tris-Cl (pH 6.8)	150 mM
SDS (20%)	6% v/v
Glycerol	30% v/v
β-mercaptoethanol (5%)	15%
Bromophenol blue	0.6% (w/v)

EMSA binding buffer:

Tris-Cl (pH 7.5)	10 mM
NaCl	50 mM
EDTA	1 mM
Glycerol	10%
BSA	1 µg/ml
$MgCl_2$	50 mM
DTT	1 mM

Transcription buffer:

Tris-Glu (pH 8.0)	20 mM

Mg-Glu	10 mM
K-Glu	50 mM

Tris-Glu buffer pH 8.0 was maintained with 10 M glutamic acid. It was prepared in 10 X concentrated solution which is diluted to 1 X for the reaction.

Denaturing gel loading buffer with dye:

Formamide	95%
EDTA	20 mM
Xylene Cyanol	0.05 gm
Bromophenol blue	0.05 gm

Non denaturing gel loading buffer with dye:

Tris-Cl (pH 7.5)	250 mM
Bromophenol blue	0.02%
Glycerol	20%

Formaldehyde agarose gel:

(For 50 ml)

DEPC treated water	43 ml
MOPS buffer	5.3 ml
Agarose	0.63 gm
Formaldehyde	2.6 ml

The above mix was boiled without formaldehyde to dissolve agarose and then at around 50ºC formaldehyde was added just before casting the gel.

40% Acrylamide solution:

Acrylamide	39 gm
Bis-acrylamide	1 gm
H_2O to	100 ml

Acrylamide solution (30%):

Acrylamide	29 gm

Bis-acrylamide	1 gm
H_2O to	100 ml

Non denaturing gel composition (50 ml):

40% acrylamide solution	5 ml
10 X TBE	5 ml
H_2O	40 ml
10% APS	250 µl
TEMED	30 µl

SDS PAGE gel (12%):

For resolving Gel (15 ml)

30% Acrylamide solution	6 ml
1.5 M Tris-Cl (pH 8.8)	3.8 ml
10% SDS	150 µl
10% APS	150µl
H2O to	15 ml
TEMED	10 µl

For stacking gel (3 ml):

30% Acrylamide solution	500 µl
1 M Tris Cl (pH 6.8)	380 µl
10% SDS	30 µl
10% APS	30 µl
H2O to	3 ml
TEMED	10 µl

Denaturing (urea) sequencing gel (6%) composition:

10 X TBE	50 ml
40% acrylamide	75 ml

Urea	210 gm (7 M)
H_2O to	500 ml

Denaturing (urea) sequencing gel (10%):

10 X TBE	50 ml
40% acrylamide	125 ml
Urea	210 gm (7 M)
H_2O to	500 ml

This was filtered through a 0.45/0.22 µ milipore filter.

For casting gel:

Sequencing gel mixure	35 ml
10% APS	150 µl
TEMED	25 µl

2.1.6 Antibiotics:

Antibiotics were used at the following final concentrations in various media as given in Table 2.4.

Table 2.4: Concentrations of antibiotics (µg/ml):

Antibiotics	LB medium	Minimal medium
Ampicillin (for plasmids)	100	50
Chloramphenicol	50	50
Kanamycin	50	25
Streptomycin	25	12.5
Tetracyclin	50	60
Trimethoprim	25	30

2.1.7 Chemicals:

Chemicals were obtained from commercial sources. Most of the chemicals such as amino acids, antibiotics, sugars, IPTG, ONPG and X-gal were obtained from Sigma Chemical Co. The media components for the growth of bacteria were mostly from Difco laboratories.

The materials used in the recombinant DNA experiments such as restriction endonucleases, T_4-DNA ligase, DNA-polymerases and DNA size markers were obtained from companies including New England Biolabs, Invitrogen, Promega, Bangalore Genei and MBI Fermentas.

RNA isolation chemicals Trizol, RNA loading buffers and dyes and RNA size markers were obtained from Invitrogen, Sigma and MBI Fermentas. Protein markers were purchased from New England Biolabs, Banglore Genei or MBI Fermentas.

Other enzymes, Exonuclease III, DNaseI were from New England Biolabs. Kits used for plasmid isolation, purification of DNA fragments, PCR amplification and DNA sequencing were from Qiagen, Life Sciences or USB Corporation. High fidelity enzymes for PCR amplification were purchased from Sigma and Stratagene.

The oligonucleotide primers used in this study were mainly synthesised by Sigma Chemical Co. or MWG Biotech. The radioactive chemicals were procured from either Amersham Biosciences or BRIT Mumbai.

2.2 Methods:

2.2.1 Genetic techniques:

2.2.1.1 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 37ºC for 20-min and the lysate was prepared in one of the following ways:

A. Broth method:

To 0.3 ml of infection mixture, 10 ml of Z-broth was added and incubated at 37ºC with slow shaking until the visible lysis of the culture occurred (in 4-6 hrs). The lysate was treated with 0.3 ml of chloroform, centrifuged and the clear lysate was stored at 4ºC with chloroform. Preparation of P1 lysates on *recA* mutant strains were also done similarly, but with a higher multiplicity of infection (i.e. 10^8 starter P1 phage).

B. Plate method

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

To quantitate the P1 phage lysate preparation, titration was done using P1 phage sensitive indicator strain such as MG1655. 100 µl each of dilution of phage (typically 10^{-5}, 10^{-6}) were mixed with 0.1 ml of fresh culture grown in Z-broth. After 15-min of adsorption at 37ºC without shaking, each mixture was added on a soft agar overlay of Z-agar plates and incubated overnight at 37ºC. The phage titer was calculated from the number of plaques obtained on the plates.

2.2.1.2 Phage P1 transduction:

To 2 ml of fresh overnight culture of recipient strain, 10^8 pfu equivalent of phage lysate was added and incubated at 37ºC without shaking for 15-min to facilitate phage adsorption. The un-adsorbed phage particles were removed by centrifugation at 4000 rpm for 5-min and pellet of bacterial cells was re-suspended in 5 ml of LB-broth containing 20 mM sodium citrate to prevent further phage adsorption. This was incubated for 30-min at 37ºC without shaking to allow the phenotypic expression of the antibiotic resistance gene. The mixture was then centrifuged, and the pellet was re-suspended in 0.3 ml citrate buffer. 100 µl aliquots were plated on appropriate antibiotic containing plates supplemented with 2.5 mM sodium citrate. A control tube without addition of P1 lysate was also processed in the same way. In the case of selection of nutritional requirement, the infection mixture was centrifuged, washed in 5 ml of citrate buffer and plated without phenotypic expression.

2.2.1.3 Transfer of *recA* allele by P1 transduction:

The strain GJ216 was employed for this purpose where the Tn*10* insertion in *srl* gene is approximately 80% co-transducible with the *recA56* mutation. Lysate prepared on this strain was transduced to another recipient strain which was to be made *recA*. Among the Tet^r transductants, *recA* colonies were identified by the UV-sensitive phenotype.

2.2.1.4 Transformation:

A. Calcium chloride method:

For routine plasmid transformations, following method which is modification of that described by Cohen et al. (1972) was used. An overnight culture of recipient strain was sub-cultured 1:100 in fresh LB-medium and grown till mid-exponential phage. The culture was chilled on ice for 15-min, and the steps thereafter were performed at 4ºC. 20 ml of culture was centrifuged and pellet was re-suspended in 10 ml of 0.1 M $CaCl_2$. After 15-min of incubation on ice, the cells were again centrifuged and re-suspended in 2 ml of 0.1 M $CaCl_2$. The suspension was incubated on ice for 30-min. To the 200 µl aliquote of the cell suspension plasmid DNA (20 to 200 ng in less than 10 µl volume) was added, incubated for half an hour on ice and given a heat shock for 90-sec at 41ºC. The cultures was rapidly chilled, mixed with 0.8 ml of LB-broth and incubated at 37ºC for 1-hr, and plated on an appropriate selective medium at various dilutions. An aliquot of cell suspension to which plasmid DNA was not added served as a negative control.

B. Preparation of high efficiency competent cells:

Competent cells for high efficiency transformations were prepared by a method of Inoue et al. (1990) with few modifications. An overnight culture of the strain (routinely DH5α) was sub-cultured into fresh sterile LB-broth in 1:100 dilutions and grown at 18ºC to an A_{600} of 0.55. The cells were harvested by centrifugation at 2500 rpm for 10-min at 4ºC. This was re-suspended in 0.4 volumes of INOUE buffer and incubated in ice for 10 min. The cells were recovered by centrifugation at 2500 rpm at 4ºC for 10-min and finally re-suspended in 0.01 volume of the same buffer. Sterile DMSO was added to a final concentration of 7%. After incubating for 10-min in ice, the cells were aliquoted in 100 µl volumes, snap frozen in liquid nitrogen and stored at –70ºC.

2.2.1.5 Scoring for phenotypes:

A. *lac* phenotype:

Lac^+ colonies were distinguished from Lac^- on X-gal containing plate or MacConkey lactose plate. X-gal is non inducing colorless substrate of β-galactosidase enzyme which upon hydrolysis yields dark blue indolyl group and hence the Lac^+ colonies on X-gal plate will appear as dark blue colonies. Similarly, on the MacConkey agar plates Lac^+ colonies will appear dark pink colonies whereas Lac^- will remain colourless.

B. UV-sensitivity phenotype:

To check the UV-sensitivity of the strains qualitatively, the strains were streaked onto duplicate LB-agar plates and one of the plates was exposed to UV-radiation from a 15-W UV-germicidal lamp at a distance of 70 cm for 30-sec. The UV-exposed and unexposed plates were incubated overnight in the dark after wrapping with aluminium foil and then growth was scored. This test could differentiate *rec*$^-$ strain from *rec*$^+$ strains.

C. Test for canavanine (CAN) sensitivity:

CAN is a toxic analog of Arg and is an inhibitor of bacterial growth. Strains were tested for sensitivity/resistance to CAN by streaking them on Minimal A glucose plates supplemented with and without 40 μg/ml CAN.

2.2.1.6 β- galactosidase assay:

β-galactosidase assay was performed according to Miller (1992). An overnight grown culture of the bacterial strain was sub-cultured in glucose Minimal A medium supplemented with amino acids and appropriate antibiotic and grown at 37ºC to an A_{600} of 0.5-0.6. Around 0.1-0.5 ml of culture was made up to 1 ml with Z-buffer and lysed

with addition of one drop of chloroform and 1-2 drops of 1% SDS solution. 0.2 ml of freshly prepared 4 mg/ml ONPG was added to start the reaction and incubated at room temperature till the color of the reaction mixture turned yellow. 0.5 ml of 1 M Na_2CO_3 was added to stop the reaction and the time duration from initial addition of ONPG to the stopping of reaction was noted. The absorbance of reaction mix was taken at 420 nm and 550 nm. The A_{600} of the culture used was also noted. The enzyme specific activity (in Miller units) was calculated using following equation:

$$\textbf{β-galactosidase specific activity} = [1000 \times A_{420} - (1.75 \times A_{550})] / t \times v \times A_{600}$$

Where t, the time period in min and v, the volume of culture used in ml.

2.2.2 Recombinant DNA techniques:

2.2.2.1 Isolation of plasmid DNA:

1.5 ml of cells from an overnight culture were pelleted by centrifuging in cold (4ºC) for 10-min at 6000 rpm. The cells were re-suspended in 200 µl solution I (50 mM glucose; 25 mM Tris-Cl, pH-8; 10 mM EDTA, pH-8) with vortexing. 400 µl of freshly prepared solution II (0.2% NaOH, 1% SDS) was added and mixed by gently inverting the tubes. Subsequently, 300 µl of solution III (prepared by mixing 60 ml of 5 M CH_3COOK, 11.5 ml glacial acetic acid, 28 ml water) was added and the tubes were inverted repeatedly and gently for homogeneous mixing followed by incubation for 5-min on ice. After centrifuging at 12,000 rpm for 15-min, supernatant was decanted into a fresh tube, an equal volume of iso-propanol was added, the precipitated nucleic acids were then recovered by centrifugation at 12,000 rpm for 30-min. The pellet was washed once with 70% ethanol, air-dried and re-suspended in 100 µl of TE-buffer. It was treated with RNase at a concentration of 20 µg/ml by incubating at 37ºC for 1-hr. It was further extracted with an equal volume of phenol:chloroform mixture followed by

chloroform:isoamyl alcolhol (24:1) mixture. After centrifugation, the clear supernatant was used for recovering the nucleic acids. The nucleic acids were precipitated with 200 µl of alcohol in presence of 0.3 M sodium acetate (Sambrook et al. 1989). In case where high purity plasmid preparations are required (DNA sequencing) the plasmid isolation was carried out with the commercially available kits following the manufacturers instruction. Plasmids were observed on 1% agarose gel.

2.2.2.2 Isolation of chromosomal DNA:

Chromosomal DNA isolation was carried out according to the method described in Current Protocols in Molecular Biology. 1.5 ml of stationary phase culture was centrifuged and cell pellet was re-suspended in 567 µl of TE buffer. To this 30 µl of 10% SDS, and 3 µl of proteinase K (20 mg/ml) were added in that order and the cell suspension was mixed and incubated at 37ºC for 1-hr. When the suspension was clear 100 µl of 5 M NaCl was added and thoroughly mixed followed by the addition of 80 µl CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 7 M NaCl). The suspension was incubated at 65ºC for 10-min, brought to room temperature and extracted with an equal volume (780 µl) of chloroform isoamyl alcohol (24:1), and aqueous phase transferred to fresh tube. The aqueous phase was further extracted successively, first with phenol:chloroform:isoamyl alcohol (25:24:1) and then with chloroform isoamyl alcohol (24:1). DNA was precipitated from the clear supernatant by the addition of 0.6 volumes of iso-propanol. The chromosomal DNA was either spooled out or pelleted at this stage and washed with 70% ethanol air dried and dissolved in 100 µl of TE-buffer.

2.2.2.3 Agarose gel electrophoresis:

The DNA samples were mixed with appropriate volumes of 6 X loading dye (0.25% bromophenol blue and 0.25% xylene cyanol and 30% glycerol in water) and subjected

to electrophoresis through 0.8 to 1 % agarose gel in TAE buffer. The gel was stained in 1 µg/ml ethidium bromide solution for 15-min at room temperature and visualised by fluorescence under UV-light in a UV-transilluminator.

2.2.2.4 Restriction enzyme digestion and analysis:

Around 0.5 to 1 µg of DNA was regularly used for each restriction digestion. 2 to 5 units of restriction enzyme were used in the total reaction volume of 20 µl containing 2 µl of the corresponding buffer supplied at 10 X concentration by the manufacturer. The reaction was incubated for 2-hrs at the temperature recommended by the manufacturer. The DNA fragments were visualised by ethidium bromide staining after electrophoresis on a 0.8 to 1% agarose gels. Commercially available DNA size markers were run along with the digestion samples to compare with and to estimate the sizes of the restriction fragments.

2.2.2.5 Purification of DNA by gel elution:

DNA fragments to be used for specific purposes like ligation or radioactive labelling were eluted from the agarose gel after electrophoresis. The gel piece containing the desired band was sliced out from the gel and the DNA was purified using commercially available purification kits for this purpose. The efficiency of elution was determined by checking a small aliquot of DNA sample on the gel.

2.2.2.6 End-fill reactions:

After the digestion, restriction enzyme was heat inactivated by incubation at 65ºC for 15-min, and the end filling reaction was then set up by supplementation with 33 µM of each dNTP and addition of Klenow DNA polymerase to a final concentration of 1 unit/µg of DNA. The reaction was carried out for 5- to 10-min at 37ºC, following which the enzyme was heat inactivated by heating at 75ºC for 10-min. In case of DNA

fragments eluted from the gel the filling reaction was carried out in 1 X Klenow buffer [10 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$ 7.5 mM DTT].

2.2.2.7 Ligation of DNA:

Typically 400-500 ng of DNA was used in each ligation reaction. The ratio of vector to insert was maintained between 1:3 to 1:5 for cohesive end ligation and 1:1 for blunt end ligation. The reaction was generally performed in 10 µl volume containing ligation buffer (provided by the manufacturer) and 0.05 Weiss unit of T_4-DNA ligase, at 16ºC for 14- to 16-hrs.

2.2.3 Biochemical techniques:

2.2.3.1 Over-expression and purification of ArgP:

For preparing the ArgP protein, a derivative (designated as pHYD1705) of the plasmid vector pET21b (EMD Biosciences) was constructed which carries the PCR-amplified *argP*$^+$ fragment downstream of the phage T7-promoter, such that the encoded ArgP protein bears a C-terminal His_6-tag provided by the vector DNA sequence. The resultant plasmid was transformed into strain BL21-SI, a derivative of strain GJ1158 (Bhandari and Gowrishankar 1997) that expresses phage T7-RNAP from the salt-inducible *proU* promoter. BL21-SI harbouring plasmid pHYD1705 was grown in 500 ml of low osmolar medium (LBON), induced at A_{600} of around 0.5 with 0.3 M NaCl and 1 mM isopropyl thio-β-D-galactoside and harvested after 3-hrs of induction. Bacteial cells were recovered by centrifugation, resuspended in 10 ml of lysis buffer (50 mM Tris-Cl, pH-8; 500 mM NaCl; 10 mM DTT and 10 mM imidazole) containing 20 µg/ml lysozyme, and lysed by sonication with 20-sec pulses for 10-min. The protocol for His_6-ArgP protein purification involved (i) passing the lysate through a 5 ml Ni-NTA chromatographic column pre equilibrated with lysis buffer, (ii) washing the

column with 100 ml of washing buffer (50 mM Tris-Cl, pH-8; 500 mM NaCl; 10 mM DTT; 20 mM Imidazole), and (iii) elution of His_6-ArgP from the column with elution buffer (50 mM Tris-Cl, pH-8; 500 mM NaCl; 10 mM DTT and 250 mM Imidazole), and collection of 1.5 ml eluate fractions (10 fractions). The fractions were tested for protein by Bradford method and the protein-carrying fractions (generally tubes 2 to 5) were pooled and dialysed against 20 mM Tris-Cl, pH-8 with 10 mM DTT and 300 mM NaCl (two changes of 1 litre each). The proteins were concentrated by centrifugation to around 1 mg/ml by using Amicon filter (pore size 10-KDa) and stored at −20ºC in storage buffer (20 mM Tris-Cl, pH-8; 150 mm NaCl; and 10 mM DTT) with 20-40% Glycerol.

2.2.3.2 Protein estimation:

Protein concentrations were estimated by the method of Bradford (1976). The A_{595} was measured after complexation with Bradford reagent. Bovine serum albumin was used as standard against which the unknown protein concentrations were estimated.

2.2.3.3 Sodium dodecyl sulphate-polyacrlyamide gel electrophoresis (SDS-PAGE):

The method followed was as described in Sambrook et al. (1989). Gels of 1.5 mm thickness were casted in the commercially available small gel apparatus. Resolving gel of 12% (15 ml) and stacking gel (4 ml) was made. Gels were polymerised by the addition of TEMED and APS (1 % v/v of the gel mix). Sample preparation for gel loading was done as follows. Cell lysate or pure protein fractions (around 30 µg) was mixed with the sample buffer to 1 X and heated at 95ºC for 2-min. For direct lysis of cells to check the expression, log and stationary phase cultures were spun down, sample buffer (1 X final concentration) was added to the cell pellet and boiled for 10 min, cooled to room temperature, and after a second spin, the clear supernatant was loaded.

The gel run was started at constant current of 20 mA. When the dye front crossed the stacking gel the current was increased to 40 mA.

2.2.3.4 Optical measurements:

A. Circular dichroism (CD) spectometry:

CD spectra were recorded at 25ºC with a 1 mm cuvette in a Jasco spectropolarimeter. Each spectrum was obtained by averaging three scans from 200 nm to 270 nm in 0.5 nm steps with a band width of 1 nm and signal averaging time of 3-sec. The spectral baseline was corrected for background buffer. Molar ellipticity expressed in milli-degrees, was plotted as a function of wavelength. The secondary structural content was calculated using K2D software available online (www. Embl-heidelberg.de/~andrade/k2d/).

B. Fluorimetry and denaturation studies:

Fluorescence spectra of purified protein were recorded on a Perkin Elmer LS-3B spectrofluoremeter. The protein was excited at wavelength of 295 nm and the tryptophan emission spectrum was recorded from 310 to 450 nm at a scan speed of 50 nm/s. Around 1 μM of protein was used for each reading in 20 mM Tris-Cl buffer pH-8.0.

For denaturation studies, fluorescence spectra of ArgP (1 μM) were measured in presence of various urea concentrations from 0 to 8 M under isothermal conditions. Fluorescence intensity (relative units) was recorded as a function of wavelength (nm). The urea-induced transitions were recorded as a function of shift in wavelength maxima. The analysis was done using conventional linear least-square analysis to fit the data.

2.2.3.5 Analytical size exclusion chromatography:

The oligomeric state of ArgP was investigated by analytical size exclusion chromatography using a Superose 12 (10/30) FPLC column (Amersham Biosciences). Chromatography was performed at 4ºC in Tris-Cl (20 mM) pH 8.0 buffer containing 100 mM NaCl as running buffer. Protein elution was detected by measurement of A_{295}. The void volume, V_0 was determined using blue dextran ($2x10^6$ Daltons) and the elution parameter K_{av} was calculated from its elution volume V_e and total bed volume V_t using the equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

2.2.3.6 Co-effector binding studies

To determine the binding of Arg or Lys to ArgP, gel filtration chromatography of ArgP was carried out using Sephadex G-25 gravity column in presence and absence of ^{14}C-Arg or ^{14}C-Lys. In a 50 µl volume of running buffer containing 20 mM Tris-Cl (pH 8) and 100 mM NaCl, ArgP protein at 10 µM (monomer) was incubated with ^{14}C-Arg or ^{14}C-Lys (250 mCi/mmol) at 0.1 mM for 30-min at 4ºC before the mixture was loaded on a 4 ml Sephadex G-25 gravity column (Amersham Biosciences) and subjected to gel filtration chromatography. In each of the 0.2 ml fractions that were collected, protein was quantitated by fluorimetry (with excitation and emission wavelengths set at 295 nm and 330 nm, respectively) and radioactivity by liquid scintillation counting. In the competition experiments, ArgP was first incubated with 10 mM of the indicated unlabelled amino acid prior to addition of ^{14}C-Arg or ^{14}C-Lys.

2.2.3.7 Glutaraldehyde crosslinking experiment:

In vitro chemical cross-linking experiment of ArgP was carried out using glutaraldehyde (Sigma Chemical Co) as described in Pani et al. (2006). Reactions were

carried out in 20 mM Tris-Cl buffer (pH 8.0), 300 mM NaCl, and 12 mM glutaraldehyde. The reaction was stopped at different time points from 0 to 10-min (0, ½, 1, 2, 5 and 10 mins) by adding sample buffer containing 400 mM glycine, 50 mM Tris-Cl, pH 8.0, 3% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol. Samples were subjected to 12% SDS-PAGE and protein bands visualised by silver staining.

2.2.4 Molecular techniques:

2.2.4.1 Polymerase chain reaction (PCR):

The PCRs were normally performed using a high fidelity Accu-Taq DNA polymerase from Sigma Chemicals Co. Approximately, 1-2 ng of plasmid or 100 to 200 ng of chromosomal DNA was used as a template in a 50 µl reaction volume containing 200 µM of each dNTP, 20 picomol each of forward and reverse primer and 1.5 units of Taq DNA polymerase. In some cases, *E. coli* cells from a freshly grown plate were resuspended in 50 µl of sterile Milli-Q water to get a cell suspension (~ 10^9 cells/ml) and 4 µl from this was used as the source of DNA template (colony PCR). The samples were subjected to 30-cycles of amplification and the typical conditions of PCR were as follows (although there were slight alterations from one set of template/primer to another):

The initial denaturation was carried out at 95°C for 4-min and the cycle conditions were as given below.

Annealing	45ºC to 50°C	1-min
Extension	68°C	(1-min/kb of DNA template to be amplified)
Denaturation	95°C	1-min

After 30 cycles of PCR, the final extension step was carried out again for 10-min at 68°C. For extending the whole plasmid template, an enzyme like *pfu* with high fidelity and very high prossessivity was used. For mutagenic PCRs Taq polymerase without proof reading activity was used.

2.2.4.2 Isolation of total cellular RNA:

For isolation of RNA, cells were grown in minimal A medium supplemented with 0.2% glucose upto A_{600} of 0.6. Cells were harvested by centrifugation and total RNA was isolated by using Trizol (Invitrogen) according to manufacturer's instructions. 1 ml of Trizol was used to lyse cells equivalent of approximately 4 ml of overnight culture. Homogeneous lysis was achieved by gentle pipetting repeatedly. The homogenised samples were incubated at room temperature for 5-min to permit complete dissociation of nucleoprotein particles. Following homogenization, 0.2 ml of chloroform for each 1 ml Trizol reagent was added and vigorously shaken with hand for 15-sec and incubated further for 3-min at RT. It was then centrifuged at 12000 rpm for 10-min at 4ºC, which separates out the homogenate into lower phenol chloroform phase (red), an interphase and a colourless upper aqueous phase. The upper aqueous phase in which RNA exists exclusively, was transferred to a fresh microfuge tube and RNA was precipitated by adding 0.5 ml of isopropyl alcohol for each ml of Trizol used. Samples were incubated at 15 to 30ºC for 10-min and centrifuged at 12000 rpm for 10-min at 4ºC. RNA formed a gel like precipitate at the bottom of the tube. Supernatant was removed and RNA was washed with 75% ethanol (by adding 1 ml of ethanol per ml of Trizol employed). RNA could be stored after this step in –20 or –70ºC for more than a year. RNA pellet was air dried for 15- to 30-min following which it was dissolved in nuclease free water. The concentrations and purity of RNA samples were determined spectroscopically as well as by visual inspection on formaldehyde-agarose gel in MOPS buffer (Gallant et al.

2004). Before loading onto the gel, RNA was mixed with loading buffer and heated at 90ºC for 3-min.

2.2.4.3 DNA sequencing:

Automated DNA sequencing on plasmid templates or on PCR products was carried out with dye terminator cycle sequencing kits from Perkin-Elmer on an automated sequencer (model 377, Applied Biosystems), following the manufacturer's instructions. Manual sequencing was achieved using the Sequenase 2 plasmid or PCR product sequencing Kit from USB Corp. as described in manufacturer's instructions and the sequencing reaction products were resolved by electrophoresis on a 6% or 10% sequencing gel.

2.2.4.4 Site directed mutagenesis:

Site directed mutagenesis of plasmid DNA was carried out by using QuikChange kit (Stratagene) with a pair of complementary oligonucleotide primers carrying the necessary sequence modifications. In this process, the plasmid (around 20 –50 ng) containing the fragment of DNA where nucleotide has to be altered, was used as template and "linear PCR" of 20 cycles was set up using *Pfu* DNA polymerase to amplify the whole plasmid with extension time calculated according to a rate of 500-bp/min. The reaction mix was digested with *DpnI* for 1-hr (to destroy the original input plasmid DNA) following which it was transformed directly to a highly competent XL1 Blue cells. The mutated plasmid was confirmed by sequencing. In this study, incorporating the modifications of bases in the primers, the putative –10 sequences of *argO* promoter was modified on pHYD1722.

2.2.4.5 Generations of deletion by inverse PCR:

The deletions were obtained by a plasmid based PCR strategy as described (Rajkumari and Gowrishankar 2001), with a pair of outwardly extending primers designed to amplify the entire plasmid except for the region to be deleted, and with *EcoRI* sites incorporated in the primers to facilitate re-circularization of the deletant plasmids.

2.2.4.6 Radiolabelling of oligonucleotides and PCR products:

Oligonucleotides and PCR products were end labeled using phage T_4-polynucleotide kinase (PNK, New England Biolabs) with ^{32}P-γ-ATP. The radiolabelling reaction mixture (50 μl) contained 1 X of buffer provided by the company, 10 units of T_4-PNK and 50 μCi of ^{32}P-γ-ATP. The reaction mix was incubated for 1-hr at 37ºC and the reaction was stopped by adding 10 μl of 5 M EDTA. The labeled oligonucleotides and DNA fragments were purified by passing through Sephadex G-25 gel filtration column. Labelling efficiency was checked by scintillation counting. To achieve strand specific labelling of a DNA fragment, the fragment was first labelled at both ends and then digested with a restriction enzyme that cleaves close to one of the ends. Altenatively, the DNA fragment was digested first with restriction enzyme and end-filled with Klenow DNA polymerase using the ^{32}P-α-radiolabelled dNTPs.

2.2.4.7 Primer extension analysis:

Primer extension analysis to map the transcription start site was carried out as described by Conway et al. (1987) and Rajkumari et al. (1997), using avian myeloblastosis virus reverse transcriptase (AMV-RT). 20 pmol of primer was labelled at its 5′-end with ^{32}P-γ-ATP as described above. 10^6-cpm equivalent of labelled primer was mixed with 10 μg of total cellular RNA. Sodium acetate pH-5.5 was added to a final concentration of 0.3 M and the nucleic acids were precipitated with ethanol, washed with 70% alcohol,

air-dried and dissolved in hybridization buffer (9 mM Tris-Cl, pH-8 and 0.35 mM EDTA) and incubated overnight at 43ºC for annealing. Reverse transcriptase reaction was performed by the addition of 5 mM $MgCl_2$, 1 mM dNTP's, 1 X RT buffer, high concentration (10 units) of AMV-RT and 1 unit/µl RNasin to the mixture of annealed labelled primer and RNA. The reaction was incubated at 43ºC for 1-hr following which the nucleic acids were precipitated with absolute alcohol and 0.3 M CH_3COONa, pH-5.5. The precipitate was air dried and dissolved in water and gel-loading dye (95% formamide, 20 mM EDTA, 0.05% each of xylene cyanol and bromophenol blue) was added. The samples were heated at 90ºC for 2-min before loading on a 6% denaturing poly-acrylamide gel for electrophoretic resolution alongside a sequence ladder generated from M13 DNA template.

2.2.4.8 Electrophoretic mobility shift assay (EMSA)

EMSA reactions were performed in 20 µl reaction volume with EMSA binding buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 50 mM $MgCl_2$; 1 mM dithiothreitol; 10% glycerol; 1 mM phenyl-methyl-sulfonyl fluoride) containing (i) 5′-end-labeled DNA fragment at 0.5 nM concentration; (ii) 1 µg each of bovine serum albumin and poly(dI).poly(dC), (iii) the protein (ArgP in this case), and (iv) when required, co-effectors at 0.1 mM. The reaction mixture was incubated at room temperature for 30-min and the complexes were resolved by electrophoresis on a non-denaturing 4% polyacrylamide gel (39:1 acrylamide:bisacrylamide).

2.2.4.9 Heparin challenge experiment of *argO*-RNAP-ArgP complex:

In heparin challenge experiments (in presence or absence of NTPs at 0.5 mM,), after the incubation of *argO* regulatory DNA with ArgP and RNAP, the *argO*-ArgP-RNAP

complex obtained was treated with heparin at 200 μg/ml and incubations continued for varying times (from 0 to 60 min) at room temperature prior to gel electrophoresis.

2.2.4.10 In vitro transcription

Single-round in vitro transcription reactions were carried out in transcription buffer (40 mM Tris-glutamate, pH 8; 0.1 mM bovine serum albumin; 1 mM dithiothreitol) as previously described (Rajkumari et al. 1996). To a 5 μl volume of mix containing the linear DNA template (10 nM), ArgP (20 nM), σ^{70}-bearing RNAP holoenzyme (80 nM), and co-effector(s) (0.1 mM), pre-incubated at 37ºC for 10-min, was added an equal volume of labelled substrate-heparin mix to give final concentrations of 500 μM each of UTP, GTP, and ATP, 50 μM CTP, 200 μg/ml heparin, and 10 μCi of ^{32}P-α-CTP (>3000 Ci/mmol), and incubation continued for 15 min. The reactions were stopped by the addition of ice-cold stop solution (40 mM EDTA, 300 μg of *E. coli* tRNA per ml). After precipitation with ethanol, the nucleic acids were dissolved in 10 μl of sample buffer (80% v/v formamide, 8 mM EDTA and 0.1% each of bromophenol blue and xylene cyanol) and boiled at 90ºC before loaded onto the gel. The run off transcripts were analysed through a 6% or 10% polyacrylamide, 7 M urea sequencing gel. For abortive transcripts assays, samples were analysed on a 20% polyacrylamide, 7 M urea sequencing gel.

2.2.4.11 Arg-chase experiment:

For the Arg-chase experiment on immobilised template, the biotinylated *argO* template was incubated with RNAP (and other additives as appropriate), and labelled substrate-heparin mix was added together with streptavidin-coated magnetic beads (Promega Corp.). The primary reaction mixtures were incubated for 15-min to allow for immobilization of the biotinylated template on the beads. The beads were then

separated from the supernatant, washed with transcription buffer containing 0.1 mM Arg (or CAN, as appropriate), and then incubated in the chase step with fresh labelled substrate-heparin mix supplemented with 0.1 mM Arg or CAN for 15-min. Each of the various preparations was analysed for labelled transcription products by denaturing gel electrophoresis on a 10 % sequencing gel.

2.2.4.12 Inverse pulse labeling experiment:

The inverse pulse labelling experiment for abortive transcript formation was performed as previously described (Kubori and Shimamoto 1996, Susa et al. 2006). The pre-incubation mix containing 40 nM RNAP, 20 nM ArgP and 10 nM of template in transcription buffer was incubated at 37ºC for 10-min. A substrate heparin mix was added that contained 100 μM each of GTP, CTP, UTP and 5 μM of ATP. After various reaction times (from 0- to 60-min), around 5 μCi γ-^{32}P-ATP was added and incubated for 15-min. The transcripts were analysed on a 20% polyacrylamide, 7M urea sequencing gel. The transcripts were quantified with Amersham Biosciences Image Quant^{TM} imaging system; the data for each species were corrected for background.

2.2.4.13 Exonuclease III assay:

For the exonuclease III protection assay, the 5′-end-labeled PCR-amplified *argO* fragment was treated with exonuclease III (50 units) in a 10 μl volume of transcription buffer containing 20 nM ArgP (monomer concentration), and 80 nM RNAP, NTPs at 0.5 mM each, and Arg or Lys at 0.1 mM. After incubation for 20-min at room temperature, the DNA was phenol-extracted and analysed by electrophoresis on a 10% polyacrylamide gel with 7 M urea, along with *argO* di-deoxy sequence ladder.

2.2.4.14 DNase I footprinting:

For DNase I footprinting, *argO* fragments uniquely labeled at the 5′-ends of the bottom strand and top strand were each dissolved at 0.6 nM in 50 μl of EMSA binding buffer

and incubated with ArgP at 20 nM (monomer concentration) in the presence or absence of Arg or Lys at 0.1 mM. After 30-min incubation at room temperature, the mixture was treated with 0.2 units of DNase I for 60-sec prior to addition of 50 µl of stop solution [50 mM EDTA (pH 8), 2% sodium dodecyl sulfate]. The DNA was then phenol extracted and electrophoresed on a 10% denaturing polyacylamide gel along with *argO* di-deoxy sequence ladder.

2.2.4.15 $KMnO_4$ footprinting:

The in vivo treatment of plasmid carrying *argO* regulatory DNA was carried out as described by Sasse-Dwight and Gralla (1989). The strain carrying required plasmid was sub cultured from an overnight culture 1:100 in 0.2% glucose-Minimal A medium and it was treated with $KMnO_4$ (5 mM) for 4-min after the growth to an A_{600} of around 0.6. Wherever required, rifampicin was added to 100 µg/ml 5-min prior to the $KMnO_4$ treatment. The cells were immediately chilled and plasmid DNA was isolated using Qiagen plasmid isolation kit. The $KMnO_4$ treated plasmids were digested with *HaeIII*, to obtain a uniform end for all of the primer extension reactions.

For in vitro $KMnO_4$ modification, the experiment was carried out as described by Yang et al. (2002). DNA (10 nM of 427-bp PCR amplified fragment), RNAP (100 nM) and ArgP (20 nM) were incubated with 1 X transcription buffer in presence of NTPs for 15-min at 37ºC to allow the formation of open complex, which was then treated with $KMnO_4$ (12.5 mM) for 3-min following which the reactions were quenched by adding 2.5 µl of 2-mercaptoethanol (14.7 mM). The DNA samples were each treated at 90ºC for 3-min with phenol and purified by Qiagen PCR clean up kit.

Primer extension analysis of each of the modified plasmids was performed as described in Liu et al (2004) by PCR cycle of primer extension. An equivalent amount of digested plasmids (around 0.5 µg) was employed as templates to extend the ^{32}P-labelled primer

using PCR cycles (45 cycles) to probe the modified bases in the template strand of the open complex. The extension products were detected by phosphorimaging following electrophoresis on a 10% denaturing polyacrylamide gel alongside a sequence ladder and their image intensities were normalised (for each lane) to that of the extension product corresponding to the *HaeIII* site on the template.

2.2.4.16 Densitometry:

Band intensities in gel autoradiograms were determined by densitometry (after correction for background), with the aid of the Amersham Biosciences Image Quant^{TM} imaging system. The intensities of the bands were expressed in terms of volume and the corrected volumes of each band were expressed as percentages of intensity or radioactivity.

2.2.4.17 K^+ estimation:

To estimate the potassium ion, cells were grown in three fold diluted (1/3rd) minimal A medium (to decrease the background potassium from media) supplemented with betaine. In case of high-osmolar condition, 0.6 M NaCl was supplemented to the culture. Cells were harvested after the A_{600} of 0.4-0.5. For sample preparation, 1.4 ml of culture was mixed with 200 µl of fuser silicon oil and centrifuge at 13000-rpm for 5-min as described in (Jennifer and Russel 2002). Three layers were separated cells, oil, and water from top to bottom. Water was sucked off with a pipettement followed by oil. The cell pellets were cut off with a fine scalpel from the tube and resuspended in 2 ml of 3 N nitric acid and digested by incubating overnight at room temperature. Cell debris was removed by spinning at 12000 rpm for 15-min and supernatant was used for K^+ estimation. Finally the K^+ in the supernatant was estimated by Atomic spectroscopy. The K^+ pool was expressed per mg dry weight of the cell.

2.2.4.18 Glu dehydrogenase (GDH) activity assay:

Cultures were grown in glucose minimal A medium supplemented with glycine betaine. At around A_{600} of 0.2 they were each split into two parts. To one part (high-osmolarity grown) NaCl was added to a final concentration of 0.6 M (from a 2.4 M stock solution prepared in glucose minimal A medium). This and the other part (low-osmolarity grown) were incubated. At an A_{600} of around 0.5, cells were harvested and cell extracts were prepared as described in Gowrishankar and Pittard (1982) with few modifications. Harvested cells were washed and resuspended in 4 ml of ice-cold 50 mM Tris-Cl buffer (pH 7.6) with 10 mM β- mercaptoethanol. The cells were lysed by sonication and after centrifugation the extracts were used for enzyme activity measurements immediately. The assay method was as described by Meers et al. (1970) performed at room temperature in 0.5 ml (total volume) mixtures. Protein concentrations in cell extracts were determined by the method of Bradford. Enzyme specific activities were expressed in milliunits per milligram of protein in the cell extracts (after correction for endogenous NADPH oxidase activity); 1 U was defined as the amount of enzyme required to oxidize 1 μmol of NADPH (extinction coefficient at 340 nm, 6,220 M^{-1} cm^{-1}) per min.

2.2.4.19 Estimation of intracellular Glu level:

For estimation of intracellular glutamate, cultures grown at low and high-osmolar media were prepared in the way as described for the GDH assays. A 0.2 ml of each culture was added to 0.8 ml of ice-cold methanol as described in the no-harvest method (Kustu et al. 1994; Csonka et al. 1994; Yan et al. 1996). Cell debris in the mixture was removed by low-speed centrifugation, and the supernatant was lyophilised and resuspended in 0.05 ml of water, and filtered through a 0.45 μm-pore-size filter. Proteins in the sample were precipitated by addition of 0.05 ml of 10% trichloroacetic

acid. The supernatant obtained after centrifugation was lyophilised again. The Glu content in the sample was then estimated, after precolumn derivatization with phenylisothiocyanate, by reversed-phase high-performance liquid chromatography with a Waters PicoTag column for free amino acids (Millipore Corp.) and detection of absorbance at 254 nm as described in the manufacturer's instructions. As standards for quantitation, 250 pmol of Glu were injected after derivatization into the column.

CHAPTER 3

ArgP purification and studies of its interaction with co-effectors

3.1 Introduction:

As described in Chapter 1, previous studies have established, based on in vivo genetic evidence that ArgP regulates *argO* expression at the transcriptional level. Arginine (Arg) addition enhances the transcription of *argO* and lysine (Lys) causes it to be shut off, and both the effects are ArgP-mediated (Nandineni and Gowrishankar 2004). However it was not clear if the effects of these amino acids and that of ArgP itself on *argO* regulation are direct or indirect. Thus it was also not understood if these basic amino acids (Arg and Lys) were co-effector molecules of ArgP protein in regulating *argO* transcription. Accordingly, to study this, the binding characteristics of Arg and Lys to ArgP protein was investigated in vitro. For this purpose, the ArgP protein [with a C-terminal hexa-histidine (His_6) tag] was purified and studied some of its biochemical properties and undertook ArgP co-effectors interaction studies. This chapter describes the cloning, expression and purification of His_6 tagged ArgP for further in vitro experiments, and presents evidence for the direct interaction of co-effectors Arg and Lys with ArgP in a mutually exclusive manner.

3.2 Results:

3.2.1 Cloning, functional complementation and purification of His_6 tagged ArgP:

3.2.1.1 Cloning of *argP:*

To purify ArgP protein, the *argP* gene was amplified from the plasmid pHYD915 by PCR using JGARGP1r (5′-AGCAGACAACA*CATATG*AAACGCCCGGA-3′) as

forward primer and JGARGP3r (5′-ATTATTTGAT*CTCGAG*ATCCTGACGAAG-3′) as reverse primer. The plasmid pHYD915 is derived from a low copy number cloning vector pCL1920 and contains a 1.86 kb *SalI* fragment from the *E. coli* chromosome which carries the *argP* gene along with its promoter (Nandineni and Gowrishankar 2004). In order to enable easy and directional insertion in the pET21b vector, restriction enzyme sites were incorporated into the primers. *NdeI* and the *XhoI* sites were respectively introduced in the forward and the reverse primers which are shown in the primer sequences in italics, and the mismatched bases so created when compared with the *argP* sequence are underlined. The ATG sequence of the forward primer borne *NdeI* site corresponds to the initiation codon of ArgP, and the CGA sequence of the reverse primer borne *XhoI* site destroys the termination codon of ArgP. The 920-bp *argP* amplicon encoding the full length ArgP protein was then digested with *NdeI* and *XhoI* and cloned into the respective enzyme sites in plasmid pET21b [a medium copy number ColE1 based vector, with a strong T7 promoter and an efficient ribosome-binding site lying upstream of the *NdeI* site in the multi cloning site (MCS) region] (Novagen, EMD Biosciences), and the resulting plasmid was designated as pHYD1705. The *argP* sequence in plasmid pHYD1705 was verified by DNA sequencing. Since the stop codon of *argP* was abolished by incorporating *XhoI* restriction enzyme site in the reverse primer the reading frame of the gene has been extended in the plasmid pHYD1705 to include, and to terminate just beyond the His_6-codons situated after the MCS region of the vector. The incorporated His_6-tag at the C-terminal end of the protein enabled ArgP purification with the aid of Ni-NTA affinity chromatography. The construction of plasmid pHYD1705 is described in Fig. 3.1.

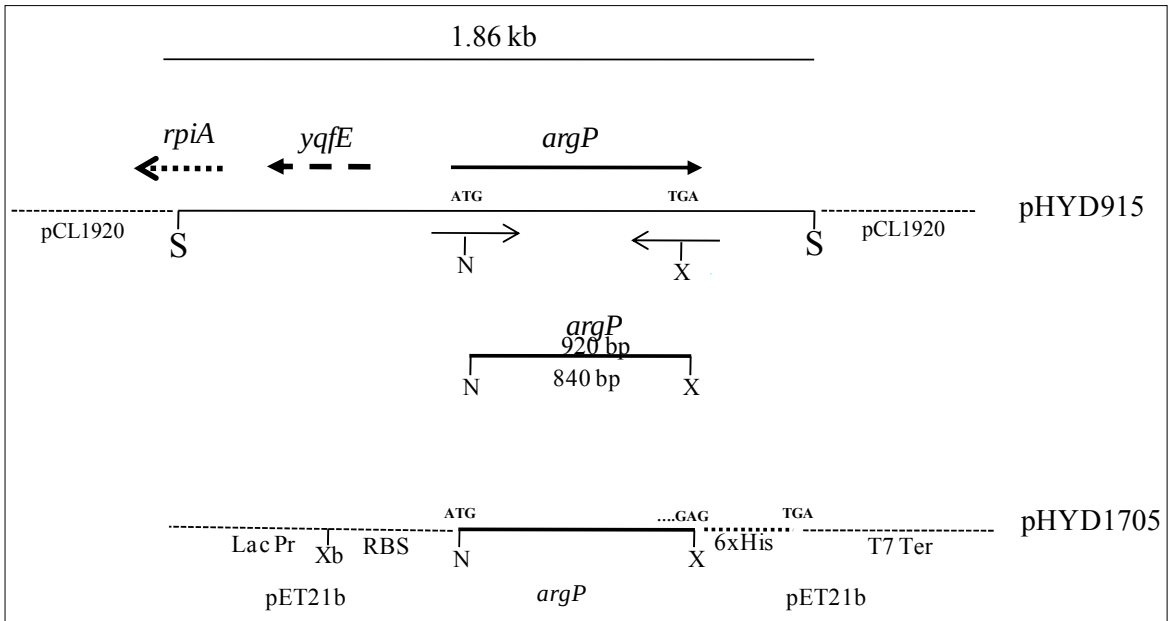


Figure 3.1: Cloning of *argP*$^+$ gene into plasmid pET21b from pHYD915 to construct pHYD1705. *argP*$^+$ gene was PCR amplified from the 1.86 kb long *SalI* fragment of *E. coli* chromosome carried on pHYD915 plasmid, using the primers shown in the Figure (not to scale) and the complete *argP* ORF was cloned in the *NdeI* and *XhoI* sites of pET21b vector. The position of various genes in the *SalI* fragment of pHYD915 is depicted in the Figure. The position of *argP* gene in pHYD1705 that is, after cloning into pET21b vector is also indicated below. N-*NdeI*, X-*XhoI*, Xb-*XbaI*, S-*SalI*, Pr-Promoter, Ter-Terminator and RBS-Ribosome Binding Site.

3.2.1.2 Functional complementation study of pHYD1705 encoding His_6-ArgP:

The His_6 tagged ArgP construct was checked for functional complementation with the test for canavanine (CAN) tolerance (Nandineni and Gowrishankar 2004) in *argP* null strain (GJ4892) in presence of 40 µg/ml CAN. In order to test this, plasmid pHYD1705 was transformed into strain GJ4892 and the ampicillin (Amp) resistant colonies were streaked on 0.2% glucose minimal A agar plates containing 40 µg/ml CAN along with MC4100 (*argP*$^+$) harbouring pET21b (positive control) and GJ4892 transformed with

pET21b (negative control). The pHYD1705 transformants of GJ4892 grew as well as MC4100 indicating that the plasmid is able to complement for ArgP function. This result also established that the His_6-tag does not affect the function of ArgP protein.

3.2.1.3 Over-expression and purification of ArgP:

Strain *E. coli* BL21-SI (Donahue and Bebee 2000, Bhandari and Gowrishankar 1997) was used for the salt-induced over expression of the His_6-ArgP protein from plasmid pHYD1705. BL21-SI is a derivative of GJ1158 (Bhandari and Gowrishankar 1997), which carries a single chromosomally integrated copy of the gene for phage T7 RNA polymerase (RNAP) under the transcriptional control of the cis-regulatory elements of the osmoresponsive *proU* operon. High osmolarity (e.g. 0.3 M NaCl) induces expression of T7 RNAP from *proU* promoter, which in turn is able to drive high level expression of a target gene cloned downstream of a T7 promoter on a plasmid (such as the *argP* gene in pHYD1705). Strain GJ1158 is in turn a derivative of BL21(DE3) in which the T7 RNAP gene is under the control of the *lac* repressor-operator system that is induced by isopropyl thio-β-D-galactoside (IPTG) (Studier and Moffatt, 1986). To overexpress ArgP, the His_6-ArgP protein, pHYD1705 was transformed into BL21-SI and an ampicilin resistant (Amp^r) colony was inoculated in LBON medium (that is, LB medium with NaCl omitted) supplemented with 20 μg/ml Amp. The overnight grown primary culture was sub-cultured in 1:100 dilution and at an A_{600} of around 0.5, was induced with 0.3 M NaCl and 1 mM isopropyl thio-β-D-galactoside. IPTG was also added for induction since the plasmid vector pET21b from which pHYD1705 was derived carries *lac* repressor gene and the *lac* operator overlapping the T7 promoter in order to reduce the basal level expression of the target gene. The culture was harvested

3 hours after the induction and processed for preparation of His_6-ArgP protein as described below.

To purify the His_6-ArgP protein, the harvested cells of BL21-SI/pHYD1705 following NaCl induction were lysed, and the lysate was passed through a Ni-NTA affinity chromatography column in which a His tagged protein is expected to be retained by non-covalent interaction (chelation) between Ni^{2+} in the column and His residues of the tagged protein. The protein(s) so immobilized were then subjected to extensive washing with washing buffer containing 20 mM imidazole (to avoid non-specific retentions), and then eluted from the column with buffer containing 250 mM imidazole (which competes with His for binding to Ni^{2+}). Figure 3.2 shows the over-expression profile and the purified fractions of ArgP as determined by SDS-PAGE analysis.

3.2.2 Evidence that ArgP is dimer in solution:

It has been shown earlier that IciA (synonymous with ArgP) exists as dimer in solution (Hwang and Kornberg 1990; Hwang et al. 1992; Azam and Ishihama 1999). To test the oligomerisation status of the purified His_6-ArgP protein (whose predicted monomer size is around 33.4 KDa) exists as dimer, glutaraldehyde crosslinking and gel filtration experiments were performed. In the crosslinking experiment, the His_6-ArgP was treated with glutaraldehyde (12 mM) for various time points (increasing from 0 to 10 min) and then analysed by non denaturing polyacrylamide gel electrophoresis. For the gel filtration experiment, around 25 μM of His_6-ArgP protein (monomer concentration) was run on a Superose 12 size exclusion chromatographic FPLC column with running buffer as described in Materials and Methods. A single peak in the gel filtration

chromatogram, corresponding to around 70 KDa when compared with the profiles of standards (Fig. 3.3*A*), and a band corresponding to the size of dimer in the crosslinking experiment (Fig. 3.3*B*), confirmed that His_6-ArgP exists as dimer in solution. Furthermore, the results indicated that the dimeric status of ArgP is unaffected by the presence of the co-effectors Arg or Lys.

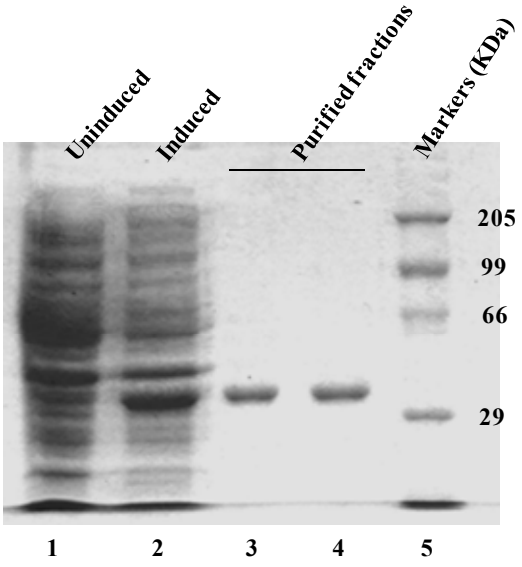


Figure 3.2: Overexpression and purification of His_6-ArgP, demonstrated by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis. Cells were induced at A_{600} of around 0.5 and they were lysed using lysis buffer containing SDS and dithiothreitol and finally boiled before loading. Lanes 1 and 2 represent, respectively, extracts of uninduced and induced cultures of the strain carrying the plasmid construct for His_6-ArgP overexpression. Lanes 3 and 4 represent purified His_6-ArgP fractions obtained by elution of proteins bound to Ni-NTA column with 250 mM imidazole. The position of the migration of His6-ArgP protein is indicated by the arrow. Lane 5, protein molecular-weight markers of sizes in kilodaltons (KDa) as indicated alongside.

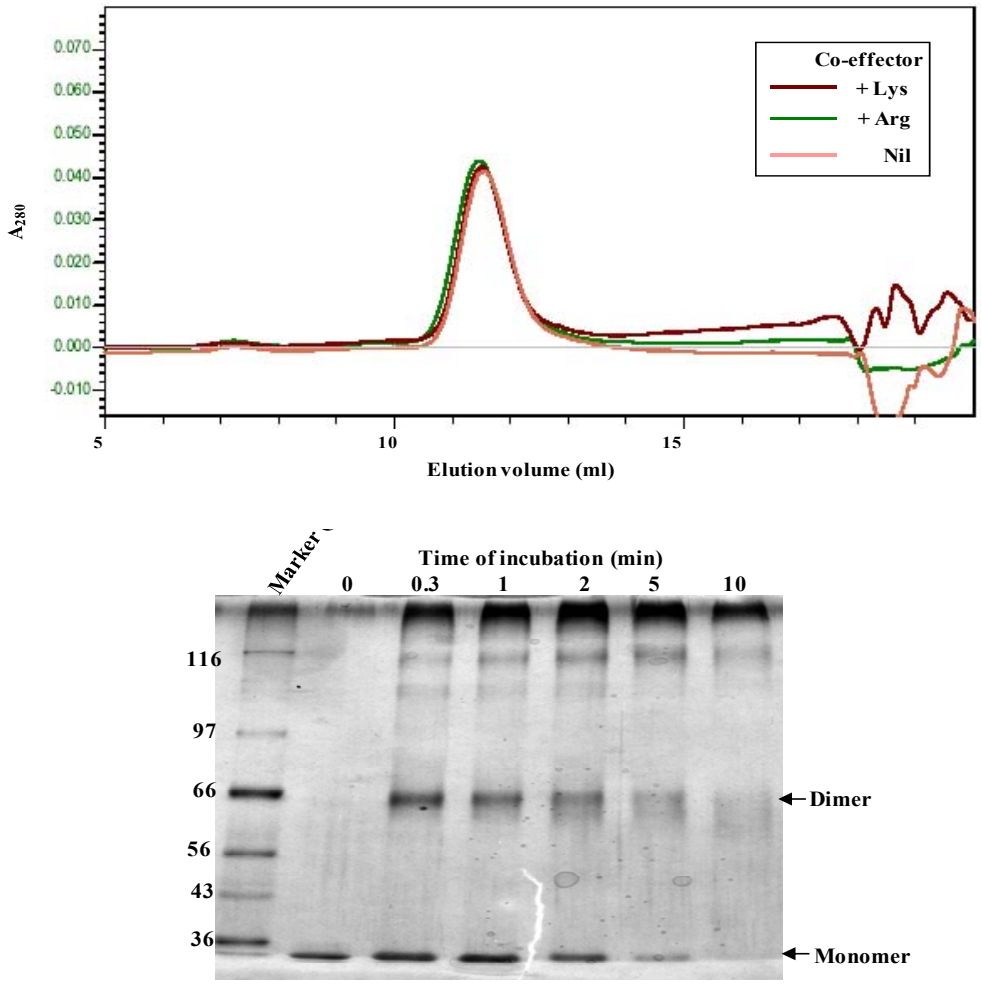


Figure 3.3. Oligomer state of ArgP. (*A*) Gel-filtration chromatography of ArgP in absence or presence of co-effectors Arg or Lys through Superose 12 (10/30) FPLC column (Amersham Biosciences) performed at 4°C with 20 mM Tris-Cl (pH8), 100 mM NaCl as running buffer. Protein was estimated by measurement of A_{280} in the eluate fractions. The size of the protein in each of the superimposed elution peaks was calculated to be approximately 70 kilodaltons (KDa), based on elution volumes of other known proteins as standards (data not shown). (*B*) Sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis (with silver staining) of ArgP following incubation with 12 mM glutaraldehyde in a 20-µl volume of running buffer for various times as indicated. Molecular-weight markers [of indicated sizes] are on lane at left. Positions of migration of proteins corresponding to ArgP monomer and ArgP dimer are marked.

3.2.3 ArgP interaction with co-effectors Arg or Lys :

Using the purified His_6-ArgP protein the in vitro binding of Arg and Lys to the protein were studied. To address this question, fluorescence and circular dichroism (CD) spectra of ArgP were measured in presence and absence of Arg or Lys .

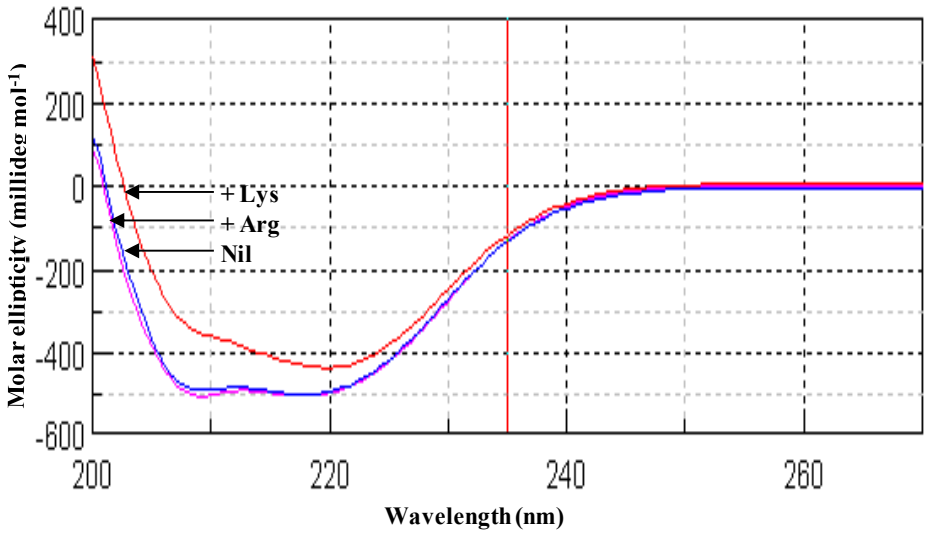


Figure 3.4. Circular-dichroism spectrum of ArgP in absence (Nil) or presence of co-effectors Arg or Lys. 5 mM of ArgP in presence and absence of 10 mM Arg or Lys in Tris-HCl buffer, pH 7.0 was used for CD spectral measurement in a Jasco Spectropolarimeter. Each spectrum was average of 3 scans from 200 nm to 270 nm in 0.5 nm steps with a band width of 1 nm and signal averaging time of 3 s and molar ellipticity was plotted as a function of wavelength. Respective spectra in presence and absence of Arg or Lys are marked in the Figure.

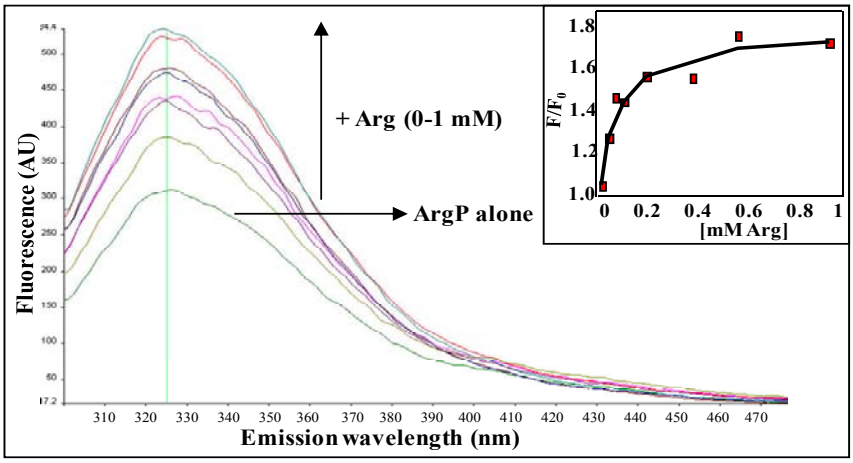
3.3.3.1 Circular dichroism (CD) spectra of ArgP in presence and absence of co-effectors:

To study the secondary structural content of ArgP and the changes, if any, that occur in the presence of co-effectors Arg or Lys, the CD spectra of purified His_6-ArgP protein with and without Arg or Lys were measured. The secondary structural content was calculated using K2D software available online (www. Embl-heidelberg.de/~andrade/k2d/). Around 5 mM of protein was used for each measurement and Arg or Lys was used at a concentration of 10 mM. The CD spectrum confirmed that more than 70% of secondary structure in ArgP is that of α-helix with signature peaks at 208 and 222 nm. There was no change in the spectrum in presence of Arg. However in presence of Lys, a slight upward shift in the spectrum indicative of the reduction in helical content (~10%) was observed (Fig. 3.4).

3.2.3.2 Fluorescence spectra of ArgP in presence and absence of Arg or Lys:

ArgP contains three tryptophan (Trp) residues in its primary structure. Fluorescence spectral changes were monitored to give a possible measure of alterations in the Trp environment of ArgP upon addition of Arg or Lys. Fluorescence emission spectra were recorded by exciting the protein at 295 nm and recording the emission spectrum in the range of 310 to 500 nm. Around 1 μM of protein was used for each measurement. The emission λ_{max} was found to be around 335 nm. Increase in fluorescence intensity (hyperchromicity) was observed on addition of increasing concentrations of Arg (Fig. 3.5*A*) or Lys (Fig. 3.5*B*). These results suggest that the Trp residues in ArgP were becoming more buried upon addition of Arg or Lys. Hence, this is one line of suggestive evidence that these co-effectors directly interact with ArgP protein.

A



B

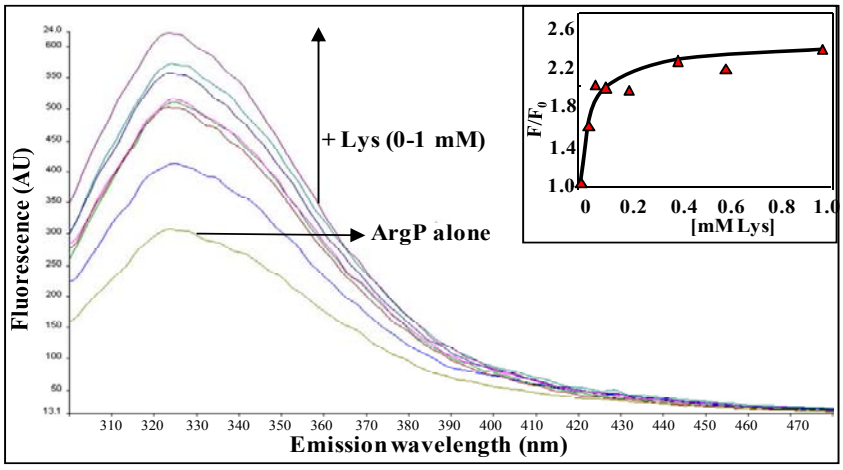


Figure 3.5: Fluorescence spectra of ArgP. Fluorescence emission spectrum of ArgP in presence of increasing concentrations of Arg (*A*) or Lys (*B*). The excitation wavelength was 295 nm where only Trp residue will absorb. The emission spectra were recorded at the range of 310-500 nm at a scan speed of 50 nm/s in a Perkin Elmer LS-3B Spectrofluoremeter. Protein used was at a concentration of 1 μM and increasing Arg *(A)* or Lys *(B)* concentrations (from 0 to 1 mM) as indicated in the figure. Hyperchromic shift in both the cases indicate the binding of either Arg or Lys to ArgP. The corresponding insets depict the curves of relative increase in fluorescence intensity F/F_0 (that is, ratio of peak fluorescence intensity with co-effector to the intensity in absence of co-effector) as a function of the co-effector concentration.

From the hyperchromicity curves, the binding affinities of the co-effectors to His_6-ArgP were also sought to be determined. For this the ratio F/F_0 was calculated for any given concentration of co-effector added, where F is the peak fluorescence intensity of ArgP at λ_{max} in presence of Co-effector (Arg or Lys) and F_0 is the peak fluorescence intensity of ArgP without any co-effector. The plot of F/F_0 as a function of co-effector concentrations are depicted in the insets of Fig. 3.5*A* and Fig. 3.5*B*. The curves were fitted using Boltzman Fit, which shows hyperbolic curves for both the co-effector additions. The analysis of the curves was achieved by linear least square analysis and K_d was calculated from the curves.

These data suggest that there is non-cooperative binding (or one to one binding) of Lys to ArgP and of Arg to ArgP. When the fluorescence spectrum of ArgP was measured with addition of non specific amino acids such as alanine (Ala) or histidine (His), no significant changes in the fluorimetric properties of ArgP were observed (data not shown). This indicates that the interaction of ArgP with Arg or Lys is specific.

From each inset curve in the Fig 5*A* and *B*, binding constant (K_d) was calculated as that concentration of co-effector at which the protein exhibits half-maximal F/F_0. The dissociation constant was calculated to be 150 μM for Arg to ArgP and 70 μM for Lys binding to ArgP (Fig. 3.5*A* and *B*). These data also suggest slight preference for binding of Lys over Arg to ArgP.

3.2.4 Evidence to show that Arg and Lys compete with each other for binding to ArgP:

The above experiments suggested that ArgP interacts directly with both Arg and Lys with somewhat higher affinity for Lys than for Arg. To confirm that the two amino

acids and to test further whether Arg and Lys compete for such binding or whether they share different binding sites on the protein, gel filtration experiments were performed after mixing ArgP with ^{14}C-labelled Arg or Lys as follows. The ArgP protein was identified in the elution fractions by measuring fluorescence at an emission wavelength of 335 nm following excitation at 290 nm and the radioactivity by scintillation counting.

When the experiment was performed with ArgP and either of the labelled amino acids, two distinct peaks of elution were seen from the radiocounting as depicted in Figure 3.6 (panels *D* and *E*). The first peak corresponds to the ArgP eluate fraction (in the void volume) which was concord with the elution peak of pure ArgP obtained from the fluorescence intensity reading (Fig. 3.6*A*) while the other peak corresponds to the elution peak for free (unbound) amino acid (shown in Fig. 7*B* and *C,* for ^{14}C-Arg and ^{14}C-Lys controls, respectively). These peaks are therefore taken to represent respectively, the ArgP bound state of co-effector, and the free co-effector (Fig. 3.6, compare positions of twin peaks in panels *D* and *E* with that of each of the single peaks of panels *A*-*C*).

Subsequently, reciprocal competition experiments were also performed to test whether binding of ^{14}C-Arg (or Lys) to ArgP could be competed by addition of 100 fold excess of unlabelled Lys (or Arg). In either case the ArgP bound fraction of labelled amino acid was markedly reduced or absent and almost the entire label was eluted in the fraction representing free amino acid (Fig. 3.6, panels *F* and *G*, for both ^{14}C-Arg and ^{14}C-Lys, respectively). Binding of the labelled amino acid to ArgP was also competed by 100-fold excess of unlabelled CAN (Fig. 3.6*I*), but not by a non-specific amino acid such as Ala (Fig. 3.6*H*). These results showed that the interaction of Lys and Arg with ArgP is specific and mutually exclusive, suggesting that they bind ArgP at a single site.

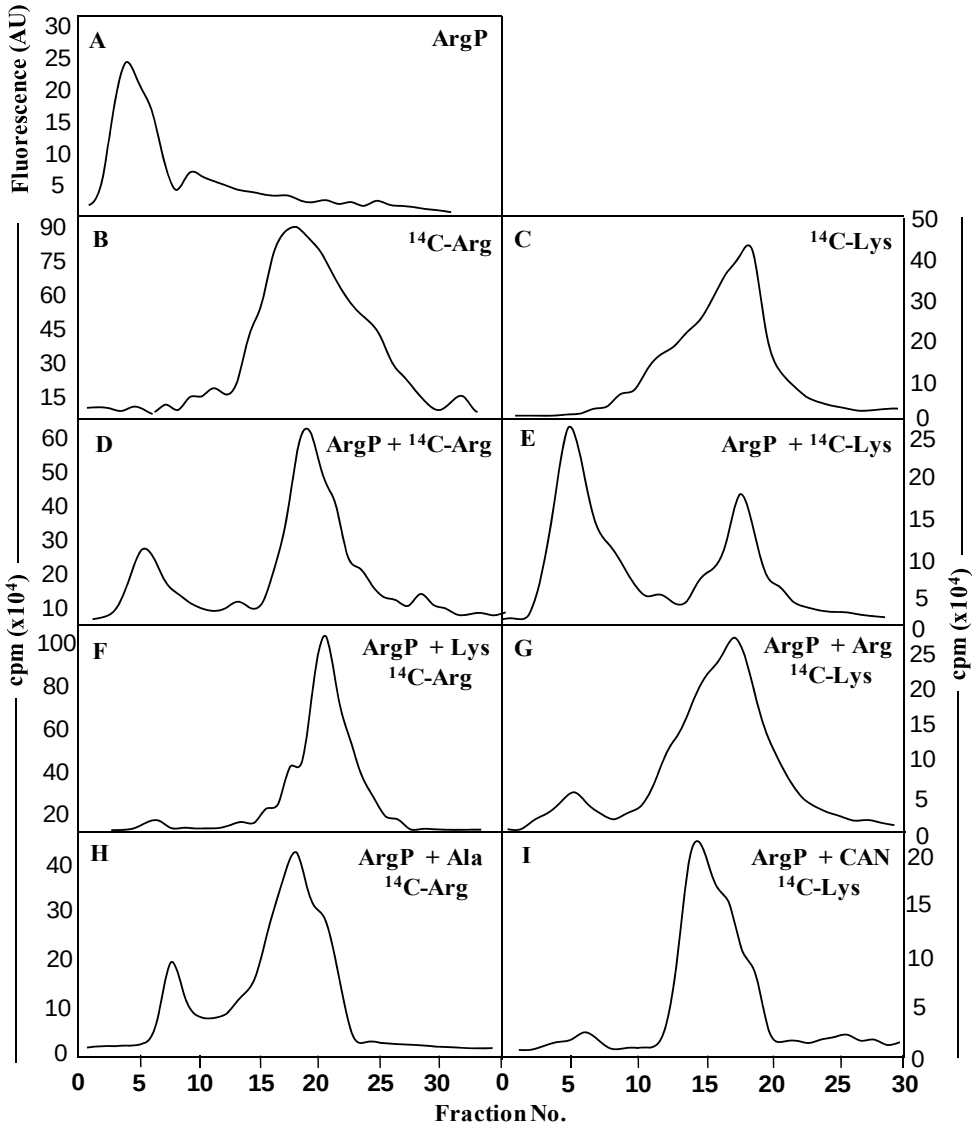


Figure 3.6: Competitive binding of co-effectors to ArgP. Indicated in each chromatogram panel is the material (protein, labelled amino acid, and/or unlabelled competitor amino acid in 100-fold excess) that was loaded on the column. The left panel is with ^{14}C-Arg and right panel is with ^{14}C-Lys. Panel *A* represents the plot of fluorescence intensity (in arbitrary units, AU) in the various fractions; all other panels represent plots of radioactivity measurements (in counts per min, cpm) in the fractions. Panel B and C are free amino acids without ArgP. Panel D and E, is ArgP with Arg or Lys where two peaks one corresponding to A and the other to panel B and C are seen. F and G, competition with heterologous unlabelled amino acid and panel, H is competition with non specific amino acid, Ala and I, with Arg analogue, CAN.

3.2.5 Co-effector binding and general characteristics of ArgP:

In order to study if the interaction of ArgP with its co-effector Arg or Lys causes alterations in the properties of ArgP, some of the basic properties of ArgP such as oligomerisation status and biophysical properties like free energy change of ArgP on Arg or Lys addition was investigated.

3.2.5.1 Biophysical properties of ArgP and the co-effector binding:

To further study the effect of co-effector binding on the biophysical properties of ArgP, the thermodynamic parameters like free energy change (ΔG_D), melting concentration (C_m) and stability were measured by urea denaturation study. The His_6-ArgP protein was denatured in presence or absence of Arg or Lys with increasing concentration of urea from 0 to 8 M and the unfolding was measured from the fluorescence spectral changes of ArgP at each concentration of urea. For this purpose ArgP was used at 1 μM and Arg or Lys at 0.1 mM for each reaction. The protein was excited at 295 nm and the fluorescence emission spectrum was recorded from 310 nm to 500 nm as described in the Section 3.2.3.2. A decrease in fluorescence intensity (hypochromic shift) was observed with increasing concentration of urea, indicative of the degree of unfolding with urea addition; and at around 8 M urea the spectral line comes almost the baseline, suggesting that the protein has been fully denatured. A change in the λ_{max} towards longer wavelength region (blue shift) was also observed, which too is a characteristic of protein unfolding leading to exposure of Trp residues from the buried hydrophobic environment of protein interior to the hydrophilic water environment.

When the Trp intrinsic fluorescence is measured versus denaturant concentration, the information on protein conformational changes can be obtained from shifts in either

peak wavelength or intensity (D'Alfonso et al. 2002). Here the λ_{max} (peak wavelength) value was plotted as a function of increasing urea concentration. The peak wavelength (reported in Fig. 3.7), shifts from the native state value of 335 nm to denatured solvent exposed state value of 355 nm. The analysis was done using conventional linear least square analysis to fit the data and to calculate the various thermodynamic parameters (Ahmad 1984; Ahmad and Bigelow 1982; Rajendrakumar et al. 2005). The transition curves are shown in Figure 3.7. In presence of co-effector Arg or Lys, compared to that without addition of co-effectors there was little or no change in the unfolding transitions from native to denatured state induced by urea. The transition data were converted into stability parameters like standard Gibbs free energy change (ΔG_D^0) as described in (Ahmad 1984; Ahmad and Bigelow 1982) using the following equation;

$$\Delta G_D = \Delta G_D^0 - m_d[\text{urea}] \qquad \ldots\ldots\ldots\ldots\ldots\ldots \textit{(1)}$$

Where ΔG_D (free energy change) is obtained from the relation,

$$\Delta G_D = -RT \ln \{ (y - y_N) / (y_D - y) \} \ldots\ldots\ldots\ldots\ldots\ldots \textit{(2)}$$

where y is the observed optical property (here, the λ_{max} value observed in presence of denaturant urea) and y_N and y_D are, respectively, the properties of the native and denatured protein molecules (here the λ_{max} values of native state and the denatured state of ArgP) of under the same experimental condition in which y has been determined; m_d, a constant given by the derivative, $d(\Delta G_D) / d[\text{denaturant}]$. The ΔG_D^0 values calculated are given in the Table 3.1. The results indicate a standard free energy change value (ΔG_D^0) for ArgP of 5.35 kcal mol^{-1}. In presence of Arg there is little change in ΔG_D^0 value whereas Lys leads to decrease, albeit insignificant, in ΔG_D^0 (from 5.3 to 4.7 kcal mol^{-1}). In all cases there was no change in the C_m value (melting concentration). This result suggests that the addition of Lys or Arg addition does not affect stability and other thermodynamic parameters of ArgP protein.

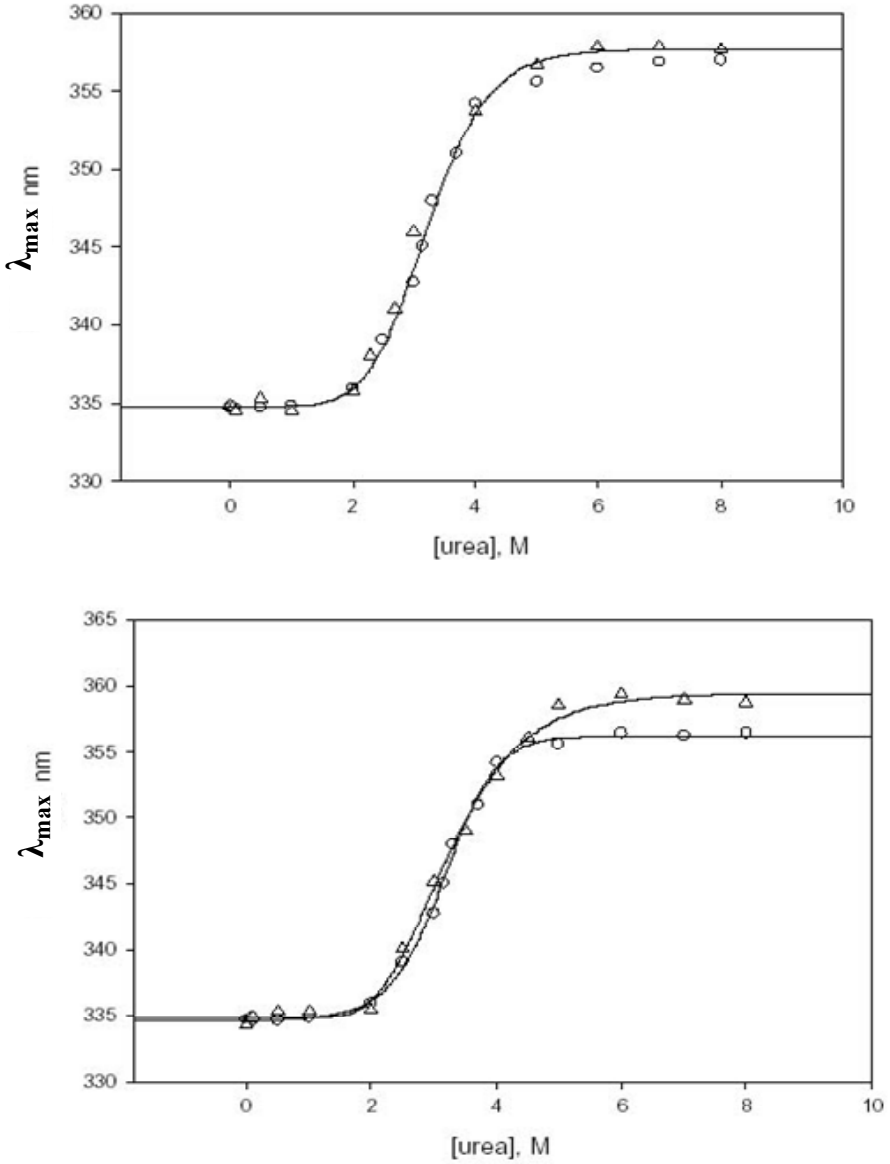


Figure 3.7: Transition (denaturation) curve for ArgP with and without Arg (*A*) and with and without Lys (*B*). Symbols O and Δ, represents respectively the data for ArgP alone and for ArgP in presence of Arg/Lys. The transition data for native to unfolded state was derived from fluorescence spectra of ArgP in presence and absence of Arg or Lys with increasing concentration of urea (from 0 to 8 M).

Table 3.1 Parameters characterizing the urea denaturation of ArgP in presence and absence of Arg and Lys.

The constant $m_{d,}$ in the equation 1 above is given by the derivative d (ΔG_D) / d [denaturant], and are obtained from the plots of ΔG_D versus [denaturant]. Cm, is the molar concentration of urea at which $\Delta G_D = 0$; and ΔG_D^0 is the extrapolated value of ΔG_D at [denaturant] = 0.

Sample	C_m (Molar Concentration)	ΔG_D^0 (kcal mol^{-1})
ArgP	3.0	5.35
ArgP + Arg	3.1	5.53
ArgP + Lys	2.9	4.70

3.3 Discussion:

In this study, the *E. coli* ArgP was cloned and over-expressed such as to obtain a purified preparation of ArgP with a His_6 tag at C-terminal end. It was shown that the six extra His residues at the C-terminal end does not affect the functional properties of ArgP and the construct encoding the His_6-ArgP product was able to complement the CAN^{SS} phenotype of an *argP* null strain. As reported earlier in the literature this study has also shown that the purified ArgP exist as dimer in solution; the addition of co-effectors Arg or Lys had no effect on the oligomeric state of the protein. Both the two co-effectors bind directly and specifically to ArgP; however they compete with each other for ArgP binding suggesting perhaps that they share a single binding site in ArgP. The data also suggest that Lys is preferred over Arg for binding to ArgP.

Despite the finding that Arg and Lys bind ArgP directly, isothermal titration calorimetry (ITC) experiment failed to detect the binding (data not shown). Although

the reasons for this falure of detection are not known, one possibility is that whereas ITC measures the change in enthalpy (heat change associated with the binding of ligand to a protein), the interaction of Arg or Lys with ArgP protein may be entropy driven so that it is associated with very little enthalpy change which does not surpass the non-specific heat change associated with the reaction.

The binding of both the co-effector causes hyperchromic shift in the fluorescence spectrum of ArgP. Lys, but not Arg, binding perturbs the secondary structure of ArgP, causing a slight decrease in its helicity. Finally co-effector binding did not cause any significant biophysical alteration to ArgP as ΔG_D^0 or Cm values remain unchanged upon Arg or Lys binding. It can therefore be concluded that the overall basic properties of ArgP are not drastically affected by co-effector binding.

CHAPTER 4

Studies of ArgP interaction with argO regulatory DNA

4.1 Introduction:

The studies described in Chapter 3 had shown that co-effectors, Arg and Lys directly interact with, and compete with one another for binding to ArgP [a member of the LysR family of regulator proteins (Schell 1993)]. Moreover, previous genetic evidence and in vivo studies had suggested that transcription of *argO* is under the strict control of ArgP, such that its expression is induced by 1 mM Arg as well as by its toxic analog canavanine (CAN, a plant-derived naturally occurring antimetabolite) (Nandineni and Gowrishankar 2004). Furthermore, *argO* expression in vivo is completely shut off upon addition of 1 mM Lys (Nandineni and Gowrishankar 2004). However, no study had been done in vitro, to demonstrate the regulation of *argO* expression by ArgP. Also unknown was the mechanism by which Lys supplementation leads to the shutting down of *argO* transcription, as to the mechanism of possible interplay between co-effectors Arg and Lys with ArgP for the regulation of *argO* transcription and the physiological significance of such regulation.

In other studies, ArgP (IciA) was reported to activate the transcription from *nrdA* (Han et al. 1998) and *dnaA* (Lee et al. 1996, 1997) promoters and also bind to AT-rich 13-mer sequences of *E. coli oriC* (Hwang and Kornberg 1990, 1992). However, no consensus binding sequence for ArgP has been reported. Regarding *argO*, no promoter and regulatory sequences had been annotated at the start of this study.

In the absence of data for consensus ArgP binding sequence, and the annotated regulatory elements of *argO*, it was considered that the first step towards understanding the mechanism of *argO* transcription and to reconstitute it's regulation in vitro will be to investigate the start site of *argO* transcription and its *cis* acting elements. This Chapter describes studies on the mapping of transcription start site and the putative

promoter elements of *argO* and verification of the sequence. Additionally, this Chapter also describes experiments that seek to study the possible binding sequence of ArgP to the regulatory region of *argO*.

4.2 Results

4.2.1 Mapping of *argO* transcription start by primer extension analysis*:*

To investigate the ArgP-mediated transcriptional regulation of *argO*, primer extension analysis on total cellular RNA was first performed to determine the transcription start site of *argO*. In this experiment, an oligonucleotide, JGARGO6r, whose sequence is complementary to the region from +57 to +29 in *argO* sequence (relative to its translation start site) shown in Figure 4.2*B*, 5′-end labelled with γ-^{32}P-ATP and polynucleotide kinase was subjected to a primer extension reaction with reverse transcriptase after its annealing to total RNA prepared from the cultures of derivatives of *argP*$^{+}$, chromosomal *argP* mutant strain GJ4892 transformed either with the *argP*$^{+}$ plasmid pHYD915 or, *argP*^{d} (S94L) plasmid pHYD926. The reaction products were resolved by electrophoresis on a 6 % urea denaturing polyacrylamide gel and visualid by phosphorimaging alongside a sequencing ladder generated from the phage M13 DNA. As depicted in the Figure 4.1, a prominent extension product was visible with RNA preparations from the derivative harboring *argP*^{d}-S94L plasmid pHYD926 (lane 2) whereas such an extension product was very faint or not visible in case of derivative with the *argP*$^{+}$ plasmid pHYD915 (lane 1) in which the activity of *argO* transcription is expected to be much lower. This extension product was therefore inferred to represent the 5′-end of *argO* mRNA, and from the size it was deduced that the start site of *argO* transcription corresponds to the "A" residue situated 28 bases upstream of the predicted translation start-site of the structural gene of *argO*. From the *argO* sequence analysis, the putative promoter elements (–10 and –35 sequences) were then identified.

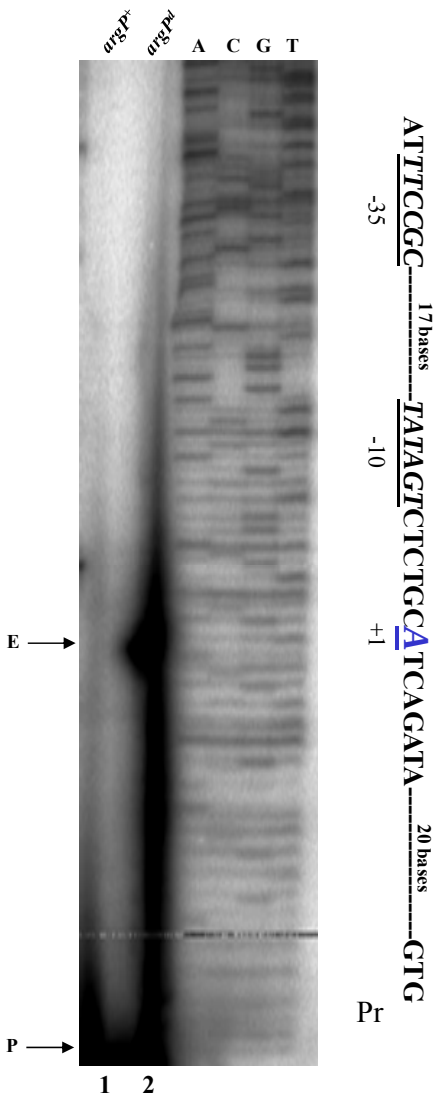
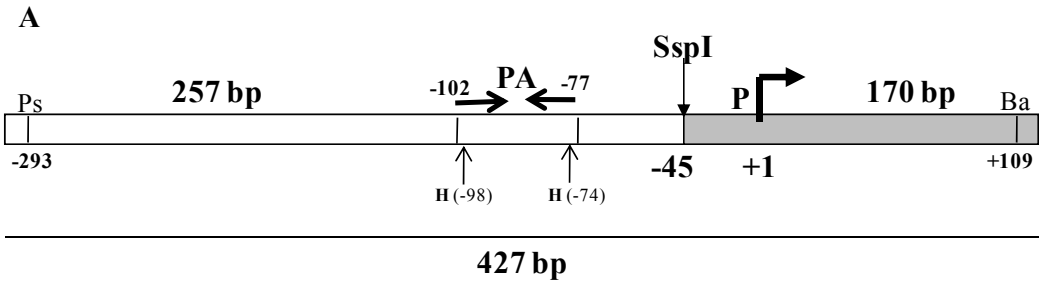


Figure 4.1. Detection of *argO* transcription start-site by primer extension analysis. Primer GJARGO6r was extended from the mRNA isolated from cultures of *argP*$^{+}$ and *argP*^{d} strains grown to mid-exponential phase in 0.2% glucose-minimal A medium. The reaction products were resolved by electrophoresis on a 6% denaturing polyacrylamide gel and visualid by phosphorimaging. P and E represent bands corresponding to labelled primer and extension product, respectively. Lanes A, C, G, T represent a di-deoxy sequence ladder of phage M13, that was employed to determine the size of the prominent primer extension product (denoted by arrow) in the *argP*^{d} strain. The start site (+1), –10, and –35 sequences are indicated in the Figure along with the primer position. Pr- primer.

The existence of additional promoter/transcription start site (if any) was further investigated with similar primer extension reaction using an upstream oligonucleotide (JGARGO7r), which is complementary to a region from −39 to −15 on *argO* template (relative to the translation start site) shown in the Figure 4.2. No extension products were observed from this primer (data not shown), suggesting that *argO* has only one promoter. The +1 transcription start site and the putative promoter sequences are depicted in the Figure 4.1 (also see Fig. 4.2*A* and *B*). In the remainder of this thesis, nucleotide numbering in *argO* has been reported relative to the start-site of transcription identified above (taken as +1).

4.2.2 *argO* regulatory DNA fragment :

A 427-bp fragment, comprising the region of *argO* extending from –293 to +109 as mentioned above, relative to the start-site of transcription, taken as +1 and expected to encompass all the *cis* regulatory sequences was PCR-amplified using, respectively, forward and reverse primers GJARGO8f (5′-GTGCGC*CTGCAG*GAACTTGGTG-3′) and GJARGO9r (5′-GTATGCCC*GGATCC*ATCACAAAA-3′). The primers were so designed as to incorporates *PstI* and *BamHI* sites at the upstream and downstream ends respectively, of the *argO* amplicon (shown in italics in the sequences in parenthesis). As shown in Figure 4.2*A*, this amplicon has one *SspI* site at the position –45 and a nearly perfect palindrome of 26-bp centred around –90 and flanked on either end by *HaeIII* sites. The *SspI* site divides this 427-bp fragment into two halves: a 257-bp upstream fragment and a 170-bp downstream fragment. The 427-bp *argO* fragment was used for various in vitro experiments including electrophoretic mobility shift assay (EMSA), footprinting, enzyme protection, and run off transcription assays.



B

GAACTTGGTGCATCGTCGATTAATTTCGTGGTCCGCGTCTGGAGCAACAGCGGCGATCT
–290 –270 –250

GCAAAACGTGTACTGGGATGTGCTGGAGCGTATTAAACGTGAATTTGATGCCGCCGGTA
–230 –210 –190

TCAGCTTCCCGTACCCGCAAATGGATGTGAACTTTAAGCGGGTGAAAGAAGACAAAGCT
–170 –150 –130

GCGTAATCAACGCTGAGGCCAGATAATACTCTCTGGCCTCTCTCTTATTAGTTTTTCTGA
–110 –90 –70

TTGCCAATTAATATTATCAATTTCCGCTAATAACAAT|CCCGCGATATAGTCTCTGCATCA
–50 –30 –10 +1

*GATACT*TAATTCG*GAATAT*CCAACGTGTTTTCTTATTACTTTCAAGGTCTTGCACTTGGGG
+10 +30 +50

CGGCTATGATCCTACCGCTCGGTCCACAAAATGCTTTTGTGATG
+70 +90 +109

Figure 4.2. A 427-bp amplicon comprising a region of *argO* promoter extending from –293 to +109; (*A*) Diagrammatic representation. The position of *SspI, HaeIII* sites, palindromic sequence, (+1) start site are indicated. PA-Palindrome, Ps-*PstI*, Ba-*BamHI*, P-promoter, H-*HaeIII*. (*B*) Sequence of 402 nucleotides from –293 to +109 of the *argO* regulatory region used in this study, numbered with respect to the A residue (in bold) representing the start-site of transcription (taken as +1). Promoter –35 and –10 motifs, and GTG triplet representing start-site of translation, are overlined. Inverted-repeat sequence is marked by overhead arrows. The ArgP binding region from around –85 to –20 (as described in Section 4.2.5.5 of this Chapter) is underlined and bracketed.

From the 427-bp amplicon, a 402-bp fragment encompassing region of *argO* promoter from –293 to +109 (Fig. 4.2*B*) was obtained after digestion with *PstI* and *BamHI* and was cloned in the *PstI* and *BamHI* sites of plasmid vectors pCU22 and pMU575 as described in Materials and Methods. The resultant plasmids were designated as

pHYD1722 and pHYD1723 respectively. Plasmid pCU22 (Ueguchi and Mizuno 1993) is a derivative of pUC19 that is useful for preparing supercoiled DNA template for in vitro transcription reactions. This plasmid has strong tandem phage *fd* transcription terminators flanking the multiple-cloning-site (MCS) to ensure both that transcripts from the cloned promoter region in the MCS will not extend to vector DNA nor that the transcript originating from vector-borne promoters will interfere with the activity of any promoter cloned in MCS. Plasmid pMU575 (Andrews et al. 1991) is IncW-based, single-copy-number promoter cloning vector in whose MCS is upstream of a promoterless *lacZ* gene allowing the in vivo determination of the strength and the regulation of any promoter cloned in the MCS by measuring the β-galactosidase activity in the cultures. Plasmid pHYD1722 (carrying *argO* in pCU22) was used for in vitro transcription as well as for mutagenesis to identify operator and promoter sequences of *argO*, while plasmid pHYD1723 (carrying *argO* in pMU575) was used for in vivo transcriptional reporter gene fusion assays.

In order to test whether the 402-bp fragment of *argO* from –293 to +109 confers similar properties of regulation as that reported earlier for a chromosomal *argO-lac* transcriptional fusion (Nandineni and Gowrishankar, 2004), *lacZ* reporter gene expression assays were done in various strains as described below that had been transformed with plasmid pHYD1723. The strains employed were the *argP* null strain (GJ4892) and its derivatives harbouring either the $argP^+$ plasmid pHYD915 or the $argP^d$-S94L plasmid pHYD926, and β-galactosidase activities were determined in presence and absence of Arg and Lys (Fig. 4.3). Notably, there was a very low level of β-galactosidase expression in *argP* mutant under all conditions (only one is shown in Figure 4.3, with Arg addition), and in the $argP^+$ strain grown in Lys-supplemented medium. Arg or CAN supplementation of the $argP^+$ derivative was associated with a

substantial induction of transcription. The expression of *argO-lac* was also rendered constitutive and independent of Arg or Lys or CAN addition in the derivative carrying the *argP*^{d}-S94L mutation. These data are consistent with the previously reported findings that *argO* is under the transcriptional regulation of ArgP in vivo and, that the addition of Arg activates and that of Lys inhibits the transcription from *argO* promoter. These findings also suggest that the fragment extending from –293 to +109 contains all the *cis* elements needed for *argO* transcriptional regulation.

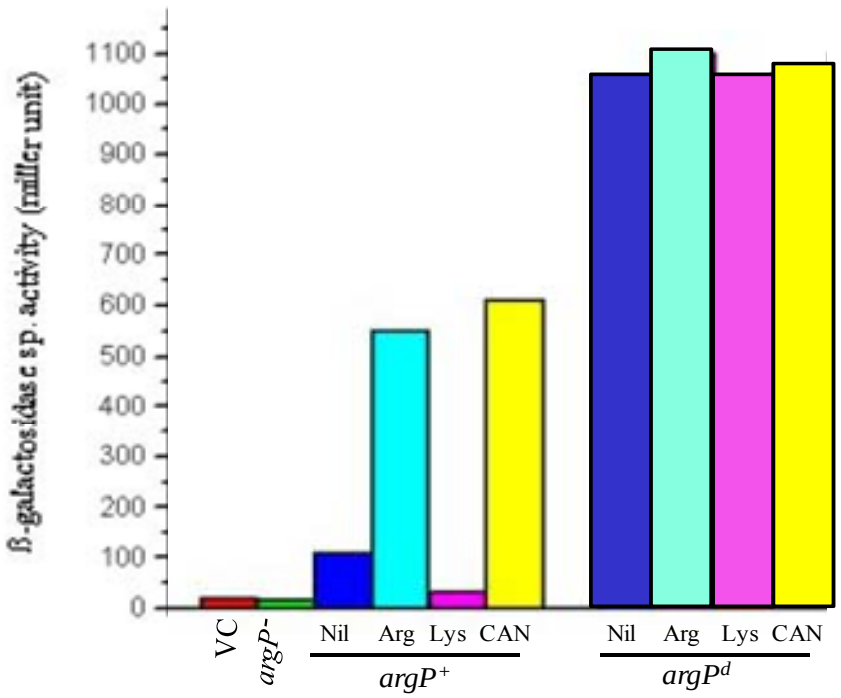


Figure 4.3. In vivo regulation study of *argO:lac* fusion using the *argO* regulatory fragment which extends from –293 to +109 with respect to +1 start site of *argO* transcription. The histograms represent the β-galactosidase specific activity (in Miller units) of the *argO:lacZ* fusion in strains described (legend in Figure). The first bar (VC) from the left represents *argP*$^{+}$ strain with pMU575 (vector control); the 2nd bar represents GJ4892 *(argP*$^{-}$) control in medium with 1 mM Arg. The next four bars represent *argP*$^{+}$ strain (GJ4892 bearing pHYD915) with the supplements as indicated in the Figure. The last four bars represent the *argP*^{d} strain (GJ4892 bearing plasmid pHYD926). Nil, in absence of co-effectors.

4.2.3 Confirmation of putative promoter sequence by site directed mutagenesis:

In the sequence upstream of *argO* transcription start site are two alternative putative −10 hexamer motifs (underlined and italicid, respectively) that are overlapping in the octamer sequence T<u>A*TAGT*</u>*CT* (Fig. 4.4*A*). Each of the motifs have the consensus invariant residues A at position 2 and T at position 6 (the consensus hexamer sequence in bacteria being TATAAT, with more than 90% conservation for the 2nd A and last T) (reviewed in McClure, 1985 and Browning and Busby, 2004). Situated seventeen nucleotides upstream of these putative –10 motifs is a nearly conserved putative −35 hexamer motif TTCCGG (the consensus for which TTGACA, the first three residues TTG are more conserved than the last three across many aligned promoter sequences). To verify the promoter sequence of *argO*, the consensus residues of the two putative –10 hexamer motifs were changed to C's by a site-directed mutagenesis approach. The corresponding nucleotides were modified on plasmid pHYD1722 template with the aid of primers where the modified nucleotides had been incorporated (shown in italics in the primer sequences below) using QuikChange Site Directed Mutagenesis kit (Stratagene) as described in Materials and Methods. Thus, the first putative –10 sequence was changed from TATAGT to *CC*TAGT, (indicated as Pm 1.1, plasmid pHYD1724) with JGYPROf1 (5′-AATCCCGCGATA*CC*TCTTCTGCATCAGATA-3′) as forward and JGYPROr1 (5′-CTGATGCAGAGAC*GG*TATCGCGGGATT-3′) as reverse primers, and to T*C*TAG*C* (indicated as Pm 1.2, plasmid pHYD1725) with primers GJYPROf2 (5′-AATCCCGCGATAT*C*GTC*C*CTGCATCAGATAC-3′) as forward and JGYPROr2 (5′-GTAT CTGATGCAG*G*GAC*G*ATATCGCG-3′) as reverse primers.

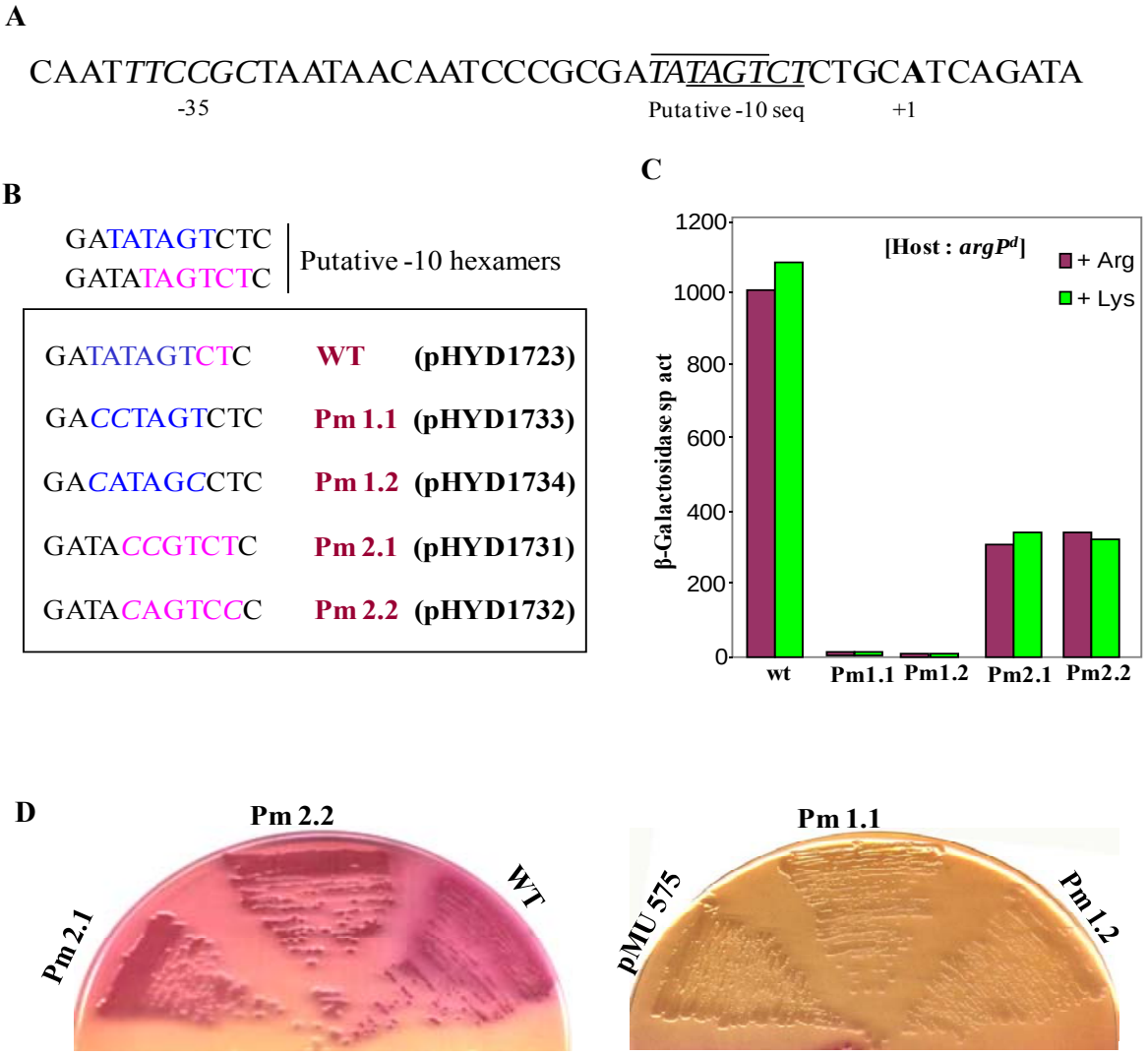


Figure 4.4. Verification of putative –10 hexamer of *argO* promoter. (*A*) *argO* promoter sequence, +1 start site, –10 and −35 hexamers are indicated; (*B*) Putative –10 hexamers of *argO* promoter and the modified sequences by site directed mutagenesis. The modified bases are italicid and the respective plasmid derivatives of pMU575 are also indicated; (*C*) *argO* promoter activity measurement by β-galactosidase assay following their transformation into GJ4892/pHYD926 (that is, with *argP*^{d}-S94L mutation) on the wild-type (WT) and mutant constructs after growth of cultures in 0.2% glucose minimal A medium supplemented with Arg or Lys as indicated. The enzyme specific activity values are in Miller units; (*D*) *argO:lacZ* activity assay with *argO* promoter mutants (as in panel *C*) on MacConkey-lactose agar plates, in comparison with pMU575 and WT control derivatives as indicated.

Similarly, the other putative hexamer sequence was modified from TAGTCT to *CC*GTCT (indicated as Pm 2.1, plasmid pHYD1726) and T*C*GTC*C* (indicated as Pm 2.2, plasmid pHYD1727) with primer pairs GJYPROf3 (5′-ACAATCCCGCGA*CC*TA GTCTCTGCATC-3′)/GJYPROr3 (5′-GATGCAGAGACTA*GG*TCGCGGGATTGT-3′) and GJYPROf4 (5′-ACAATCCCGCGAT*C*TAG*C*CTCTGCATCAGATA-3′) / GJYPROr4 (5′-TATCTGATGCAGAG*G*CTA*G*ATCGCGGGATTGT-3′) respectively.

The fragments with mutations incorporated were then sub-cloned in the *PstI* and *BamHI* sites of pMU575 vector to measure the promoter activity. The resultant plasmids of Pm 1.1 and 1.2 clones were designated, respectively, as pHYD1733 and pHYD1734. Likewise, the plasmid clones of Pm 2.1 and 2.2 were designated as pHYD1731 and pHYD1732, respectively. The list of plasmids and the changed sequence are given in the Figure 4.4*B*.

The plasmids pHYD1731, pHYD1732, pHYD1733 and pHYD1734 were transformed into *argP*$^{-}$ strain (GJ4892) harbouring *argP*-S94L (pHYD926) and the promoter activity was judged by monitoring *lacZ* expression both on the MacConckey agar plates and by by β-galactosidase assay following growth of the derivatives in 0.2% glucose minimal A medium supplemented with either Arg or Lys. Plasmids pMU575- and pHYD1723-transformed derivatives of the above strain were used as negative and positive controls, respectively.

It was observed on MacConkey plates that the transformants of plasmid pHYD1733 (Pm 1.1) and pHYD1734 (Pm 1.2) appeared Lac^{-} which was similar to pMU575 vector transformed control (Fig. 4.4*D* right panel), indicating that the promoter activity of *argO* had been abolished in these mutants. On the other hand, the strains carrying plasmids pHYD1731 (Pm 2.1) and pHYD1732 (Pm 2.2) were Lac^{+} equivalent to pHYD1723 control (Fig. 4.4*D* left panel), indicating that the promoter activity was still

preserved in these mutants. Measurements of β-galactosidase enzyme activity in these derivatives also permitted the similar conclusions. The promoter activity was abolished completely by the mutations of the first putative –10 hexamer (Pm1.1 and Pm 1.2) but not of the second putative hexamer (Pm 2.1 and Pm 2.2) (Fig. 4.4*C*). Thus, between the two alternative overlapping hexamer motifs described above, the former (TATAGT) was inferred to be the authentic –10 promoter element since site-directed alterations of the sequence to TC*TAGCCT* (Pm 1.1) or CC*TAGTCT* (Pm1.2), but not to TA*TCGTCC* (Pm 2.1) or TA*CCGTCT* (Pm 2.2), were associated with complete loss of *argO-lac* expression in the *argP*^{d} strain (Fig. 4.4). Hence the *argO* promoter –10 hexamer element can be represented as TATAGT, which is 17-bp away from –35 hexamer sequence, TTCCGG.

4.2.4 ArgP interaction with *argO*:

4.2.4.1 ArgP binds a 427-bp fragment containing *argO* region from –293 to +109

The simplest explanation to account for in vivo observation on *argO* regulation, is that of direct regulation of the *argO* promoter by ArgP. To examine this possibility, the purified ArgP protein [with C-terminal hexa-histidine (His_6) tag] was tested for its ability to bind DNA of the *argO* regulatory region. For this purpose, an electrophoretic mobility shift assay (EMSA) experiment was undertaken using the 427-bp amplicon described above that comprises the region of *argO* DNA from –293 to +109, the entire cis-regulatory elements of *argO*. The fragment was radiolabelled with ^{32}P-γ-ATP at both the 5´-ends using polynucleotide kinase as described in Chapter 2 Section 2.2.4.6, and it was incubated at 0.5 nM with varying concentrations of purified ArgP (from 0 to 15 nM) in the binding buffer for 30 minutes at room temperature in absence (Fig. 4.5*A*) or presence of Arg (Fig. 4.5*B*) or Lys (Fig. 4.5*C*), before being subjected to

electrophoresis on a 4% non-denaturing poly-acrylamide gel and then be visualid by phosphorimaging.

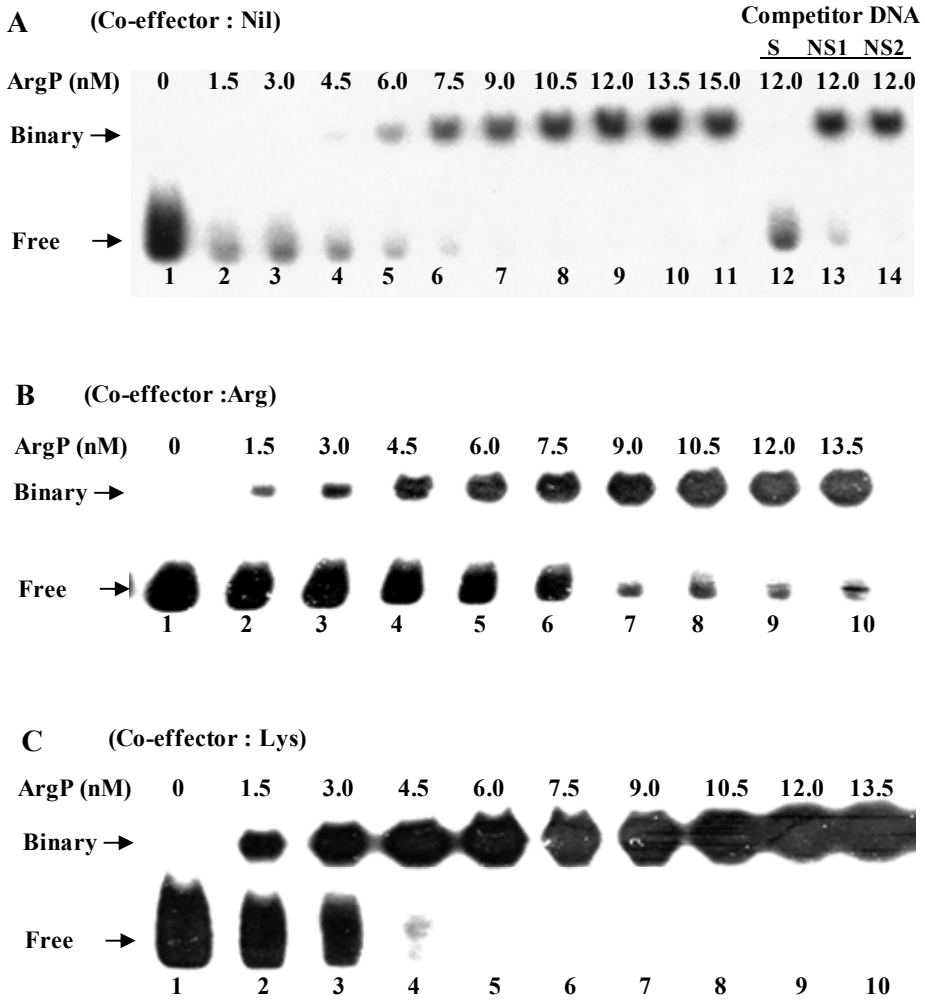


Figure 4.5. Binding of (His_6)-ArgP protein to 427-bp fragment comprising *argO* regulatory sequence extending from –293 to +109-bp by electrophoretic mobility shift assay (EMSA) in absence (*A*) or presence of the co-effectors Arg (*B*) or Lys (*C*). EMSA experiment was carried out with increasing concentrations of ArgP as indicated. Lanes 12 to 14 of panel A also depict results of addition of 100 fold excess of cold competitor DNA, either specific, that is, unlabelled *argO* fragment (S), or two different non-specific DNA fragments (NS1 and NS2), to the EMSA reactions. Free DNA (unbound) and the ArgP-DNA binary complexes are indicated. NS1 and NS2 were, respectively

It was observed that ArgP binds the radiolabelled *argO* fragment in absence of any amino acids and that the addition of either Arg or Lys did not hinder the binding. The results in Fig. 4.5 suggest that ArgP binds the *argO* regulatory DNA in vitro with half maximal binding at around 7 nM ArgP (see also Fig.4.6*A*), which is competed by excess (100 fold) of the cognate unlabelled fragment (lane 12,S) but not so by non-specific DNA fragments (lane 13,NS1 and lane 14,NS2).

4.2.4.2 Binding curves and dissociation constants for ArgP and *argO* interaction in absence and presence of co-effectors:

The distribution of radioactivity in the retarded and free-moving bands of Fig 4.5 was quantitated by densitometry of the autoradiograms, and the "% bound fraction" (which is the fraction of the total radioactivity of *argO* DNA that is shifted upon ArgP binding,) was plotted as a function of the added ArgP concentration (Fig. 4.6*B*). A sigmoidal curve was observed in absence of co-effectors Arg or Lys, suggesting that the binding of ArgP to *argO* DNA is co-operative. In presence of Arg, there was no significant change in the binding characteristics of ArgP to DNA, whereas when Lys was added, the binding curve was hyperbolic. This result suggests that the binding characteristics of ArgP to *argO* changes upon Lys addition from co-operative to non co-operative.

To obtain a quantitative estimate of binding strength of ArgP to *argO* DNA in absence or presence of Arg or Lys, the dissociation constants (K_d) were calculated from the various binding curves obtained in Figure 4.6*B*. K_d values were calculated using the equation,

$$K_d = [\text{ArgP}][\textit{argO}] / [\text{ArgP-}\ \textit{argO}]$$

When [ArgP- *argO*] equals [ArgO] (i.e., the bond fraction is 50%),

$$K_d = [\text{ArgP}]$$

Where, [ArgP] is the free ArgP concentration and is assumed to be equal to total ArgP concentration (i.e., [ArgP]>>[ArgP-*argO*]);

[*argO*] is the concentration of uncomplexed *argO* DNA; and

[ArgP- *argO*] is the concentration of the ArgP- *argO* complex

It was observed that the K_d of ArgP binding to *argO* DNA was 6.45 X 10^{-9} M in absence of Arg or Lys and 6.30 X 10^{-9} M in presence of Arg. Therefore this result again shows that there is no difference in the binding affinity of ArgP and Arg bound ArgP to *argO* DNA, as suggested above. However, in presence of Lys, the K_d was 2.4 X 10^{-9} M which is three fold lower than that in absence of any co-effector. It can be concluded that whereas Arg addition does not cause any significant change in the binding affinity of ArgP to *argO*, Lys-bound ArgP shows a considerably higher affinity of binding to *argO*. These conclusions are qualitatively supported by data from an independent EMSA experiment depicted in Fig 4.7, in which it its seen that whereas 3 nM ArgP is sufficient to cause 100% retardation of *argO* in presence of Lys, only around 20% of *argO* is retarded in presence of Arg or in absence of co-effectors.

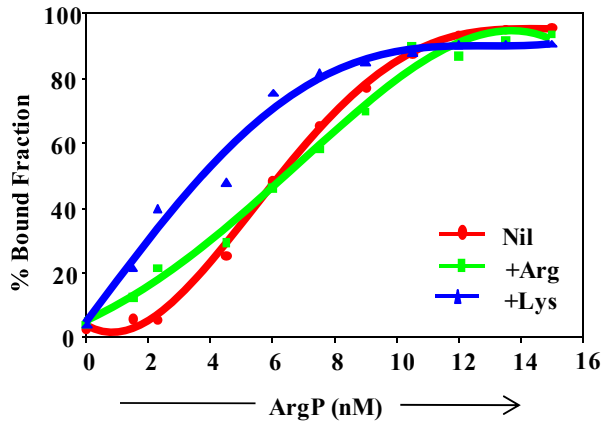


Figure 4.6. Binding curves for ArgP protein binding to *argO* DNA in presence and the absence of co-effectors Arg and Lys. These curves were established from the experiments shown in Fig 4.5. Nil, absence of co-effectors.

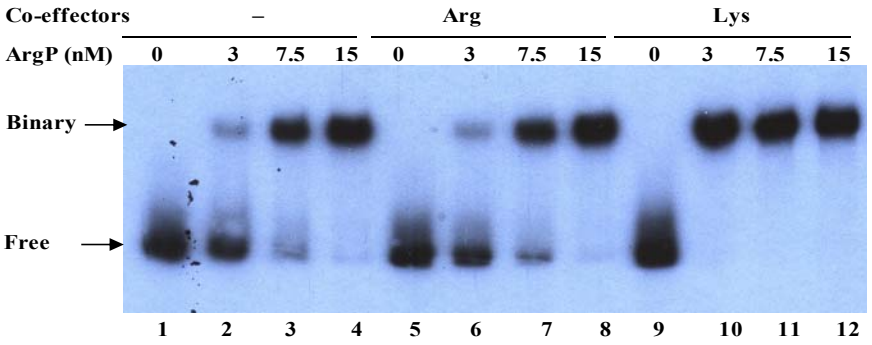


Figure 4.7. EMSA experiment of ArgP with *argO* regulatory DNA in absence and presence of Arg or Lys.

4.2.4.3 Evidence from EMSA experiment that Arg in excess out-competes Lys for ArgP binding:

In presence of equimolar concentrations of Arg and Lys, the transcription from *argO* promoter is predominantly inhibited both in vivo (data not shown) and in vitro (as described in chapter 5). Similarly in EMSA experiments as well when equal concentrations of Arg and Lys (0.1 mM each) were present, the binding affinity of ArgP to *argO* regulatory region was equivalent to that with Lys alone and higher than that either in the presence of Arg alone or absence of any co-effector. (compare Fig. 4.8*A* with Fig. 4.5*A* and *B*; Fig. 4.8*B* lane 2 and lane 4). The nature of binding and the K_d was equivalent to that of Lys addition (compare Fig. 4.8*A* with Fig. 4.5*C*; Fig. 4.8*B* lane 3 and lane 4). This suggests a predominant effect of Lys over Arg for ArgP mediated *argO* regulation.

Subsequently, it was investigated whether an excess of Arg can out-compete Lys as a co-effector molecule of ArgP as assessed by EMSA experiments with *argO* regulatory region. For this purpose, *argO* binding by ArgP (20 nM) was determined in presence of constant concentration of Lys (0.1 mM) and increasing concentrations of Arg (from 0.1 to 2 mM). At low concentration of Arg:Lys (≤ 4:1), the binding pattern was observed to

be similar to that of Lys addition alone, with complete retardation of the *argO* fragment (Fig. 4.8*B*, see lanes 3 to 6). With higher Arg:Lys ratios, there was a steady increase in the unbound *argO* fraction. Although not conclusive, these results suggest that Lys was being significantly competed out by Arg for ArgP binding as assessed by EMSA of *argO* DNA.

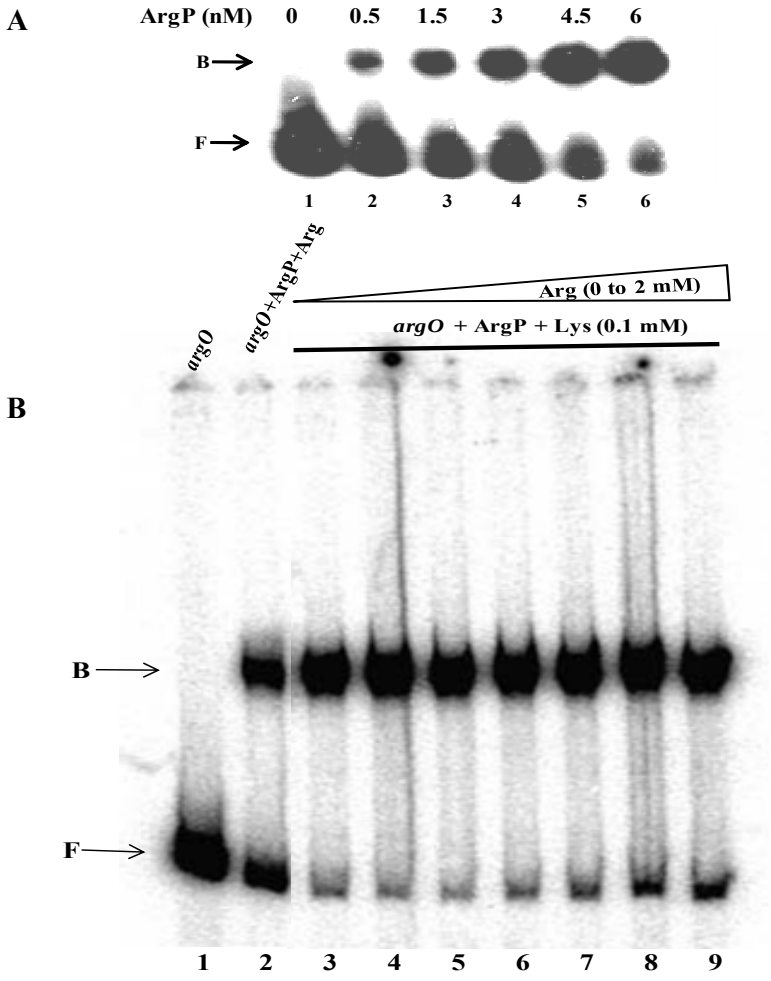


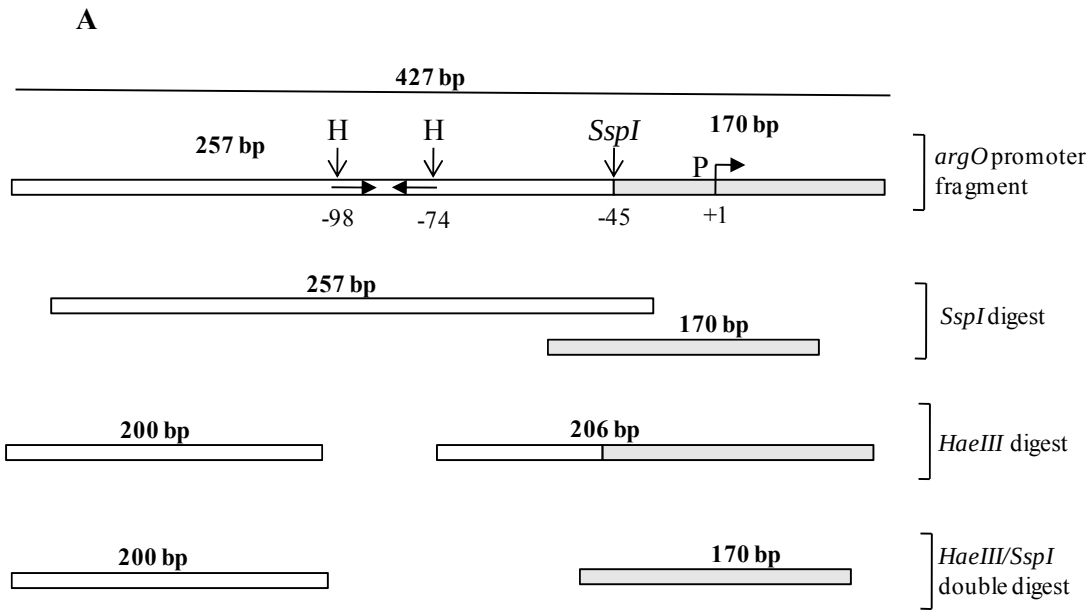
Figure 4.8. EMSA experiments of *argO* regulatory DNA with ArgP in presence of both Arg and Lys. (*A*) ArgP binding to labelled *argO* DNA in presence of equimolar concentrations (0.1 mM) of Arg and Lys with increasing [ArgP]. (*B*) ArgP binding to labelled *argO* DNA in presence of constant Lys concentration (0.1 mM) and increasing concentration of Arg (lanes 2 to 9, with [Arg] at 0, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1.0 mM and 2.0 mM respectively). F-free DNA, B-binary complex.

4.2.5 Identification of regions on the *argO* DNA that interact with ArgP:

4.2.5.1 ArgP predominantly binds 257-bp fragment upstream of *SspI* site in the *argO* regulatory region:

As depicted in Figure 4.9*A*, the 427-bp fragment comprising all the *cis* regulatory elements of *argO*, contains an *SspI* site at –45 such that the digestion with *SspI* will liberate a 257-bp upstream fragment from –293 to –45 and a 170-bp downstream fragment from –45 to +109. To investigate which of the two fragments might contain the ArgP binding sequence, the two fragments from *SspI* digestion as well as the full length 427-bp fragment as control, were labelled at their 5′-ends with ^{32}P-γ-ATP and polynucleotide kinase. EMSA experiments were carried out with the labelled fragments for ArgP binding (Fig. 4.9*B*). It was observed that ArgP binds the upstream 257-bp fragment but to a less extent than the full length *argO* region, whereas, the 170-bp downstream fragment was not retarded upon ArgP addition. This suggests that a substantial fraction of ArgP interacting sequences in the *argO* region might lie upstream of *SspI* site.

Besides, when the fragment was digested with both *SspI* and *HaeIII*, a region from 1st *HaeIII* site (–98) to *SspI* site (–45) was lost and ArgP was unable to bind the resultant fragment suggesting that the sequence between *HaeIII* and *SspI* sites are important for interaction with ArgP.



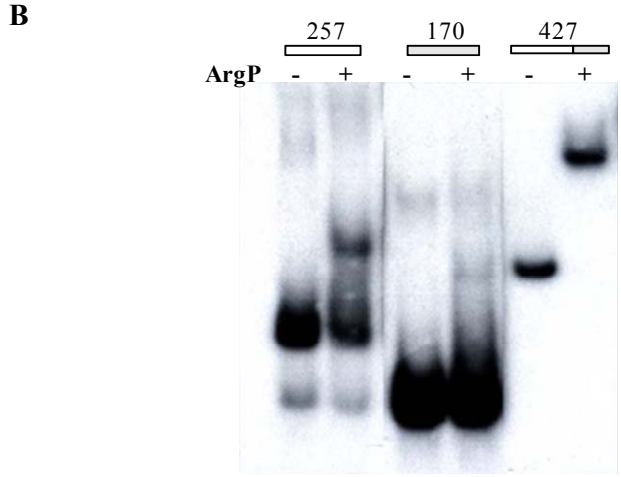


Figure 4.9. (*A*) Depiction of 427-bp amplicon comprising *argO* regulatory region from –293 to +109 and various fragments after digestion with restriction enzymes as indicated; H, *HaeIII* (*B*) EMSA experiment using *SspI* digest fragments along with the full-length 427-bp *argO* fragment (*B*). The absence (–) and presence (+) of ArgP (40 nM) are indicated.

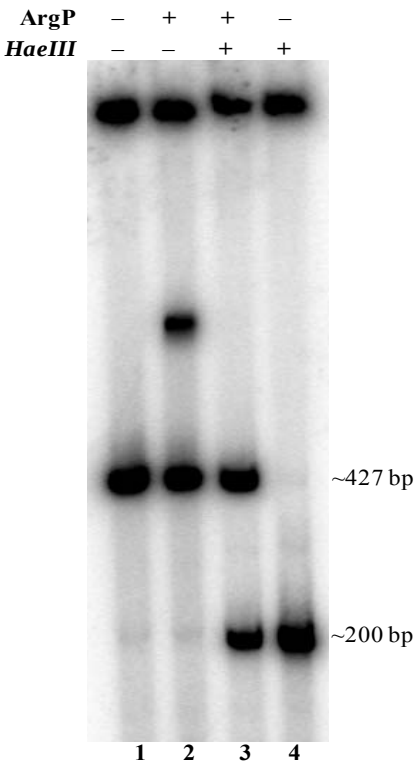


Figure 4.10. Assay for *HaeIII* protection of *argO* regulatory fragment by ArgP. Digestion with *HaeIII* after incubation of *argO* fragment with or without ArgP as indicated.

4.2.5.2 *HaeIII* Protection assays on *argO* template:

The *argO* regulatory fragment contains two *HaeIII* sites centred at –74 and –98 that flank the 26-bp palindrome upstream of *argO* promoter. The possibility was considered that ArgP may bind in this region and confer protection from *HaeIII* digestion, since LysR-type transcriptional regulator proteins recogni palindrome sequences. To investigate whether the *HaeIII* sites are protected by ArgP binding, the 5′-end labelled 427-bp *argO* DNA fragment was after pre-incubated with ArgP in *HaeIII* buffer and then treated with *HaeIII*. It was observed that ArgP does bind *argO* promoter sequence

in *HaeIII* buffer (Fig 4.10 lane 2), although with lower affinity than in the general binding buffer routinely used for EMSA experiments. When the 427-bp fragment was digested with *HaeIII*, the resulting fragments (200-bp and 206-bp; see Fig. 4.9*A*) were observed to co-migrate (Fig. 4.10 lane 4). When the digestion reaction with *HaeIII* was preceded by incubation with ArgP protein, two bands were visible, one migrating at the level of 427-bp fragment and the other with the 200-bp doublet (Fig. 4.10, lane 3). This suggested that one or both of the *HaeIII* sites in *argO* fragment were not completely protected by ArgP.

4.2.5.3 ArgP does not recognize palindrome in *argO*:

As shown in Figure 4.11*A*, upstream of the *argO* promoter there exists a nearly perfect palindrome of 26-bp between positions –102 to –77. As mentioned in Chapter 1, ArgP belongs to the LysR-type transcriptional regulator family, and most of the LysR-type regulator proteins recognize palindromic sequence in binding DNA. Since the palindrome in *argO* lies in the regulatory regions, it can be speculated that it is perhaps the binding sequence of ArgP. To study this, the 26-bp palindrome was deleted from the *argO* regulatory fragment as described in Chapter 2 Section 2.2.4.5 by a plasmid-based PCR strategy (Rajkumari and Gowrishankar 2001), with a pair of outwardly reading primers designed to amplify the entire plasmid except for the region to be deleted, and with *EcoRI* sites incorporated in the primers to facilitate re-circularization of the deletant plasmids. Primers JGAODelF (5′- CACCGGAATTCTCTCTTATTAGT TTTTCTG-3′) and JGAODelR (5′-TCCGGAATTCAGCGTTGATTACGCAGCT-3′) were extended on a plasmid template pHYD1722 (which carries the *argO* fragment from –293 to +109 cloned in pCU22 vector) by a PCR reaction, digested with *EcoRI* and religated to obtain a product lacking the 26-bp palindrome to generate plasmid

pHYD1728. Similarly overlapping 26-bp regions extending from –115 to –90 (that is, comprising the upstream half of the palindrome and adjacent 13-bp further upstream) and from –90 to –64 (that is, comprising the downstream half of the palindrome and the adjacent 13-bp further downstream) were also deleted from the plasmid pHYD1722 using primer pairs JGAUODelF (5′-CACCGGAATTCGAGGCCAGATA ATACTCT-3′) / GJAUODelR (5′-TCCGGAATTCTTTCACCCGCTTAAAGTT-3′) and GJADODelF (5′-CACCGGAATTCATTAATATTATCAATTTCC-3′) / JGAODelR (5′-TCCGGAATTCGAGGCCAGAGAGTATTAT-3′) to generate plasmids pHYD1729 and pHYD1730, respectively. The deletion plasmids were confirmed for deletion by sequencing. Three deletant *argO* fragments from pHYD1728, pHYD1729, and pHYD1730 were sub-cloned into pMU575 vector and the resultant plasmids were designated, respectively as pHYD1735, pHYD1736 and pHYD1737 (designated as Δ1, Δ2 and Δ3 in the Figure 4.11 for the respective deletions). Each of the three deleted 26-bp fragments are shown in Figure 4.11*A* (boxed).

The pMU575 derivative plasmids (pHYD1735 to pHYD1737, with deletions as above and wild type pHYD1723) were transformed into the chromosomal *argP* mutant strain GJ4892 harbouring *argP*$^{+}$plasmid pHYD915. β-Galactosidase activity measurements were performed in cultures of the transformants supplemented with Arg or Lys (Fig. 4.11*B*). It was observed that even after the deletion of either 26-bp palindrome (–102 to –77, designated as Δ1) or its overlapping upstream region of (–115 to –90, designated as Δ2), the property of *argO* transcriptional induction by Arg was still retained to a significant extent. On the other hand, deletion of the overlapping downstream region of 26-bp from –89 to –64 (designated as Δ3) nearly abolished *argO-lac* expression even in presence of Arg.

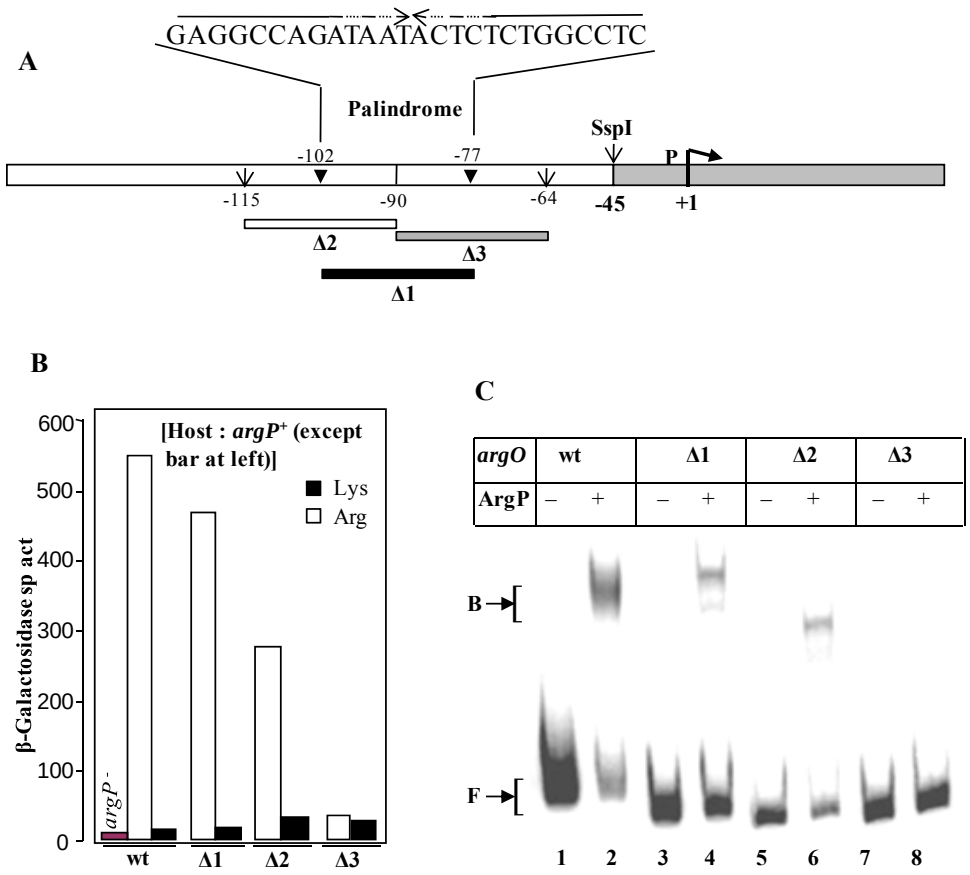


Figure 4.11. Role of 26-bp palindrome in *argO* regulatory region in ArgP binding and regulation. (*A*) Depiction of the *argO* regulatory region showing the positions of 26-bp palindrome and extent of deletion Δ1 to Δ3 described in text. (*B*) In vivo *argO-lac* expression, measured as β-galactosidase specific activity, in host strain derivatives with plasmid constructs carrying the *argO* regulatory region, either wild-type (wt; pHYD1723) or with deletions Δ1 to Δ3 (pHYD1728, pHYD1729 and pHYD1730 respectively). Host strain was *argP*$^+$ (GJ4892 with plasmid pHYD915) (except for bar at extreme left, which was from GJ4892 itself, and hence *argP* null mutation). Cultures were supplemented with Arg or Lys as indicated. (*C*) EMSA experiment with radiolabelled 427-bp *argO* fragment (wt) or its deletion derivatives (Δ1 to Δ3) in absence or presence of ArgP at 20 nM ArgP (monomer concentration). Bands corresponding to free DNA (F) and to DNA in binary complex with ArgP (B) are marked.

The in vivo results above were supported also by data from EMSA experiments that were carried out to check for ArgP binding in vitro to the deletant *argO* fragments Δ1 to Δ3 (Fig. 4.11*C*). Neither the Δ1 (palindrome, –102 to –77 deletion) fragment nor Δ2 (overlapping upstream, –115 to –90 deletion) was hindered for ArgP binding in presence or absence of co-effector (Lys), and both the mobilities were retarded by ArgP to the same extent as that of wild-type (undeleted) 427-bp *argO* fragment. However, the Δ3 (overlapping downstream, –89 to –64 deletion) *argO* fragment was substantially affected for ArgP binding. Taken together these results suggest that the 26-bp palindrome centred at –90 in *argO* is not essential for ArgP-mediated regulation of *argO*, but rather that sequences downstream to it.

4.2.5.4 ArgP binds a sequence downstream of –89 in *argO* regulatory region:

The binding of ArgP to *argO* was further investigated by EMSA experiments with double-stranded 26-mer oligonucleotide sequences corresponding to the palindrome of –102 to –77 and the overlapping upstream (from –115 to –90) and downstream (from –89 to –64) region. The double-strand fragments of the 26-mers were prepared by annealing the respective upper strand and the lower strand oligonucleotide sequences. It was observed that neither the double-stranded 26-mer sequence from –102 to –77 (palindrome) nor that from –115 to –90 (overlapping upstream) exhibited any binding to ArgP in EMSA, whereas that from –89 to –64 (overlapping downstream) bound the protein, albeit very weakly, both in absence and presence of Lys (Fig. 4.12, left panel). This results lent further support to the suggestion that the *argO* regulatory region upstream of –89 is not required for ArgP binding.

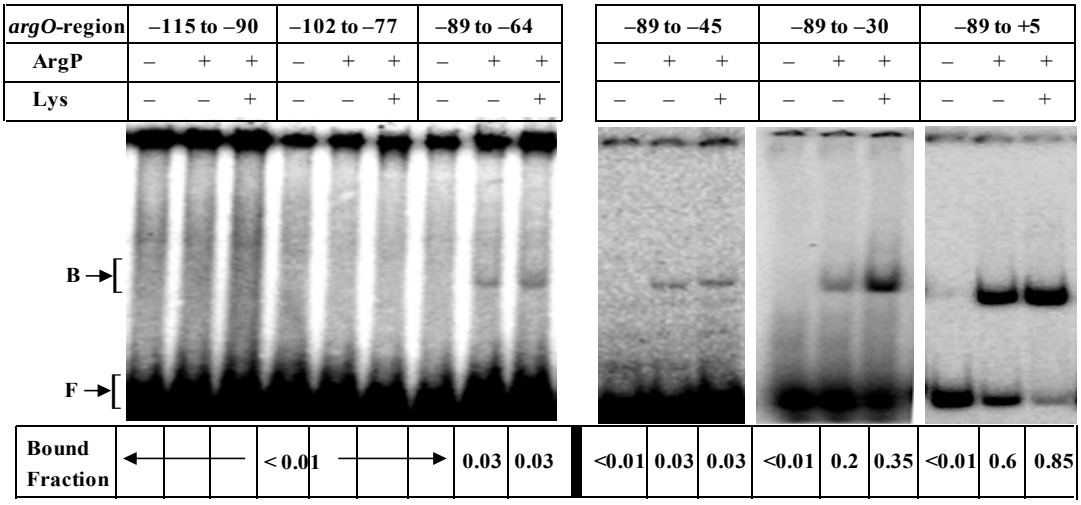


Figure 4.12. Binary interaction of ArgP with *argO* regulatory region. EMSA of double strand radiolabelled fragments of the 26-bp palindrome and similarly deleted overlapping fragments in presence and absence of Lys (Left panel). EMSA of radiolabelled fragments corresponding to indicated regions of *argO* in absence or presence of ArgP (20 nM monomer) and Lys (Right panel). Beneath each lane is given the value, obtained from densitometry, for the fraction of labelled DNA bound by the protein. Bands corresponding to free DNA (F) and to DNA in binary complex with ArgP (B) are marked.

To further investigate the extent of ArgP binding sequence on *argO* promoter, various fragments of *argO* regulatory region of increasing length downstream of –89 were tested for ArgP binding in the EMSA experiment. Sequences from –89 to –45, –89 to –30, –89 to +5 were prepared either by PCR or by annealing of respective oligonuceotide sequences of upper and the lower strands, and tested for ArgP binding. The affinity of ArgP binding to the sequence from –89 to –45 remained weak, but it was higher for the fragment from –89 to –30 and even more so for that from –89 to +5;

in the latter two cases, further increases in ArgP binding affinity were also observed in the presence of the co-effector Lys (Fig. 4.11, right panel). These results suggest that the ArgP binding sequence resides between –89 to +5 on *argO* promoter DNA.

4.2.5.5 DNase I footprinting to determine the ArgP binding region on *argO*:

Dnase I footprinting experiments were then performed to map the region on *argO* that is protected by ArgP binding in absence or presence of Arg or Lys. The 427-bp amplicon of the *argO* regulatory region from –293 to +109 (having *PstI* site at its upstream end and *BamHI* site at its downstream end) was end-labelled by ^{32}P-γ-ATP and polynucleotide kinase and digested with *PstI* enzyme so as to obtain an *argO* fragment end-labelled uniquely at the 5′ end of the bottom (template) strand towards +109. To obtained an *argO* uniquely labelled on the 5′-end of upper strand, a 5′-end-labelled 234-*bp argO* fragment (with sequence from –115 to +109) that had been digested with *BamHI* (whose recognition site is present in the *argO* reverse primer used in PCR) was used to obtain a fragment uniquely labelled at the end corresponding to –115 (that is, 5′-end of top strand). The 427-bp fragment was itself not used for 5′-end labelling of the top strand at –293 since preliminary experiments had shown that the labelled end was too far away from the site of ArgP binding for good resolution to be obtained in the footprinting experiment.

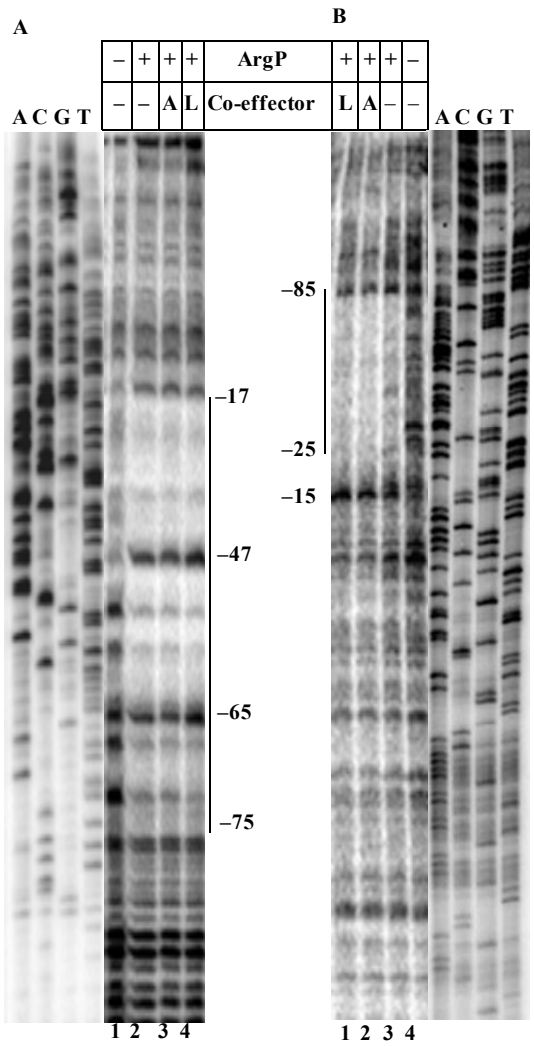


Figure 4.13. Determination of ArgP binding site on *argO.* DNase I digestion patterns were determined for radiolabelled *argO* fragments that had been uniquely 5′-end-labelled (as described in Materials and Methods) on either the top strand at –115 (*A*) or the bottom strand towards +109 (*B*), in absence or presence of ArgP and co-effectors Arg (A) or Lys (L). The corresponding di-deoxy sequence ladder of *argO* is represented alongside the numbered lanes in each of the two panels. Vertical lines denote the regions of ArgP footprint, and nucleotide positions of interest are also marked.

After incubation with ArgP protein in presence or absence of Arg or Lys, the labelled fragments were subjected to limited digestion with DNase I and then resolved by denaturing polyacrylamide gel electrophoresis on a 10% sequencing gel. It was observed that there was prominent protection by ArgP of the stretch from around −75 to −17 on the top strand (with intervening regions of no protection and hypersensitivity, respectively, around −65 and −47) (Fig. 4.13*A*), and from around −85 to −25 on the bottom strand (with a hypersensitive band at −15) (Fig. 4.13*B*), both in the absence and presence of Arg or Lys. Small differences in band patterns for the bottom strand (Fig. 4.13*B*) were observed between reactions done with ArgP without co-effector (lane 3) and those with Arg or Lys (lanes 1 and 2) such as, for example, around positions −18, −50, and −135, but importantly, there were no differences between the patterns obtained on both strands for ArgP in presence of Arg and those in presence of Lys. Thus, although the EMSA experiments had shown that ArgP exhibits different affinities of binding to *argO* depending upon whether Arg or Lys is added as co-effector, the DNase I footprint on the DNA remains invariant in the two cases.

4.2.5.6 EMSA experiment with ArgP and *argO* sequence from –85 to –20 on:

Based on observation from the DNase I footprinting experiments, the binding of ArgP was tested in an EMSA experiment to an *argO* fragment from −85 to −20 (Fig. 4.14), and the results indicated that this fragment exhibits similar affinities of binding to ArgP in both the absence and presence of Lys as those of the full-length 427-bp fragment from –293 to +109 (Fig. 4.14). The data therefore suggest that most, if not all, of the important sequences or contacts for ArgP lie within the *argO* region from approximately –85 to –20 (whose sequence is underlined and bracketed in Fig. 4.1*A*).

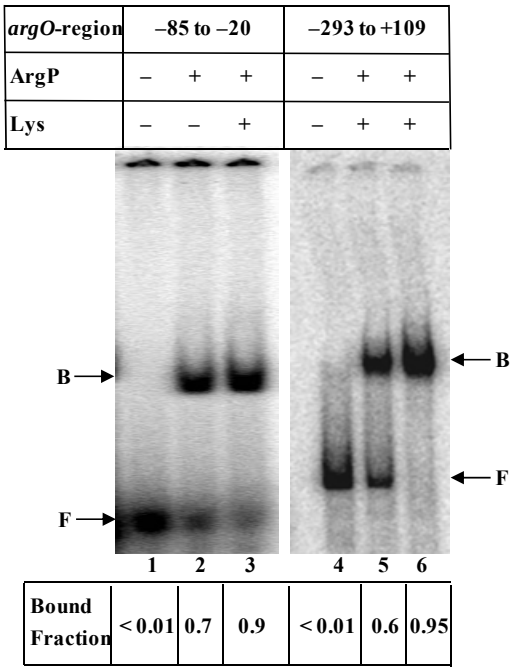


Figure 4.14. Comparison of electrophoretic mobility of *argO* fragment from –85 to –20 with that of the 427-bp fragment (from –293 to +109), in absence or presence of ArgP (20 nM monomer) and Lys. F, free DNA; B, ArgP-DNA binary complex. Beneath each lane is given the value, obtained by densitometry, for the fraction of labelled DNA bound by the protein.

4.2.6 Sequence alignment of the *argO* regulatory region:

To investigate how conserved are the elements of *argO* regulatory region, an alignment was undertaken for *E. coli argO* with regulatory sequences of *argO* orthologous genes taken from the sequenced genome of various bacterial species, using the the Clustal X (1.82) program.

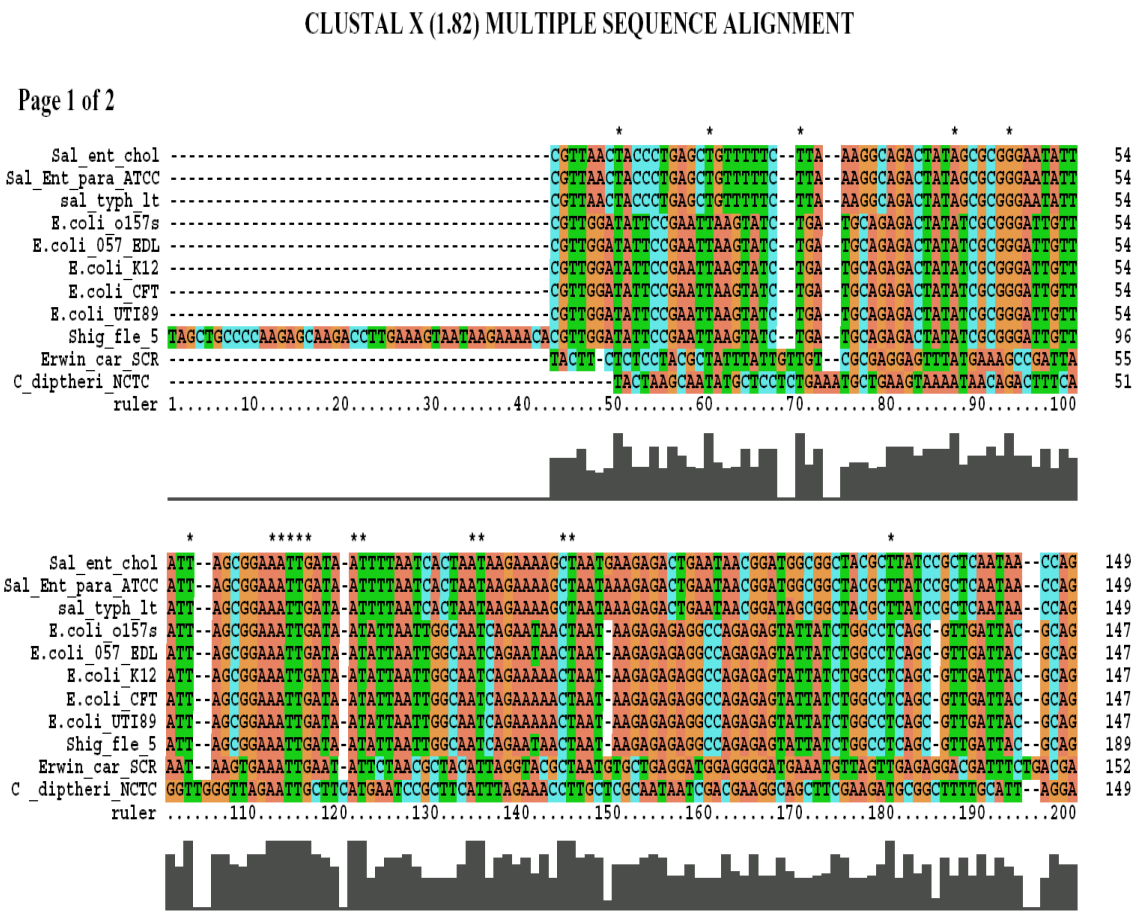


Figure 4.15. CLUSTAL X (1.82) alignment of *E. coli argO* regulatory region (from –270 to +30) with those from orthologous genes from related organisms. The various bacterial strains taken are: Sal_ent_chol, *Salmonella enterica* subsp. *Choleraesuis* str. SC-B67, Sal_ent_para_ATCC, *Salmonella enterica* subspecies *Paratyphi* str. ATCC 9150, sal_typh_lt, *Salmonella typhimurium* LT2, E. coli_0157s, *Escherichia coli* 0157:H7 str. *Sakai*, E. coli_0157_EDL, *Escherichia coli* 0157:H7 str. *EDL933*, E.coli_K12, *Escherichia coli* K12, E. coli_CFT, *Escherichia coli* CFT073, E.coli_UT189, *Escherichia coli* UT189, Shig_fle_5, *Shigella flexnery* 5 Str. 8401, Erwin_car_SCR *Erwinia corotovora* subsp. *atroseptica* SCRI1043, and C_diptheri_NTCC, *Corynebacterium diptheri* str. NTCC 13129. The vertical bars represent the score of conservation and the residues which are completely conserved are indicated by asterisks. Residue positions in the alignment corresponding to various *argO cis* acting elements are 75 (+1), 82 to 87 (–10 sequence), 94 to 164 (Region protected by ArgP).

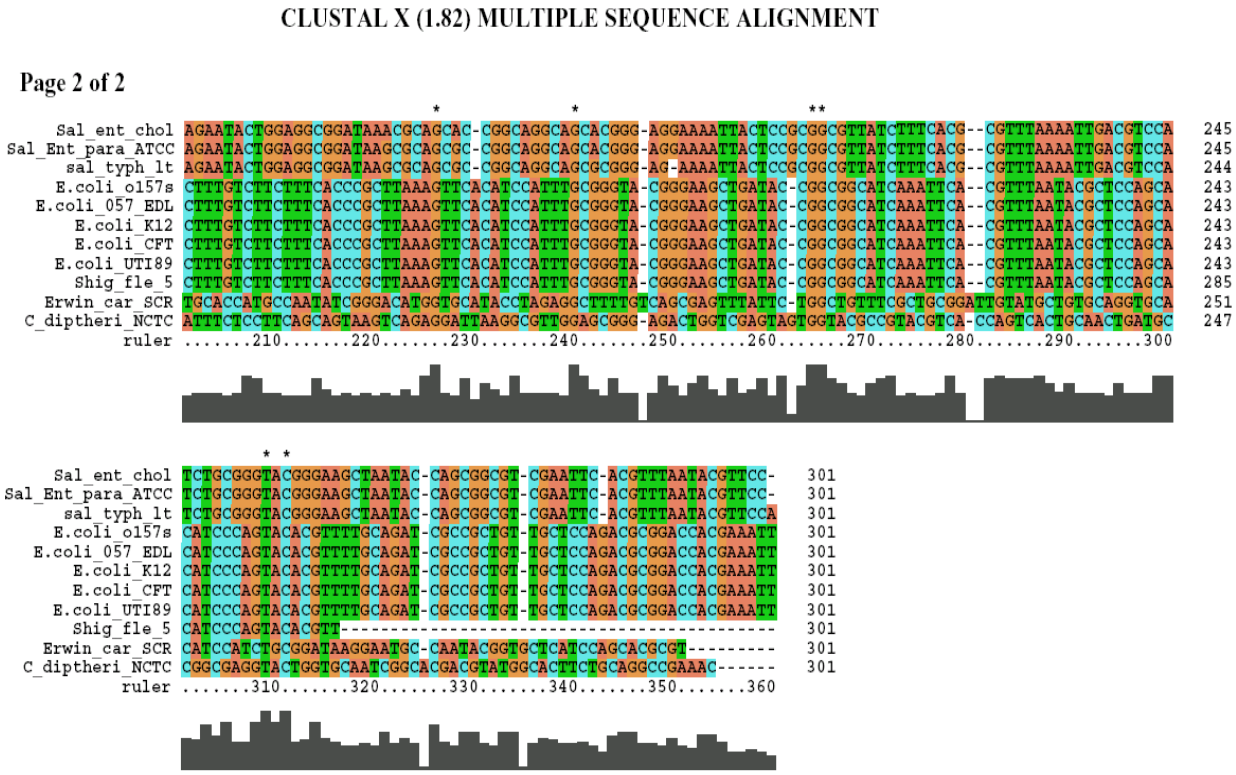


Figure 4.15. CLUSTAL X (1.82) alignment of *E. coli argO* regulatory region (from –270 to +30) with those from orthologous genes from related organisms. The various bacterial strains taken are: Sal_ent_chol, *Salmonella enterica* subsp. *Choleraesuis* str. SC-B67, Sal_ent_para_ATCC, *Salmonella enterica* subspecies *Paratyphi* str. ATCC 9150, sal_typh_lt, *Salmonella typhimurium* LT2, E. coli_0157s, *Escherichia coli* 0157:H7 str. *Sakai*, E. coli_0157_EDL, *Escherichia coli* 0157:H7 str. *EDL933*, E.coli_K12, *Escherichia coli* K12, E. coli_CFT, *Escherichia coli* CFT073, E.coli_UT189, *Escherichia coli* UT189, Shig_fle_5, *Shigella flexnery* 5 Str. 8401, Erwin_car_SCR *Erwinia corotovora* subsp. *atroseptica* SCRI1043, and C_diptheri_NTCC, *Corynebacterium diptheri* str. NTCC 13129. The vertical bars represent the score of conservation and the residues which are completely conserved are indicated by asterisks. Residue positions in the alignment corresponding to various *argO cis* acting elements are 75 (+1), 82 to 87 (–10 sequence), 94 to 164 (Region protected by ArgP).

The region from –270 to +30 was chosen for the alignment (Fig. 4.15). In the alignment sequence, the residue number 75 corresponds to the transcription start (+1) site, 82 to 87 correspond to –10 promoter sequence and 94 to 164, the ArgP binding region (–20 to –85) on *argO* regulatory DNA with respect to the *E. coli* K12 sequence. The alignment data suggest that the regulatory regions are indeed conserved to substantial extent in the sequenced genomes of bacterial strains closely related to *E. coli* K12, for example, sequences of the *E. coli* strains used for alignment (*E. coli* CFT, *E. coli* UTI89, *E. coli* 0175 etc), *Salmonella enterica, S. typhimurium* etc. Sequence conservation was observed especially in the promoter regions and the regulator protein interacting regions (ArgP binding region in case of *argO*). However, conservation was decreased with the sequences from more distantly related bacteria, for example, *Erwinia corotovora*, *Corynebacterium diptheri* etc.

4.3 Discussion:

In this study, the start site of *argO* transcription was mapped by primer extension analysis to an "A" residue situated 28 bases upstream of the predicted translation start-site (GTG) of the structural gene. The predicted –10 hexamer motif of the promoter was verified to be TATAGT by site directed mutagenesis. Therefore, the promoter sequence of *argO* can be represented as start site (+1) A (in CAT) with upstream hexamer TATAGT (–10 sequence) separated by 17-bp from the –35 hexamer motif TTGACA. A 427-bp fragment containing a region of *argO* extending from –293 to +109 (relative to the start-site of transcription, taken as +1) was shown to encompass all the *cis* regulatory elements of *argO*, as determined by in vivo *lacZ* reporter gene expression assays.

In EMSA experiments, ArgP was shown to bind specifically to the *argO* fragment comprising the sequence from –293 to +109 with a K_d of 6.5 nM (monomer concentration). Binding affinity was unaffected in presence of Arg, whereas it was increased 2- to 3-fold (K_d, 2.5 nM) in presence of Lys. Thus, despite the fact that Lys inhibits transcription of *argO* in vivo, ArgP binding to *argO* in vitro was enhanced in presence of Lys, suggesting that the inhibition of *argO* transcription in vivo was not simply by preventing ArgP from recognizing the *argO* operator.

Although the *argO* regulatory region contains a near-palindomic 26-bp sequence from –102 to –77, data from a variety of in vitro and in vivo experiments established that this is not the binding site of ArgP unlike the situation with several other LysR-type regulators. Thus deletion of this sequence did not affect ArgP-mediated *argO* regulation in vivo nor ArgO binding to *argO* in vitro. Subsequent experiment involving both EMSA and DNase I footprinting led to the conclusion that the binding site for ArgP lies between positions –89 to –20 in *argO*.

The DNase I footprinting experiments also revealed hypersensitive sites at –15 on the top strand and –47 on the bottom strand of *argO*. This suggests that ArgP binding to *argO* DNA causes bends (or kinks) in the DNA thereby exposing certain residues to be more accessible to DNase I action. This study also showed that there was no difference in the footprint pattern of ArgP on *argO* in presence of Arg or Lys despite the fact that ArgP exhibits an enhanced affinity for *argO* in presence of Lys.

Sequence comparison of the ArgP binding region in *argO* with those of DNA sequences at other loci in *E. coli* previously reported to bind ArgP (IciA) did not reveal any evident similarity and it is as yet not possible to establish any consensus DNA sequence for ArgP binding. Celis (1999) pointed out a partially dyadic nucleotide

sequence with a conserved T-N_{11}-A motif in all the reported binding sequences of ArgP (*argK*, *dnaA*, and three 13-mers of *oriC*), but it is clear that this motif is not sufficiently discriminatory. For example, when the ArgP protected region on *argO* was checked for the existence of this motif, as many as twelve such possible motifs exist within the 55-bp ArgP protected region in *argO* itself. Thus, the only feature common to all the DNA sequences reported to be bound by ArgP is that they are AT-rich, and this is true for the binding site in *argO* as well. Finally, sequence alignment of the regulatory regions of the *argO* orthologous genes from diverse bacterial species has revealed that this sequence is conserved among the closely related bacterial species of *E. coli* K12.

CHAPTER 5

Mechanism of ArgP- and Arg- or Lys-mediated transcriptional regulation of argO

5.1 Introduction:

It was described in the previous Chapter that ArgP binds the *argO* regulatory region, suggesting a direct regulation of *argO* transcription by ArgP protein, and that Lys addition increases the affinity of ArgP binding. Besides, it had been demonstrated in Chapter 3 that Arg and Lys compete with each other for a common binding site in ArgP. In addition, an earlier report (Nandineni and Gowrishankar 2004) had also established that the transcription of *argO* in vivo is ArgP dependent and that its expression is induced by exogenous Arg and repressed by Lys.

In this Chapter, reconstitution of the transcriptional regulation of *argO* in vitro in presence of ArgP (with and without Arg or Lys) and a σ^{70}-bearing holoenzyme of *E. coli* RNA polymerase (RNAP) is described. Further, the studies described in this Chapter have sought to investigate the four steps of transcription initiation at *argO* promoter so as to understand which step(s) is/are regulated by ArgP in absence or presence of co-effector (Arg or Lys). These studies were aimed towards understanding the mechanism of ArgP mediated regulation of *argO* transcription and the difference in the mechanism in presence of Lys (which causes repression) and in presence of Arg (which causes activation) of *argO* transcription.

5.2 Results:

5.2.1 Dependence on buffer composition for ArgP binding to *argO* and transcription:

In attempting reconstitution of *argO* transcription in vitro, various reaction buffers were tested. In the commonly used Tris-glutamate (Glu) transcription buffer (described, for example in Cheeran et al. 2005 and Pani et al. 2006, whose composition is 20 mM Tris-

Glu, 10 mM MgGlu, and 50 mM KGlu; pH 8.0), or Tris-HCl transcription buffer (described, for example, in Rajkumari et al. 1996, whose composition is 50 mM Tris-HCl, pH 7.8; 3 mM $Mg(CH_3COO)_2$; 0.1 mM EDTA; 50 mM NaCl; 0.1 mM dithiothreitol), no transcription from the *argO* promoter was obtained under any of the conditions tested. On further investigation, it was found that the binding of ArgP to *argO* DNA was comparatively weaker under these buffer conditions than it was in the EMSA buffer described in Chapter 4 (Fig. 5.2), which could perhaps explain the failure of the transcription reactions.

These results suggested that ArgP binding to DNA (at least for *argO* promoter) is dependent on buffer conditions. This was further tested with various restriction enzyme buffers (New England Biolabs) for enzyme such as *SspI, HaeIII, BamHI*, and in all the buffers tested, ArgP exhibited very weak binding to *argO* DNA (data not shown; see also Section 4.2.5.2, Chapter 4). This could perhaps be attributed to the differences in, and/or the concentrations of, salts in the buffers.

Therefore, ArgP binding to *argO* promoter was tested in EMSA experiment using Tris-Glu buffer either neat or that had been modified by dilution or potassium glutamate (KGlu) supplementation, as shown in Figure 5.1. Less than $1/3^{rd}$ of the labelled *argO* fragment was retarded with the Tris-Glu transcription buffer (lane 3) as compared to the control EMSA buffer (lane 2). In half-strength Tris-Glu buffer, the intensity of the retarded band was increased, indicating a significant increase in binding of ArgP to *argO* under this condition (lane 4). The binding of ArgP to *argO* was also increased with increase in the concentration of KGlu as seen from lanes 5 (10 mM KGlu) to 10 (100 mM KGlu) where there was steady rise in the *argO* bound fraction. Thus the highest binding was observed with either half-strength Tris-Glu buffer (lane 4) or with the Tris-Glu buffer supplemented with 100 mM KGlu (lane 10). When the EMSA

experiment was undertaken with Tris-HCl buffer (Rajkumari et al. 1996), only feeble binding was observed (lane 11). Since the half-strength Tris-Glu buffer and Tris-Glu buffer with 100 mM KGlu exhibited significant binding of ArgP to *argO* DNA, in vitro transcription reactions were reconstituted in these buffer conditions, and both were successful in giving run off transcript products from *argO* promoter whereas these reactions had failed with Tris-Glu buffer (data not shown). Following these findings, the half-strength Tris-Glu buffer was used for all the in vitro transcription reactions in this entire work.

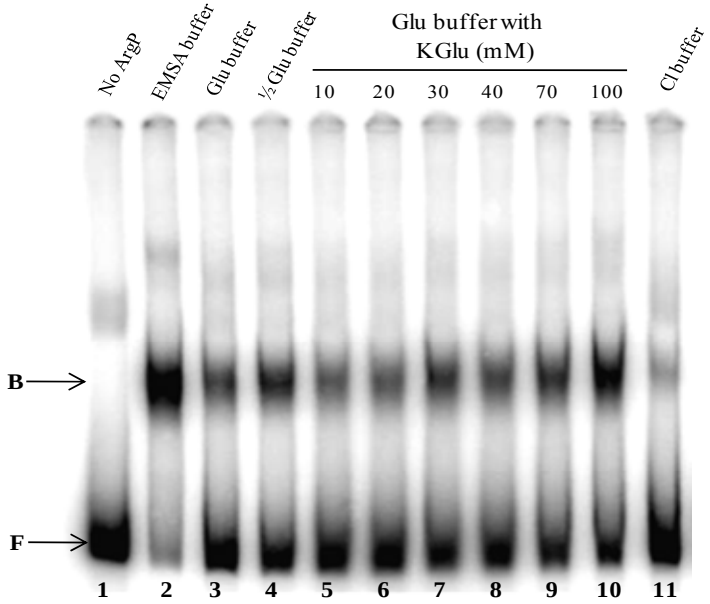


Figure 5.1. ArgP binding to *argO* promoter under various buffer conditions. ArgP (20 nM) was added to all the lanes except at the leftmost lane. EMSA buffer is the buffer used for all the EMSA reactions described in Chapter 4. Glu buffer (Tris-Glu) and Cl buffer (Tris-HCl) are as described in the text. ½ Glu buffer is the half-strength Tris-Glu buffer. The free DNA (F) and the binary complex with ArgP (B) are marked. KGlu-Potassium glutamate.

5.2.1 Transcriptional regulation of *argO* by ArgP in vitro:

ArgP-mediated regulation of *argO* transcription in presence and absence of various co-effectors was then tested in a defined in vitro single round run-off transcription system (with half-strength Tris-Glu buffer as described in the previous Section) using σ^{70}-bearing RNAP holoenzyme and a linear DNA template, the 427-bp amplicon (described in previous Chapter) that encompasses the *argO* region from –293 to +109. Run-off transcription from the *argO* promoter on the 427-bp template fragment was expected to yield a 110-bp transcript. As depicted in Figure 5.2, the data obtained from these experiments were consistent with those from in vivo studies in that they demonstrated (i) a low basal level of *argO* transcription in the absence of ArgP (lane 1); (ii) substantial ArgP-dependent activation occurring in presence of Arg or CAN but not Lys (compare lanes 3 and 5 with lanes 1, 2 and 4); and (iii) reversal of such activation upon addition of Lys but not of a non-specific amino acid such as histidine (His) (compare lanes 6 and 7). These results confirm that *argO* is under the direct transcriptional control of ArgP, and that Arg addition activates while Lys inhibits the expression of *argO*, and that the inhibitory effect of Lys is dominant over the activating effect of Arg.

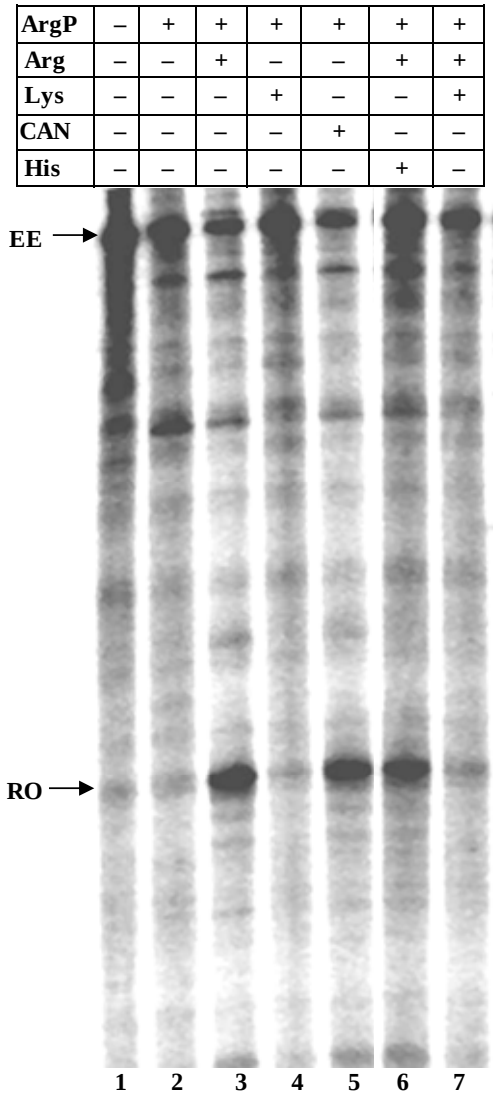


Figure 5.2. ArgP-mediated regulation by Arg, Lys, and CAN of *argO* transcription. Radiolabelled in vitro transcription products obtained with the 427-bp *argO* template (with sequence from –293 to +109), in reaction mixes with various additives as indicated, and subjected to electrophoresis on denaturing 10% polyacrylamide gel. RO, run-off transcript from *argO* promoter; EE, end-to-end transcription product; His, histidine.

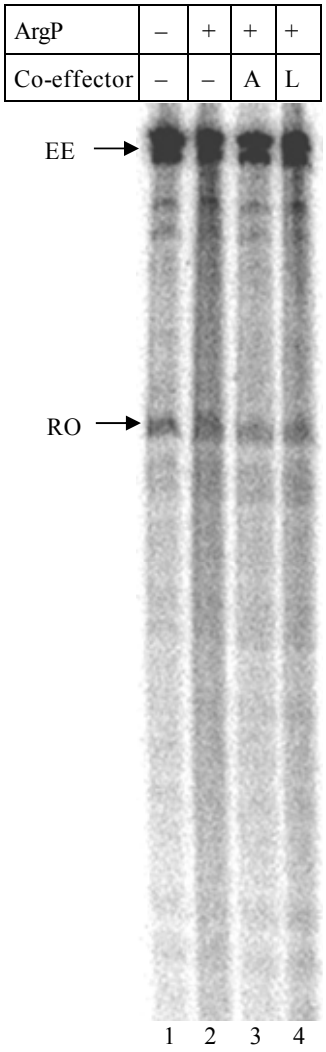


Figure 5.3. Absence of effect of ArgP and co-effectors on a control promoter. Radiolabelled in vitro transcription products were obtained with linear template bearing the phage T7A1 promoter, in reaction mixes with various additives as indicated, and subjected to electrophoresis on denaturing 10% polyacrylamide gel. RO, run-off transcript from T7A1 promoter (215 bases); EE, end-to-end transcription product (330 bases); A, Arg; L, Lys.

5.2.3 Specificity of the ArgP mediated *argO* regulation:

To investigate if the transcriptional regulation of *argO* by ArgP and the opposite effects of co-effectors (Arg and Lys) were specific to *argO* promoter, in vitro transcription

experiment was undertaken with control T7A1 promoter template in presence and absence of ArgP and the co-effectors Arg or Lys. The results revealed that transcription from the T7A1 promoter is unaffected by ArgP or the co-effectors (Fig 5.3). A band corresponding to the run-off transcript from T7A1 promoter (215-bp) was observed in all lanes either in presence (lanes 2 to 4) or absence (lane 1) of ArgP. No difference was observed in the intensity of band in absence or presence of ArgP with or without co-effectors, Arg and Lys. Besides, as described above, the transcription reaction with *argO* template had also revealed that other amino acids like His had no affect on transcription of *argO* (Fig. 5.2). These findings, hence, established that ArgP, as well as Arg and Lys, act specifically and directly in regulating *argO* transcription.

5.2.4 Mechanism of *argO* regulation - Steps of transcription initiation:

As described in Chapter 1, Section 1.3.1.1, transcription initiation has four distinct steps; viz, recruitment and closed complex formation by RNAP on promoter, isomerisation from closed to open complex, initial transcribing complex formation, and promoter escape to undergo elongation. The various steps were investigated at *argO* promoter to study which step(s) is being regulated by ArgP in presence of Lys or Arg.

5.2.4.1 Ternary Complex (closed) formation by RNAP at *argO* promoter:

It is known that repressor proteins commonly act by steric hindrance of RNAP binding to promoters and activators act by "recruiting" RNAP to the promoters (Ptashne and Gann 1997; Browning and Busby 2004). Therefore the first step of transcription initiation i.e. the step of RNAP recruitment is the most common step at which regulation of gene expression is mediated. Accordingly, the simplest model in case of *argO* would be that ArgP in presence of Arg (or CAN) recruits RNAP to *argO* and that ArgP in presence of Lys occludes RNAP from the *argO* promoter.

In order to examine if there is any effect of ArgP protein on binding of RNAP to *argO* promoter in presence and absence of Arg, CAN or Lys, the ternary complex formation of RNAP, ArgP and *argO* regulatory DNA was investigated by EMSA experiment. 80 nM of RNAP (holoenzyme), 20 nM ArgP, and 0.5 nM of radiolabelled *argO* DNA (427-bp PCR-amplified fragment comprising *argO* region from –293 to +109) were used in the binding reactions. ArgP was first allowed to bind the *argO* operator DNA for 5-min and then RNAP was added and further incubated for 30-min before resolving in a 4% native polyacrylamide gel. Arg, CAN or Lys was added at a concentration of 0.1 mM whenever required.

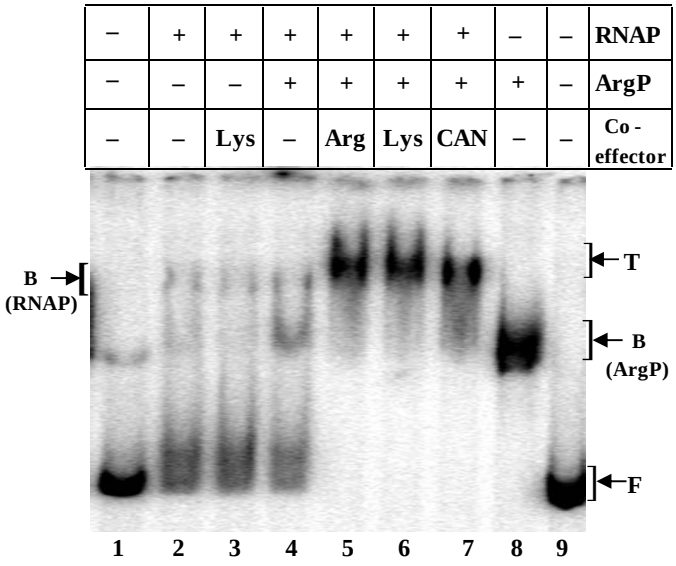


Figure 5.4. Ternary complex formation of ArgP and RNAP with *argO* regulatory region. Electrophoretic mobility of radiolabelled *argO* fragment in absence or presence (as indicated) of 20 nM ArgP (monomer), 80 nM RNAP, and co-effectors Arg, Lys, or CAN at 0.1 mM. Bands corresponding to free DNA (F), DNA in binary complex with ArgP [B(ArgP)] or RNAP [B(RNAP)], and that in ternary complex with ArgP and RNAP (T), are marked. (It may be noted that the aliquot of *argO* DNA preparation used in lane 1 of this panel appears to have been inadvertently contaminated with a small amount of ArgP protein.)

As also shown earlier in Chapter 4, with ArgP alone addition, all radioactivity in *argO* was shifted to a sharp band of retarded mobility, indicative of formation of a binary complex that was stable during gel electrophoresis (Fig. 5.4, lane 8). On the other hand, with RNAP added in absence of ArgP, only a faint retarded band representing binary complex of *argO* with RNAP was observed, but the band corresponding to free *argO* had also become diffuse indicating that although a substantial proportion of the binary complex had indeed formed in solution it had then dissociated during gel electrophoresis (Fig. 5.4, lane 2); this pattern remained unaffected by addition of Lys to the mixture (Fig. 5.4, lane 3).

In the mixtures containing both ArgP and RNAP, the mobility shift patterns were determined by the presence or absence of co-effectors. In the absence of any co-effector, the radioactivity was distributed in three bands, two of which coincided with the pattern observed with RNAP in absence of ArgP (that is, corresponding to RNAP-DNA binary complex and to its dissociation to free DNA), while the third (intermediate) band representing the stable ArgP-DNA binary complex displayed reduced intensity (compared to that observed in absence of RNAP) (Fig. 5.4, lane 4). These findings indicated that, in the absence of any of the co-effectors, RNAP competes with ArgP for binding to *argO*, and that the formation of binary complexes of *argO* with RNAP on the one hand and with ArgP on the other are mutually exclusive with no evidence for occurrence of a ternary complex.

On the other hand, in the presence of RNAP and ArgP with any one of the co-effectors Arg, Lys, or CAN, the pattern of distribution of radioactivity indicated that the mobility of 100% of the *argO* molecules had been retarded, with a prominent band that corresponded to the ternary complex of DNA, ArgP, and RNAP leading into a diffuse smear that terminated abruptly at the position corresponding to the ArgP-DNA binary

complex (Fig. 5.4, lanes 5-7); The smear is interpreted as an evidence for conversion of a fraction of the ternary complex (RNAP-ArgP-DNA) to the stable binary complex (ArgP-DNA) during electrophoresis.

From densitometric analysis of the gel of Figure 5.4 (data not shown), it was estimated that at the protein concentrations employed (8-fold higher molar RNAP concentration compared to that of ArgP dimer) approximately 70% of the DNA forms a largely unstable binary complex with RNAP in either the absence of ArgP or its presence without co-effectors, and that the remaining 30% forms the binary complex with ArgP under the latter conditions. In presence of a co-effector, the entire DNA is engaged in the ternary complex which is largely stable, since only around 25% dissociates during electrophoresis to the ArgP-DNA binary complex. It can therefore be concluded that the co-effectors promote both the formation and stability of the ternary complex, and that the three co-effectors Arg, Lys, and CAN are more or less similar to each other in this regard. These observations suggest that the expectation is correct as far as Arg-bound ArgP is concerned that it helps in the recruitment of RNAP to the *argO* promoter. However, the intriguing finding was that Lys-bound ArgP was also proficient for RNAP recruitment to *argO* although the transcription of *argO* is known to be repressed under these conditions.

5.2.4.2 Open complex formation by RNAP at the *argO* promoter in presence of ArgP:

The finding that Lys supplementation was associated with absence of productive transcription despite evidence for RNAP recruitment to the promoter and the formation of stable ternary complex on the *argO* template raised the need to compare the nature of the *argO*-ArgP-RNAP complex obtained in the presence of Arg with that in the

presence of Lys, and to test the possibility that RNAP is trapped at *argO* in presence of ArgP and Lys. Trapping of RNAP to a promoter has earlier typically been described at any one of three different steps (reviewed in Hsu 2002a), namely those of isomerisation from closed to the open complex, transition from abortive transcription (or a moribund complex) to productive transcription, or pausing of RNAP in the initial transcribed sequence (ITS). These three models are exemplified by, respectively, (one mode of) Lac repressor action at the *lac* promoter (Straney and Crothers 1987), synthesis of abortive transcripts at a variety of promoters (Hsu 2002a), or, at moribund complex (Kubori and Shimamoto 1996; Susa et al. 2006) and the ternary transcription complex at the phage λ P_R promoter that serves as a substrate for modification by the λ Q protein (Roberts et al. 1998). Here, the requirement of open complex formation at the *argO* promoter and the impact thereon of Arg or Lys addition were first investigated.

5.2.4.2a Heparin sensitivity of the RNAP-ArgP-*argO* ternary complex:

A complex of RNAP and promoter DNA becomes heparin-resistant when the promoter –10 region is melted and irreversibly stabilised in an open complex; on the other hand, in a reversible closed complex of promoter and RNAP, heparin will compete with RNAP for interaction with promoter DNA leading to heparin-sensitivity of the complex (McClure 1985; Ninfa et al. 1987; Lucia et al. 1997; Leoni et al. 2000). To check whether the ternary complex formed at *argO* promoter is a closed complex or an open complex, heparin challenge experiment was carried out. After the ternary complex was formed in absence of nucleoside tri phosphate (NTP), it was challenged with heparin (200 μg/ml) for various time points from 0 to 60 min in presence of either Arg or Lys. It was observed that on heparin treatment the ternary complex of ArgP-*argO*-RNAP in presence of either Arg or Lys was dissociated to the binary complex of ArgP-DNA (Fig

5.5). Thus, both with Arg and with Lys supplementation, the ternary complex of ArgP and RNAP assembled on *argO* (represented by the super-shifted band in EMSA) exhibited roughly similar patterns of heparin-sensitivity (dissociating to the binary complex of *argO* and ArgP) in the absence of nucleoside triphosphates (NTPs) (Fig. 5.5, compare lanes 3-6 with lanes 7-10). This demonstrates that the RNAP binding to *argO* promoter is sensitive to heparin in the absence of nucleotides, representative of a reversible closed complex of RNAP and *argO* promoter.

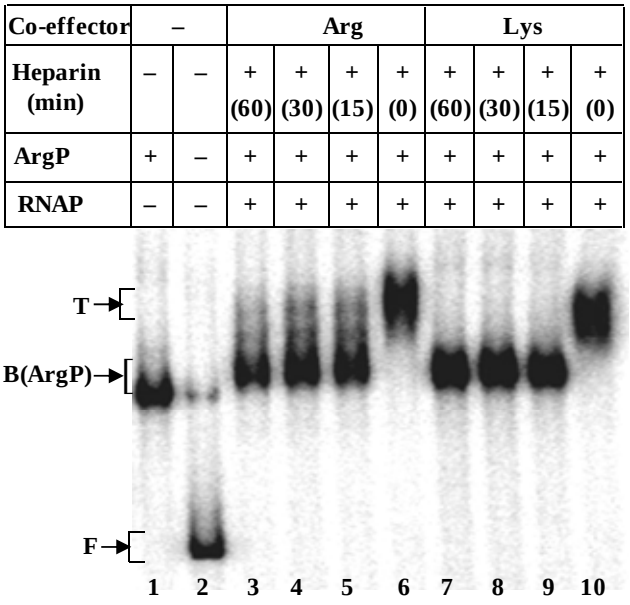


Figure 5.5. Electrophoretic mobility experiment of radiolabelled *argO* fragment in absence or presence (as indicated) of 20 nM ArgP (monomer), 80 nM RNAP, and co-effectors Arg, Lys, or CAN at 0.1 mM., in absence of or following treatment with heparin at 200 μg/ml for time periods indicated in parentheses. (Note that the samples of free DNA probe preparations used lane 2 of this panel appear to have been inadvertently contaminated with a small amount of ArgP protein.) Bands corresponding to free DNA (F), DNA in binary complex with ArgP [B(ArgP)] or RNAP [B(RNAP)], and that in ternary complex with ArgP and RNAP (T), are marked.

A close examination and densitometric analysis (data not shown) of the autoradiograph depicted in Figure 5.5 did indicate a small but significant accentuation of heparin-sensitivity for the Lys-associated ternary complex compared to that of the Arg-associated one in the absence of NTPs. Thus, a smear extending from the region of the ternary complex band to that of the binary (ArgP-DNA) complex was seen in the lanes for Arg-supplementation (lanes 3-5) but not in those for Lys-supplementation (lanes 7-9). These results indicate that, in the absence of NTPs, the Lys-associated ternary complex had completely dissociated in solution itself upon heparin addition whereas a significant fraction (20%) of the Arg-associated ternary complex had persisted in solution and underwent dissociation only during electrophoresis. Thus, although in broad terms the ternary complex assembled in presence of either Arg or Lys was heparin-sensitive without addition of NTPs, the sensitivity was more pronounced with Lys than it was with Arg.

5.2.4.2b Addition of NTPs renders ternary complex of ArgP-RNAP-*argO* resistant to heparin:

Evidence exists for various promoters wherein addition of NTPs stabilizes melting of –10 region and hence the open complex formation (Ross et al. 1999; Leoni et al. 2000). Reports have also suggested that NTPs especially that corresponding to +2 position drives the open complex formation and provides the space for +1 NTP to be incorporated and hence stabilizing the open complex in T7 promoter (Stano et al. 2002). There are *E. coli* promoter, which are dependent on NTP for stable open complex formation, and *leuV* is one such example where the promoter forms an unusually heparin-sensitive complex with RNAP which is stabilised with the addition of NTPs (Ross et al. 1999).

In order to investigate if there is any dependency on NTPs for the opening of DNA during transcription initiation and stable open complex formation in case of *argO* promoter, the heparin challenge experiment (15 and 30 min) was undertaken of ternary complex assembled in presence of NTPs. Both with Arg and with Lys addition, the ternary complex of ArgP and RNAP assembled on *argO* (represented by the super-shifted band in EMSA) exhibited heparin-resistance in presence NTPs, and the patterns in the two cases were roughly similar. [all four NTPs were added in case of Lys, but only three (ATP, UTP, CTP) in case of Arg supplementation, to prevent run-off transcription in the latter instance] (Fig. 5.6, compare lanes 2-4 with 7-9).

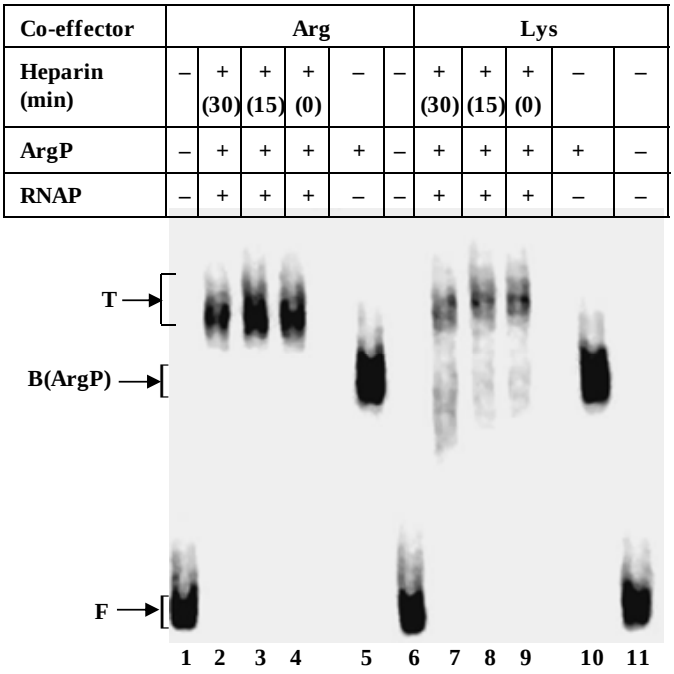


Figure 5.6. Heparin challenge experiment in presence of NTPs. Electrophoretic mobility experiment of radiolabelled *argO* fragment in absence or presence (as indicated) of 20 nM ArgP (monomer), 80 nM RNAP, and co-effectors Arg or Lys at 0.1 mM., in presence of 0.5 mM NTPs in all reactions. Bands corresponding to free DNA (F), DNA in binary complex with ArgP [B(ArgP)] or RNAP [B(RNAP)], and that in ternary complex with ArgP and RNAP (T), are marked.

As described in the previous Section for heparin-sensitivity in absence of NTP addition, in the experiments involving heparin addition in presence of the NTPs as well (Fig. 5.6), it was observed that the Arg-associated ternary complex was somewhat more heparin-resistant than the Lys-associated one. Thus, the band patterns were suggestive of greater instability during electrophoresis of the ternary complex assembled in presence of Lys (see lanes 7-9, in which diffuse bands corresponding to the ArgP-DNA binary complex are visible and the bands representing the ternary complex are less intense) compared to that in presence of Arg (lanes 2-4). It was estimated from densitometric analysis (data not shown) that approximately 25% and <5%, respectively, of the Lys- and Arg-associated ternary complexes assembled in presence of NTPs underwent dissociation during gel electrophoresis following treatment with heparin. Nevertheless, these data broadly support the conclusion that the Lys-associated ternary complex is indeed largely resistant to heparin in presence of NTPs, hence rendering unlikely the possibility that RNAP trapping in the presence of ArgP and Lys is at any step prior to formation of the open complex.

Experiments were attempted to determine the patterns of heparine-sensitivity/resistance at *argO* in the presence of RNAP, ArgP, Arg, or Lys as co-effector, and various concentrations of one, two or three added NTPs. However the results obtained from these experiments were not clearly interpretable, one possible reason of which could be that the NTP preparation were not sufficiently pure and were contaminated with other NTP species.

5.2.4.2c $KMnO_4$ probing for open complex formation at *argO* promoter:

As described above and Chapter 1, open complex formation is a major rate-determining step in the process of transcription initiation with *E. coli* RNAP (reviewed in McClure

1985 and Browning and Busby 2004), and a large number of promoters are regulated at this step (McClure 1985; Green and marshall 1999; Yang et al. 2000; Caramel and Schnetz 2000). $KMnO_4$ has been widely used as a probe to detect open complex formation by RNAP binding to promoters (Sasse-Dwight and Gralla 1989, 1991; Sullivan et al. 1997). $KMnO_4$ reacts preferentially with pyrimidines when the DNA strands in the duplex are melted (that is, rendered single stranded). $KMnO_4$ footprinting provides direct information as to location and the degree of distortions in the open complex.

Following the finding above that ArgP and RNAP bind concurrently to the *argO* promoter to form a ternary closed complex in presence of Arg or Lys and that the complex becomes heparin-insensitive in presence of NTPs, the $KMnO_4$ probing experiment was first undertaken in vivo to investigate the open complex formation at *argO* promoter. For this purpose, cultures of the *argP* null strain and wild-type (*argP*$^+$) strain that had been grown in presence and absence of Arg or Lys were treated with rifampicin (in order to trap RNAP at open complexes), treated with $KMnO_4$, and the extracted DNA was digested with *HaeIII* and subjected to primer extension analysis in the *argO* promoter region as described in Chapter 2 Section 2.2.4.15.

As shown in Figure 5.7, the results from these experiments indicated that melting of the –10 region of the *argO* promoter is more prominent in the *argP*$^+$ strain with sensitive sites were at –13, –11, –9, +1 and +4 than in the *argP* mutant (compare lane 1 with lanes 2-4). More significantly, for the *argP*$^+$ strain, the extent of opening of the –10 region in cells grown with 1 mM Lys supplementation (when *argO* transcription is inactive) was similar to that in cells grown with 1 mM Arg supplementation (when *argO* transcription is induced) (compare lanes 3 and 4). (It may be noted that the

pattern observed in lane 2, for the culture grown without Arg or Lys supplementation, would represent the effects of the endogenous amino acid pools in the cells.) These results (obtained on *argO* in vivo) provided strong support to the conclusion drawn from the heparin-resistance studies in vitro that RNAP is not impeded for the formation of an open complex at *argO* in presence of ArgP and Lys.

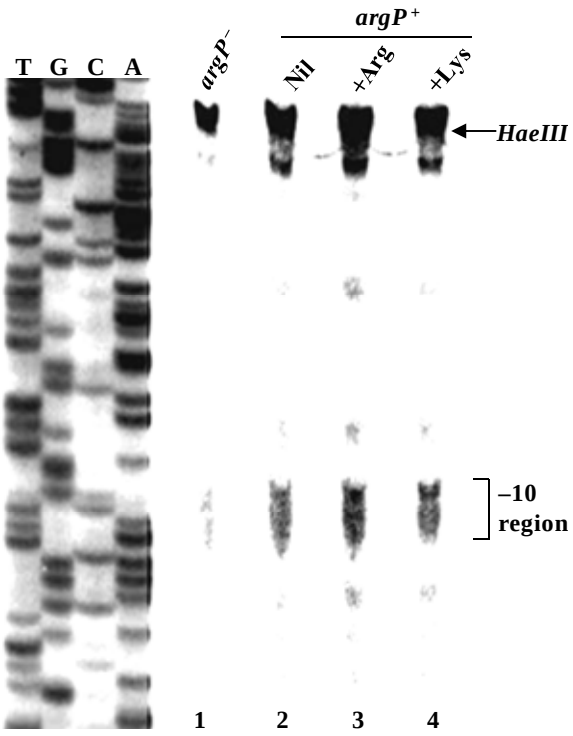


Figure 5.7. Probing of open complex formation at *argO* by in vivo $KMnO_4$ footprinting. The experiment was performed with the cultures of *argP* mutant (*argP*$^-$) and *argP*$^+$ strain grown in 0.2% glucose-minimal A medium without (Nil) or with 1 mM Arg or Lys supplementation as indicated, and then treated with rifampicin. The corresponding di-deoxy sequence ladder of *argO* is represented alongside, and the –10 region of the *argO* promoter as well as the *HaeIII* truncation site at –80 are marked. (Since DNA extracted from the cultures was digested with *HaeIII*, the bulk of the primer extension products were terminated at the –80 *HaeIII* site in *argO*, and this band at –80 was used to normalize for the amount of DNA in the different lanes.)

To further confirm the findings above, the $KMnO_4$ modification and probing experiments were also undertaken in vitro, in presence of either Arg or Lys. Similar results to those from the in vivo experiments were obtained. The opening of –10 region of *argO* in presence of NTPs and ArgP along with either Arg or Lys was observed, and there was no difference in the band pattern between the two co-effector additions (data not shown). These results suggested that the open complex formation in *argO* promoter is independent of whether Arg or Lys is added as co-effector to ArgP.

5.2.5 In vitro transcription of *argO* in presence of dinucleotide, ApU:

As described earlier, the start site of *argO* transcription was deduced to be at "A" (sandwiched in the sequence CAT), in the *argO* promoter sequence. In such a case, transcription should be able to start by dinucleotide ApU in absence of UTP and is expected to generate a short transcript of six nucleotides which are italicised in the *argO* sequence C*ATCAGA*TACT (start site underlined). In order to investigate this, a transcription reaction was set up with ApU, ^{32}P-α-CTP, ATP, and GTP in absence of UTP, using a biotin-labelled *argO* template immobilised on streptavidin-coated magnetic beads. After the transcription reaction, an aliquote of the mixture was fractionated into pellet (magnetic beads and associated DNA with bound macromolecules) and the supernatant fraction by magnetic separation. The pellet was chased with a mixture of four NTPs without further addition of RNAP and ArgP, and the products were resolved on a 20% sequencing gel. It was expected that the transcription reaction had been initiated under this experimental condition, a six-nucleotide transcript in the supernatant fraction and the full length transcript in the chased pellet fraction would be obtained. However, as depicted in Figure 5.8, many short transcripts (upto ~40-mers) were observed when either the (unchased) pellet and

supernatant mix (lane 1) or the supernatant alone (lane 2) were loaded, so that the evidence could not be regarded as conclusive. The possible explanation for the generation of various short transcripts can be that the ApU used might have been contaminated with UTP. This was supported by the data in lane 3 (of the pellet following chase with NTPs), wherein full length products were observed along with small amounts of shorter transcripts indicating that the RNAP had been bound and that the transcript synthesis had already been initiated in the pre-chase mixture.

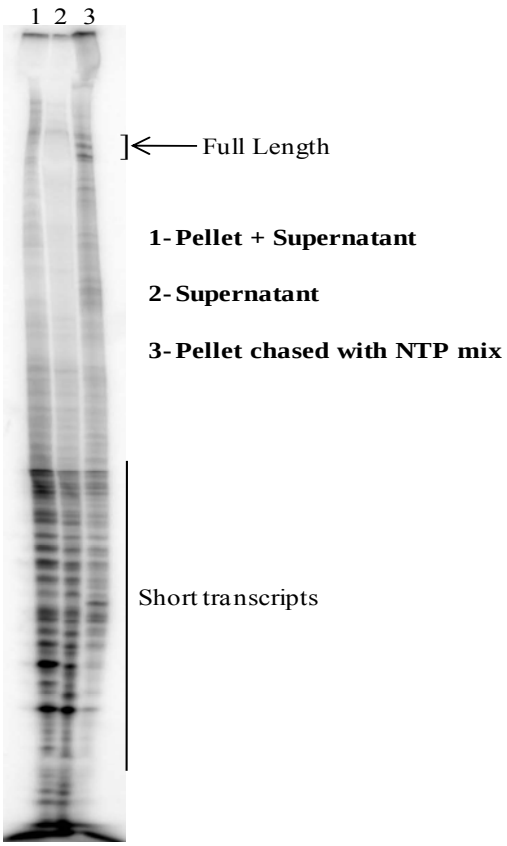


Figure 5.8. In vitro transcription with dinucleotide, ApU. The transcription reaction was carried out with biotin-labelled *argO* template in presence of streptavidin-coated magnetic beads. For lanes 1 and 2, transcription reaction was carried out in presence of ApU, ATP, GTP and α-^{32}P-CTP but without UTP. For lane 3, the pellet of lane 1 was chased with NTPs (GTP, ATP, UTP and α-^{32}P-CTP).

5.2.6 The Promoter Escape Step:

5.2.6.1 Abortive RNA synthesis at *argO* promoter:

Following open complex formation, the next step in transcription initiation is the iterative synthesis and release of abortive products, which precedes the escape of RNAP from the promoter (reviewed in Hsu 2002). Abortive transcription is one of the major steps at which regulation can be mediated by other accessory proteins. A majority of the examples wherein hindrance of transcription at promoter clearance occurs are associated with accumulation of abortive transcripts. To determine why the strong binding of RNAP and open complex formation at *argO* promoter in presence of ArgP and Lys result in no productive RNA synthesis, abortive transcription products from the promoter were analysed. In presence of ArgP and Arg, short transcription products with two prominent abortive transcripts were observed (probably 6-mer and 8-mer) (Fig. 5.9). Surprisingly, there was no accumulation at all of abortive products in presence of ArgP and Lys (compare lanes 3 and 4 in Fig. 5.9). Therefore, notwithstanding the evidence above for open complex formation at the *argO* promoter by RNAP in presence of ArgP and Lys, the in vitro transcription experiments revealed that the absence of run-off transcript synthesis is also accompanied by the absence of synthesis of any short paused or abortive products under these conditions, whereas both abortive as well as full-length transcripts were obtained in presence of ArgP and Arg.

Figure 5.9. Abortive transcription at *argO* promoter. In vitro transcription products (abortive, run-off from *argO* promoter [RO], and end-to-end [EE], as marked) from the 427-bp *argO* template (with sequence from –293 to +109) in reaction mixes with RNA, NTPs, and various additives (ArgP and co-effectors Arg or Lys) as indicated were subjected to electrophoresis on denaturing 20% polyacrylamide gel.

5.2.6.2 Inverse pulse labeling experiment to check the existence of moribund complex:

Shimamoto and coworkers have suggested the existence of a branch pathway characterised by persistent abortive product synthesis, in addition to the normal pathway of productive transcription. In the branch pathway, the majority of the RNAP-DNA complexes are proposed to be retained in a non-productive form called as moribund complexes (which produce only abortive transcripts but no full-length transcript) whose fate could be either inactivation by further converting into a dead end complex that retains short transcript but has no elongation activity, or reactivation by conversion into a productive complex (Kubori and Shimamoto 1996; Sen et al. 2001; Susa et al. 2002, 2006).

To test the possibility trapping of RNAP in moribund inactive complex in case of *argO* promoter in presence of ArgP and Lys, the abortive synthesis was studied by an inverse pulse labelling experiment as described in Chapter 2, Section 2.2.4.12. This experiment, which had been reported to be a more sensitive means to detect the presence of moribund RNAP-promoter complexes synthesizing abortive transcripts and in which the labelled nucleotide is added at different time points to an in vitro transcription mix already containing all four NTPs (Kubori and Shimamoto 1996; Susa et al. 2006), also failed to detect abortive products from the *argO* template in the presence of ArgP and Lys (data not shown) suggesting that the inhibition mediated by ArgP and Lys at *argO* promoter is not through a moribund complex.

5.2.7 Experiments to test whether Lys-bound ArgP traps RNAP at the initial transcribed sequence (ITS):

The data obtained so far had indicated that the inactive transcription ensemble obtained at the *argO* promoter in presence of ArgP and Lys is explained neither by a defect in isomerisation from closed to open complex nor as a moribund complex that is unable to transit from abortive to productive transcription. The possibility was therefore considered that it represents a paused complex in the ITS, analogous to that characterised in detail for the late gene promoters of the lambdoid phages which are the substrates for action by the cognate antiterminator Q proteins (Roberts et al. 1998; Ko et al. 1998; Marr et al. 2001), as well as for the *lac*UV5 and additional promoters in *E. coli* (Nickels et al. 2004; Brodolin et al. 2004). One feature that lent support to this possibility was the presence of two promoter –10-like hexamer motifs starting, respectively, at +6 and +18 in the ITS region of *argO* (sequence motifs italicised in Fig. 5.11), reminiscent of the situation in the lambdoid phage and *lac*UV5 promoters in each of which a –10-like element in the ITS binds σ^{70} in RNAP and leads to pausing of the holoenzyme. The ITS region and the –10-like elements are depicted in the Figure 5.10.

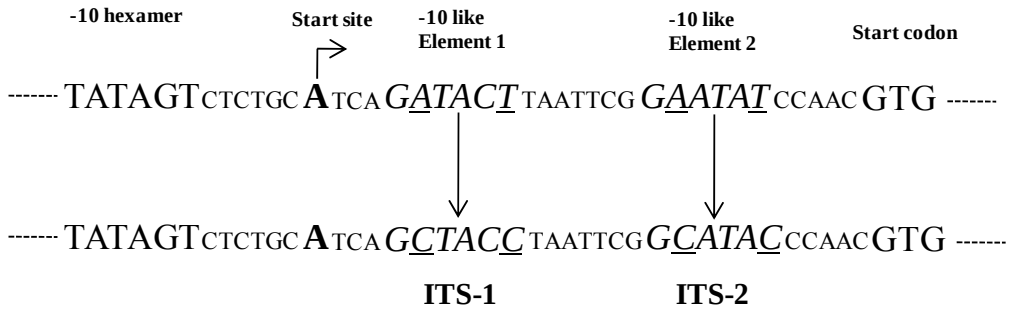


Figure 5.10. Initial transcribed region (ITS) of *argO* from the –10-sequence to the translation start codon of *argO*. The sequence changes to the –10 like elements are indicated in the lower panel (ITS-1 and ITS-2). The –10 like hexamer motifs are italicised along with the changed sequences underlined.

Accordingly, the highly conserved residues A at position 2 and T at position 6 in each of the two –10-like elements of the *argO* ITS were mutated so as to convert one hexamer from GATACT to G*C*TAC*C* (designated ITS-1) and the other from GAATAT to G*C*ATA*C* (designated ITS-2) using QuikChange site-directed mutagenesis kit as described for other mutagenesis experiments in Section 4.2.3, Chapter 4 in order to test whether Lys-mediated inactivation of *argO* transcription is abolished in them. The residues were modified on the plasmid pHYD1722 with pairs of primers, JGA10Lf (5′-AGTCTCTGCATCAGCTACCTAATTCGGAATATCC-3′) and JGA10Lr (5′-GGATATTCCGAATTAGGTAGCTGATGCAGAGACT-3′) for ITS-1 and the generated plasmid was designated as pHYD1735; and with primers JGA10L2f (5′-ATACTTAATTCGGCATACCCAACGTGTTTTC-3′) and JGA10L2r (5′-GAAAACACGTTGGGTATGCCGAATTAAGTAT-3′) for ITS-2 and the resultant plasmid was designated as pHYD1736. The modified fragments from the resultant plasmids were sub-cloned into *PstI* and *BamHI* sites of the *lacZ* reporter gene-bearing promoter cloning vector pMU575 to generate the plasmids pHYD1737 and pHYD1738, respectively. These plasmids were transformed into *argP* mutant strain GJ4892 harbouring the *argP*$^+$ plasmid pHYD915, and the promoter activities were measured in vivo by determining the β-galactosidase acivity with the cultures grown in presence of Arg or Lys, along with appropriate controls. It was observed that neither the mutation represented by ITS-1 nor that by ITS-2 caused any significant loss of ArgP- and Arg- or Lys-mediated transcriptional regulation of *argO* in vivo (Fig. 5.11). With either of the mutant plasmid-bearing strains, the β-galactosidase activity observed was similar to the strain with plasmid pHYD1732 wild-type *argO-lac* in presence of both Arg and Lys, and there was no change in the Lys-mediated inhibition of *argO* transcription.

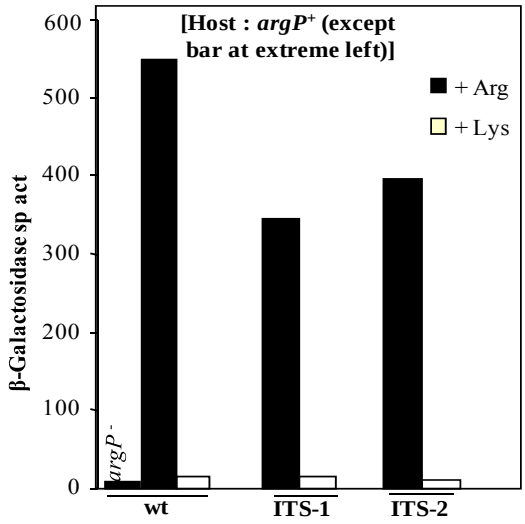


Figure 5.11. In vivo *argO-lac* expression studies using plasmid derivatives carrying mutations in the –10-like hexamer motifs (ITS-1 or ITS-2) of the *argO* regulatory region along with wild-type 427-bp *argO* carrying plasmid. Host strain was *argP*$^+$ (except for bar at extreme left, which was from host with *argP* null mutation).

Likewise, the runoff transcription assay was reconstituted with the mutant fragments in presence of ArgP and Arg or Lys. The results from both in vitro transcription experimments (Fig. 5.12), however, demonstrated that Lys-supplementation in the presence of ArgP leads to a drastic reduction in *argO* transcription even in the case of the two ITS mutants. The Lys mediated inhibition was also not abolished in vitro by the mutation of these –10-like hexamer motifs in ITS of *argO*. These results suggest that Lys-mediated inhibition at *argO* promoter is not by promoter proximal pausing of RNAP at –10-like hexamer motifs in the ITS region of *argO*.

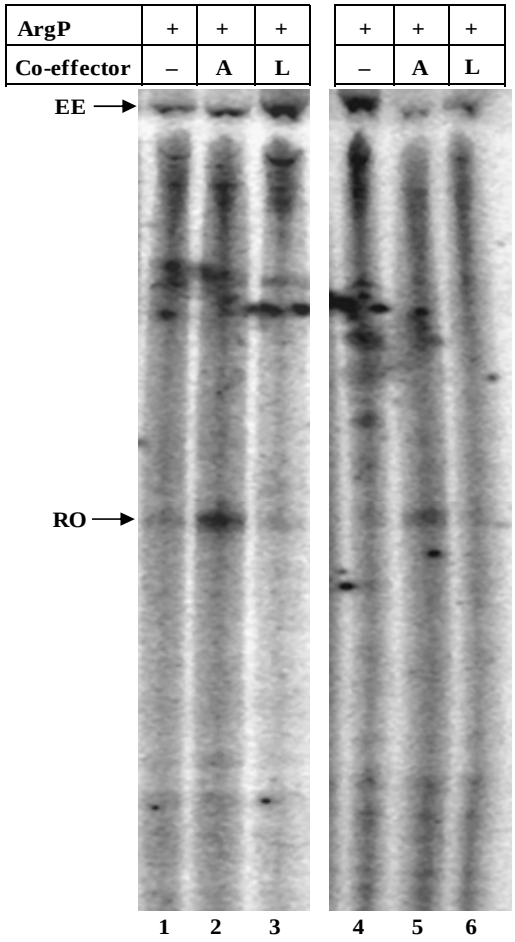


Figure 5.12. Absence of effect of mutations in *argO* ITS on ArgP-mediated *argO* transcriptional regulation in vitro. In vitro transcription reactions were performed on 427-bp *argO* templates (with sequence from –293 to +109) bearing the ITS-1 or ITS-2 mutations described in the text, with various additives as indicated (A, Arg; L, Lys). Transcription products were subjected to electrophoresis on denaturing 10% polyacrylamide gel. RO, run-off transcript from *argO* promoter; EE, end-to-end transcription product.

5.2.7.1 Absence of pause transcripts in the Lys-mediated inactive complex:

To investigate if short transcripts of around 14-nucleotides or 20-nucleotides (which will result from the trapping of RNAP in the –10-like sequences GATACT and

GAATAT in the ITS region of *argO*) were formed, in vitro transcription experiments were carried out in presence of ArgP and Lys and the transcripts were observed on a 20% denaturing sequencing gel. No paused transcripts (of 14 or 20-nucleotides) were detected suggesting also that RNAP is not trapped in the ITS region of *argO* in presence of ArgP and Lys.

5.2.7.2 Effect of GreB addition on the Lys mediated inhibition of *argO* transcription:

Further evidence that RNAP is not trapped in the *argO* promoter proximal region at the −10-like hexamer motifs of the ITS (in presence of ArgP and Lys) was obtained from transcription experiments performed in presence of GreB protein. As shown in Figure 5.13*A* for the in vitro transcription reaction of *argO* in presence of RNAP, ArgP and Lys, GreB (50 nM) addition did not relieve the ArgP- and Lys-mediated RNAP trapping at *argO* (Fig. 5.13*A* compare lanes 3 and 4), contrary to the situation described for the lambdoid and *lac*UV5 promoters (Roberts et al. 1998; Ko et al. 1998; Nickels et al. 2004, Brodolin et al. 2004).

5.2.7.3 $KMnO_4$ footprinting in presence and absence of rifampicin: For a normally transcribing gene, in vivo $KMnO_4$ footprinting patterns in presence and absence of rifampicin are similar whereas in a model of RNAP pausing at the ITS, the two patterns are expected to be different. In case of the *argO* promoter region in an *argP*$^+$ strain grown in cultures supplemented with 1 mM Lys, $KMnO_4$ footprinting in the absence of rifampicin gave a similar pattern to that with rifampicin addition (Fig. 5.13*B*). This is also consistent with the above observations showing that RNAP is not paused in the *argO* promoter proximal region at the −10-like hexamer motifs in the ITS.

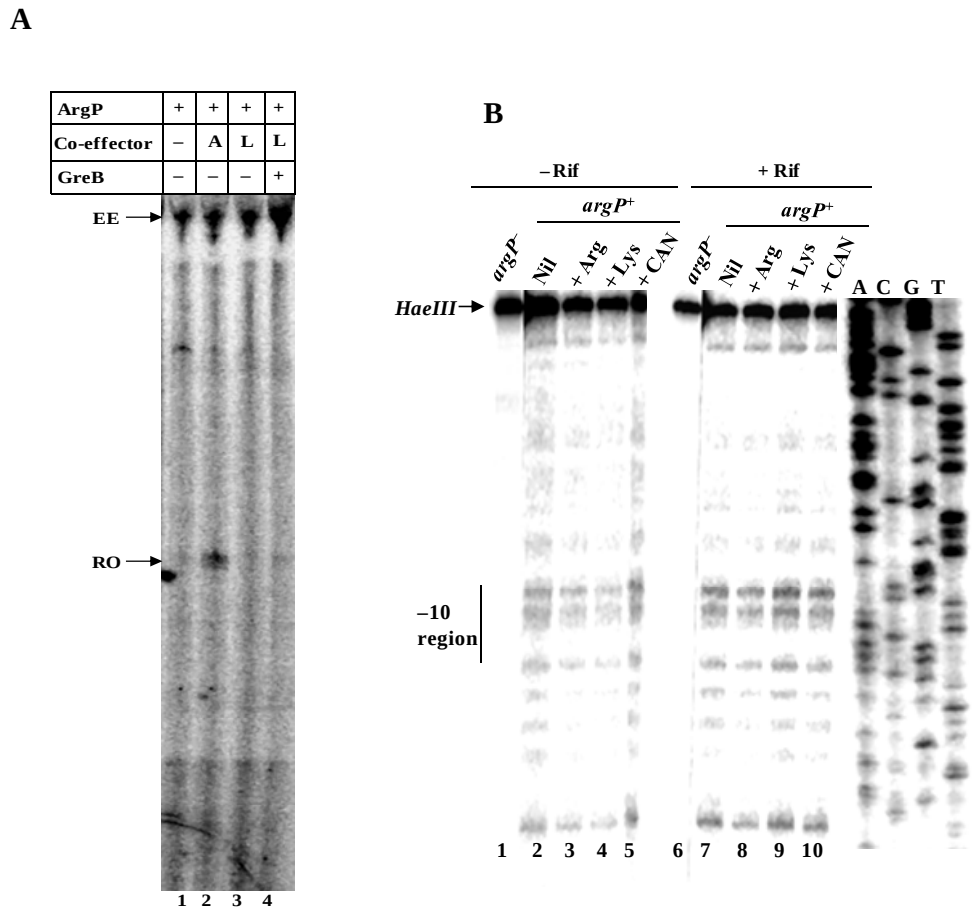


Figure 5.13. Lys mediated inhibition at *argO* promoter is not by promoter proximal pausing of RNAP at the –10 like hexamer motifs in the ITS. (*A*) Absence of effect of GreB on ArgP- and Lys-mediated inhibition of *argO* transcription in vitro. A, Arg; L, Lys; RO, run-off transcript from *argO* promoter; EE, end-to-end transcription product. (*B*) In vivo $KMnO_4$ footprinting at *argO* regulatory region in absence or presence of rifampicin (Rif) at 100 μg/ml, as indicated. Strains, other experimental details, and symbols are as described in the legend to Fig. 4*D*; where indicated, CAN was added to a final concentration of 40 μg/ml. The image shown is of a single gel with samples loaded in the order as marked, but the brightness/contrast of different lanes have each been individually adjusted to normalize for the intensity of the extension product corresponding to the *HaeIII* truncation site.

5.2.8 A novel inactive ternary transcription complex is assembled on the *argO* promoter with ArgP and RNAP in presence of Lys:

Based on the results described in the earlier Section of this Chapter, it could be surmised that the inactive transcription ensemble obtained at the *argO* promoter in presence of ArgP and Lys is explained neither by a defect in isomerisation from closed to open complex nor as a moribund complex that is unable to transit from abortive to productive transcription. Besides the possibility was also ruled out that it represents a paused complex in the ITS, analogous to that characterised in detail for the late gene promoters of the lambdoid phages which are the substrates for action by the cognate antiterminator Q proteins (Roberts et al. 1998; Ko et al. 1998; Marr et al. 2001), as well as for the *lac*UV5 and additional promoters in *E. coli* (Nickels et al. 2004; Brodolin et al. 2004). Therefore it could be envisaged that the paused complex mentioned above, in presence of ArgP and Lys at *argO* promoter, represents a novel inactive ternary complex which has hitherto not been reported so far at any promoter. The following experiments were then undertaken to examine in more detail the nature of this paused complex at the *argO* promoter.

5.2.8.1 Exonuclease III protection to map the RNAP trapped position at *argO* promoter:

To delineate the position of trapped RNAP in the complex assembled in *argO* in presence of ArgP and Lys, exonuclease III protection experiments were undertaken with a pair of DNA fragments spanning the *argO* regulatory region that had been 5′-end-labelled on either the bottom strand towards +109 (Fig. 5.14*A*) or the top strand at –115 (Fig. 5.14*B*) (to detect, respectively, the upstream and downstream edges of protection by protein binding).

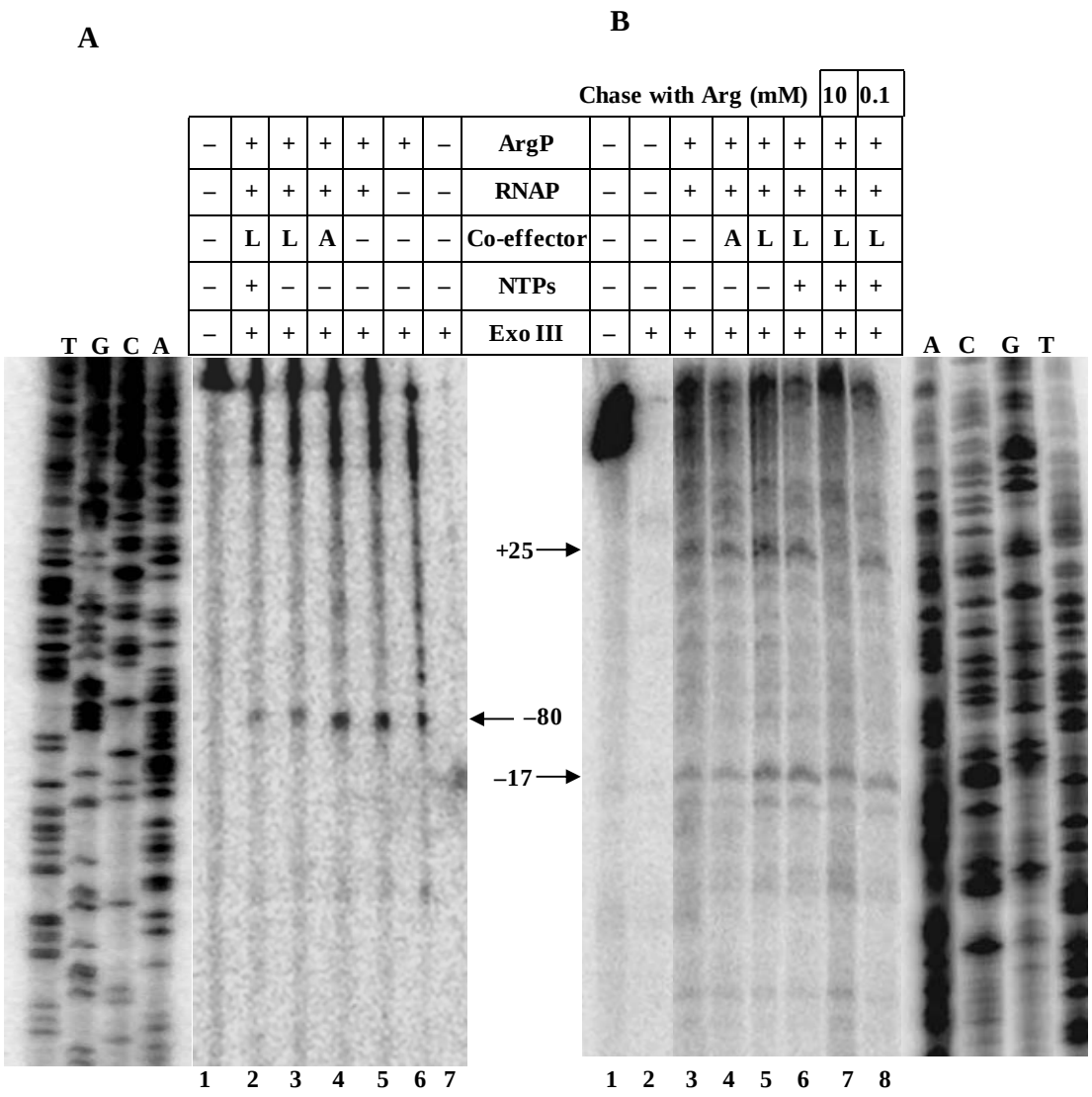


Figure 5.14. Exonuclease III (Exo III) protection assays to determine upstream and downstream edges of protection on *argO* by ArgP and RNAP. *argO* fragments that had been uniquely 5′-end-labelled (as described in *Materials and Methods*) on either the bottom strand towards +109 (*A*) or the top strand at –115 (*B*) were used in reaction mixes with various additives as indicated (A, Arg; L, Lys). The corresponding di-deoxy sequence ladder of *argO* is represented alongside the numbered lanes in each of the two panels. Arrows denote protected bands at the indicated nucleotide positions in the two panels. Lanes 7 and 8 in panel *B* represent the reactions involving chase with Arg (0.1 mM and 10 mM, respectively).

With ArgP alone, the upstream and downstream edges of protection of *argO* were at approximately –80 (Fig. 5.14*A*, lane 6) and –17 (data not shown), respectively. These bands were visible also in reaction mixtures comprising ArgP along with other components (Fig. 5.14*A*, lanes 2-5, and Fig. 5.14*B*, lanes 3-8), suggesting the existence of a subset of molecules in binary complex with ArgP under all the other conditions tested. In addition, another downstream edge of protection at around +25 was observed for reaction mixes with ArgP and RNAP in the absence of co-effectors or with Arg (without NTPs), which represents the footprint of RNAP (Fig. 5.14*B*, lanes 3 and 4). Interestingly, an identical downstream edge of protection at around +25 was also observed for the mixes containing ArgP with RNAP and Lys (both with and without the four NTPs added) (Fig. 5.14*B*, lanes 5 and 6), suggesting that even in the Lys-associated ternary complex which is inactive for both abortive and productive transcription, RNAP is trapped at the step of the open complex itself or immediately thereafter.

5.2.8.2 Arg-chase experiment to test for the conversion of the Lys-associated inactive ternary transcription complex at *argO* to a productive form:

It was further tested whether the inactive transcription complex formed at the *argO* promoter in presence of ArgP and Lys is reversible and can be converted to a productive form upon Arg addition (that is, upon Arg-chase). In one approach where the chase was undertaken in solution, to the pre-incubated mixture of *argO* template (from –293 to +109) with ArgP, RNAP and Lys, was added the labelled substrate-heparin mix to generate a primary reaction mix which was inactive for transcription (Fig. 5.15*A*, lane 3). An aliquot taken from this mix was then chased with an excess (10

mM) of Arg; the presence of heparin in the mix ensured that new binding of RNAP to the template did not occur following secondary Arg addition, and yet productive transcription was obtained (Fig. 5.15*A*, lane 4); in the control experiment in which Lys was omitted from the primary reaction mix, the secondary addition of Arg failed to elicit productive transcription (Fig. 5.15*A*, lane 2).

The exonuclease III protection pattern was also determined following addition of (that is, chase with) either 0.1 mM or 10 mM Arg to a solution mix of *argO* template DNA (5′-end-labelled on the top strand at –115) with ArgP, Lys (0.1 mM), RNAP, the four NTPs, and heparin. Although the band at +25 (representing the downstream edge of RNAP at the *argO* promoter) was still visible upon chase with 0.1 mM Arg, it was abolished upon addition of excess (10 mM) Arg (Fig. 5.14*B*, compare lanes 7 and 8). These data are consistent with the notion that under the latter conditions, RNAP molecules from the entire population of ArgP- and Lys-associated inactive ternary complexes had been released to undergo productive transcription.

In the second approach to test the effect of an Arg-chase, a biotinylated *argO* template (from –293 to +109) was initially incubated with ArgP and RNAP in the absence or presence of Arg or Lys, immobilised on streptavidin-coated magnetic beads, and treated with heparin and the four NTPs. An aliquot of this primary reaction mix was saved for analysis; from the remainder, the beads and supernatant were separated, following which the beads were washed (with Arg-supplemented transcription buffer) and incubated (without fresh ArgP or RNAP addition) with Arg and the four NTPs.

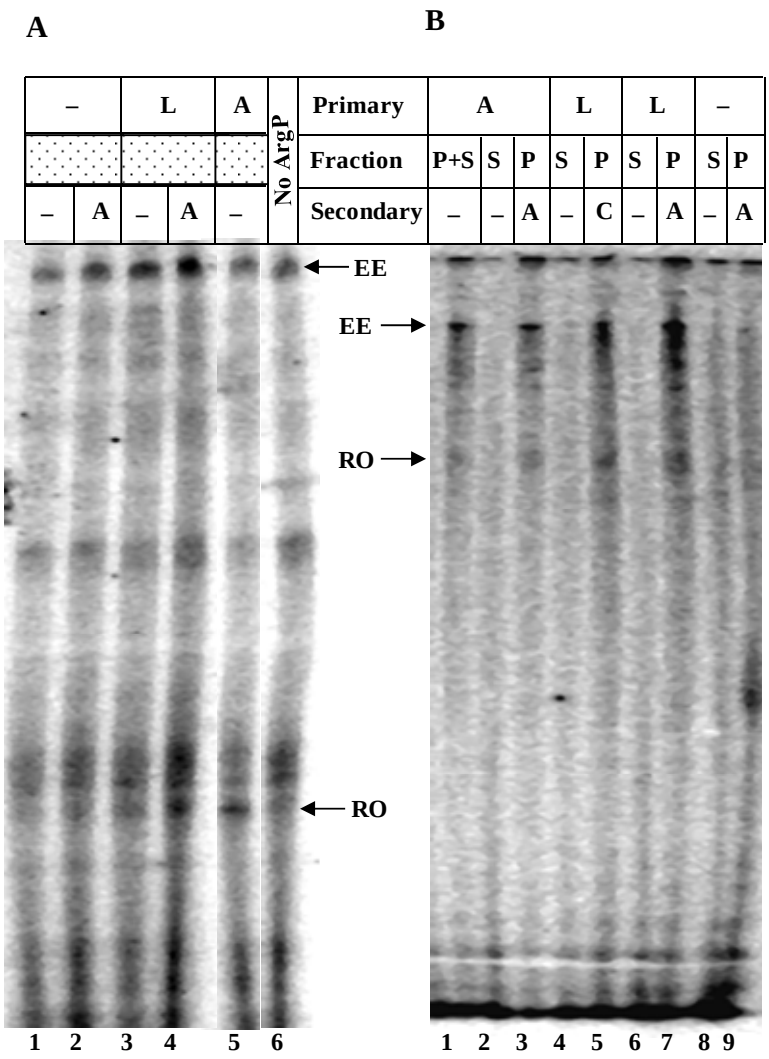


Figure 5.15. Arg (or CAN)-chase, of ArgP-RNAP-*argO* complex assembled in presence of Lys and NTPs, either in solution (*A*) or on streptavidin-coated beads (*B*). For details, see text. Unless otherwise indicated, ArgP was added to all reaction mixes. Other additions were as indicated above the lanes: the primary additives were those that were present in the initial reactions, and the secondary additives were those that were added during the chase step. A, Arg; L, Lys; C, CAN; RO, run-off transcript from *argO* promoter; EE, end-to-end transcription product. In panel *B*, the fractions from the primary reaction mixes are indicated (P, pellet; S, supernatant; P+S, unseparated primary reaction mix) that were used for preparation of samples for loading on the corresponding lanes of the gel. Transcription products were resolved on denaturing 6% (panel *A*) or 10% (panel *B*) polyacrylamide gels.

As expected, both run-off (*argO* promoter-encoded) and end-to-end transcription products were obtained in the total primary reaction mix (that is, of beads and supernatant together) containing Arg (Fig. 5.15*B*, lane 1 and Fig. 5.16, lane 1); however, little if any of these products was released into the supernatant under the conditions of our experiment (Fig. 5.15*B*, lane 2), which is consistent with data from other groups that long RNA products are preferentially retained in the pellet after in vitro transcription on immobilised templates (Kubori and Shimamoto, 1997; R. Sen, personal communication). Again as expected, in the case of each of the total primary reaction mixes with either Lys or no amino acid added, only the end-to-end transcription product (and no promoter-specific run-off transcripts) was obtained (Fig. 5.16, lanes 2 and 3), which too was not released into the supernatant (Fig. 5.15*B*, lanes 4, 6 and 8). On the other hand, both run-off and end-to-end transcripts were observed following secondary addition of Arg and NTPs to the pellet fractions obtained from the primary mixes that had contained Arg or Lys (Fig. 5.15*B*, lanes 3 and 7), but the same did not occur for similarly treated pellet fractions from the primary mix that had neither amino acid added (Fig. 5.15*B*, lane 9). It is possible that, in the case of the sample from the Arg-containing primary mix, at least some of the transcripts observed were those that had been synthesised in the primary reaction itself and retained in the pellet; however, those observed for beads from the Lys-containing primary mix must represent new synthesis following Arg addition in the secondary reaction. Such activation by an Arg-chase could be demonstrated even for complexes that had been incubated with Lys for up to 2 hours in the primary mix (data not shown). CAN addition was also moderately effective in eliciting run-off transcription from the *argO* promoter on immobilised template first treated with Lys and ArgP (Fig. 5.15*B*, lane 5). It is concluded that the transcription complex formed in the presence of ArgP and Lys is in

an inactive yet reversible state and that it can be chased into the productive form with Arg (or CAN) addition.

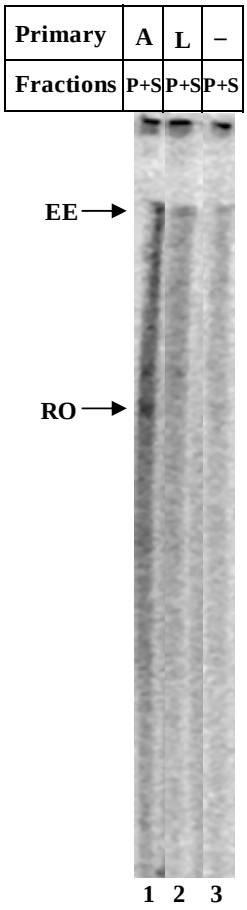


Figure 5.16. In vitro transcription from *argO* template immobilised on streptavidin-coated beads in presence of ArgP, without or with co-effectors as indicated. Symbols are as described in legend to Figure 5.15*B*.

5.3 Discussion:

In vitro transcription data provide confirmatory evidence for the regulation of *argO* by ArgP and indicate that ArgP activates *argO* transcription in presence of Arg and inhibits it in presence of Lys. The various observations can be summarised as follows:

(i) Arg-bound ArgP mediates the recruitment of RNAP to form a ternary complex at *argO* promoter that is proficient for initiation of productive transcription. (ii) Lys-bound ArgP is also enabled for RNAP recruitment at *argO* promoter its isomerisation to open complex which is heparin resistant in presence of NTPs but the complex is associated with neither productive nor abortive transcript synthesis (iii) Lys- and ArgP-mediated inhibition of *argO* is reversible upon an Arg-chase.

5.3.1 A model for ArgP-mediated *argO* transcriptional regulation:

The model of ArgP-mediated regulation of *argO* transcription which emerges from the results described above is the following. In the absence of ArgP, RNAP engages with the *argO* promoter to form a relatively unstable binary complex, resulting in just a low basal level of productive *argO* transcription. The ArgP dimer has a common binding site(s) for its ligands Arg and Lys (and probably also CAN), and is able to bind the sequence between approximately –85 and –20 of *argO* to form a stable binary complex in both its liganded and unliganded states (Chapter 4). However, the binding of unliganded ArgP and of RNAP to the *argO* regulatory region appear to be mutually exclusive, and it is only ArgP in its ligand-bound form which is able to co-operatively bind along with RNAP at *argO* to establish formation of a stable open complex. [It has earlier been speculated that ArgP (IciA) contacts the α-subunit of RNAP in the process of recruiting the latter to different promoters (Lee and Hwang 1997).] Whereas the open complex established by RNAP through the mediation of Arg (or CAN)-bound ArgP is proficient for productive transcription, that mediated by Lys-bound ArgP is trapped in a paused and inactive state. The latter, however, can be chased into the productive state by competitive replacement of the Lys ligand which is bound to ArgP by Arg. That this model is valid for the situation in vivo is suggested by the evidence

obtained for ArgP-dependent open complex formation at the *argO* promoter in cells grown in Lys-supplemented medium, despite the absence of productive *argO* transcription under these conditions.

5.3.2 Rip van Winkle ensemble at *argO* promoter in presence of Lys:

The step after open complex formation at which RNAP is trapped at the *argO* promoter by Lys-bound ArgP is different from other examples of RNAP trapping at bacterial promoters described earlier (reviewed in Hsu 2002), even as a recent report suggests that productive transcription fails to occur from nearly one-quarter of all RNAP-bound promoters in vivo (Reppas et al. 2006). One mechanism of RNAP trapping previously characterised is that involving promoter-proximal pausing of RNAP which is mediated by the binding of σ^{70} to –10-like hexamer motifs present in the ITS region (Roberts et al. 1998; Ko et al. 1998; Marr et al. 2001; Nickels et al. 2004; Brodolin et al. 2004), and as shown above, the Lys-induced transcriptional inactivation at *argO* does not fall into this category. All the other known examples of RNAP trapping (many of which are caused by increased affinity between promoter and RNAP) are associated with increased synthesis of abortive transcripts, and it has been shown in several cases that the GreA/GreB factors are able to revert the paused complex to the productive form (Hsu 2002; Ellinger et al. 1994; Hsu et al. 1995, 2006; Sen et al. 2001; Liu et al. 2004; Susa et al. 2006). On the other hand, no abortive products were observed for the *argO* promoter in the presence of ArgP and Lys, nor was the GreB factor able to restore productive transcription. It can be suggested that, under these conditions, RNAP exists in a dormant "Rip Van Winkle" ensemble after melting of the promoter duplex at the –10 region, at a step that precedes, and is common to, both abortive and productive transcription in which RNAP is reversibly trapped prior to the step of promoter escape.

5.3.3 Possible mechanisms of ArgP- and Lys-mediated RNAP trapping at *argO* promoter:

Results published from some recent studies may provide clues to the nature and mechanism of action of the paused ternary complex of ArgP-RNAP-*argO* assembled in presence of Lys. The deletion of the tip of the σ^{70} region 3.2 loop has been shown to result in defective promoter clearance of the cognate holoenzyme associated with reduced synthesis of abortive transcripts (Kulbachinskiy and Mustaev 2006), and one possibility is that Lys-bound ArgP triggers a similar, but reversible, conformational change in wild-type RNAP in the open complex at *argO* as that observed in binary open complexes with the mutant holoenzyme. Since the role of the σ^{70} 3.2 loop region in transcription initiation appears to be to reduce the K_m for binding of the second templated nucleotide to the *i*+1 site of RNAP (Kulbachinskiy and Mustaev 2006), this model may also explain the findings of this study (Fig. 4*B*, *C*) that the Lys-associated ternary initiation complexes assembled on *argO* are more heparin-resistant in the presence of NTPs than in their absence.

A second model, not mutually exclusive, is based on the recent identification of the phenomenon of DNA scrunching in the transcription initiation (Kapanidis et al. 2006; Revyakin et al. 2006) which is described below. These authors showed that initial transcription proceeds by a scrunching mechanism which invokes contraction of DNA within the open complex (the model for scrunching is shown in Figure 5.17 as depicted in Revyakin et al. 2006). According to this mechanism, for each cycle of initiation, RNAP unwinds downstream DNA and pulls it into itself (1-bp per phosphodiester bond formed) towards the active centre accommodating the pulled DNA as single strand bulges in the unwound region. For each base-pair pulled, a base-pair is broken on the

DNA strand (shown by the additional looped-out region on each of the two DNA strands within the open complex in the right half of Fig. 5.17).

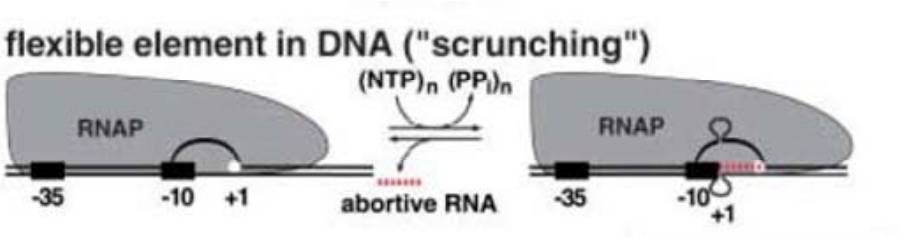


Figure 5.17. Model of DNA scrunching in transcription initiation (from Revyakin et al. 2006)

The scrunched complex represents a "stressed intermediate" in initial transcription wherein the energy stored in the intermediate provides the driving force for abortive initiation as well as for promoter escape and productive transcription. The stress in this complex is resolved in one of the two ways; one, by release of the abortive RNA product, after extrusion of the scrunched DNA and reformation of the unstressed open complex by retaining interaction of RNAP with the promoter; and second, by retaining the RNA product and breaking the interactions of RNAP with promoter, leading to enzyme translocation and transition to the elongation complex (promoter escape) (Kapanidis et al. 2006; Revyakin et al. 2006). Based on this model, it can be suggested that the RNAP complex at *argO* in presence of ArgP and Lys is blocked in the step of DNA scrunching and therefore, that both the abortive and productive transcripts are not synthesised.

5.3.4 Physiological relevance of *argO* regulatory mechanism

What is the likely physiological importance of this mode of *argO* transcriptional regulation, wherein ArgP mediates *argO* induction in presence of Arg and reversible

inactivation in presence of Lys? The answer is at present unclear, but it was earlier proposed that the ArgO exporter performs two functions in *E. coli*, namely, that of exporting CAN which is a plant-derived naturally occurring antimetabolite, and that of excreting Arg so as to maintain an optimal ratio of the intracellular Arg to Lys pools (rather than simply to keep the cytoplasmic Arg level below an arbitrary absolute value) (Nandineni and Gowrishankar 2004). Both functions are expected to be self-limited by feedback regulation, since the reduction in Arg or CAN concentrations consequent to their export through ArgO would be in turn lead to the shut-off of *argO* transcription.

One could speculate that, with the particular mechanism of Lys-mediated regulation of *argO* transcription that has been identified in this study, the cells are poised to switch rapidly between transcription-activating and -inactivating complexes at the *argO* promoter depending upon instantaneous changes in the intracellular Arg to Lys ratios. If, in such a situation, the ArgO exporter were also to exhibit a rapid turnover, then the transcription switch would result in instantaneously tunable rates of Arg export out of the cells. The findings from the present study, that the K_d for Arg binding to ArgP is relatively high (around 150 μM) (Chapter 3), is consistent with the notion of avoidance of futile cycling of Arg uptake and export across the cytoplasmic membrane, so that Arg export would occur only when the twin conditions of high intracellular Arg and low intracellular Lys concentrations are simultaneously satisfied.

CHAPTER 6

Characterisation of canavanine resistant argP mutations

6.1 Introduction:

Previous Chapters had shown that ArgP controls the transcription of *argO* (which codes for an Arg exporter protein that also exports CAN) in vitro and, that Arg activates and Lys inhibits the expression of *argO* (mediated through ArgP). The finding that ArgP controls *argO* expression explains the earlier report that null mutation of *argP* (*argP*::Kan) exhibit more sensitivity to CAN [CAN supersensitive (CAN^{SS})] than the wild-type $argP^+$ strain.

Nandineni and Gowrishankar (2004), isolated seven gain of function *argP* missense mutations which render the strain CAN resistant (CAN^R). All but one mutation were reported to be trans-dominant for the CAN^R phenotype over the wild-type $argP^+$. The list of plasmids carrying the CAN^R *argP* mutations are described in Table 6.1.

In this Chapter, it is sought to characterize the various CAN^R *argP* mutants. This Chapter also describes the study of one representative CAN^R mutant protein $ArgP^d$-S94L in vitro to investigate the mechanism of *argO* regulation by mutant $ArgP^d$.

Table 6.1 List of previously isolated CAN^R *argP* alleles[a]

Plasmid	Dominance/Recessivity	Amino acid change
pHYD926	D	S94L
pHYD927	D	P108S
pHYD928	D	V144M
pHYD929	D	P217L
pHYD930	D	L294F
pHYD931	R	R295C
pHYD932	D	A68V

a- These mutations are described in Nandineni and Gowrishankar 2004.

6.2 Results:

6.2.1 CAN^R *argP* alleles and the *argO* expression:

In order to investigate the effect of CAN^R *argP* mutants on *argO* expression in vivo, β-galactosidase assays were performed in derivatives of strain GJ4892 (that has an *argP* null mutation on the chromosome) carrying the *argO-lac* fusion on plasmid pHYD1723 and the various CAN^R *argP* mutant plasmids (pHYD926 to pHYD932); a derivative with the *argP*$^+$ plasmid pHYD915 was used as control in this experiment. The β-galactosidase specific activities are shown in Table 6.2.

Table 6.2: β-galactosidase specific activity (in Miller Unit) of *argO:lacZ* in presence of various CAN^R *argP* mutant alleles in presence and absence of Arg or Lys

argP plasmid in GJ4892/pHYD1723 (allele)	β-galactosidase Sp act (U) in medium with			Inferred mutant Class
	Nil	Arg	Lys	
pHYD915	90	390	10	WT
pHYD926	1020	980	1150	I
pHYD927	300	330	180	III
pHYD928	1000	1050	100	II
pHYD929	900	860	600	III
pHYD930	950	980	895	I
pHYD931	120	130	95	IV
pHYD932	710	900	680	I

The control strain with the plasmid pHYD915 showed the expected regulation of *argO* by ArgP-dependent induction by Arg or CAN and repression by Lys. The CAN^R *argP*

mutant allele, *argP*^{d}-S94L on plasmid pHYD926, also conferred the expected phenotype in that it rendered the *argO* expression constitutive and independent of co-effectors Arg or Lys. In strains with the other CAN^{R} *argP* mutant plasmids, *argO* expression levels varied over a wide range, although one common feature of all the mutants was that the values of β-galactosidase specific activity were higher than that in the *argP*$^{+}$ control (Fig. 6.1).

The *argP*^{d} mutants could be devided into three classes on the basis of sensitivity of *argO* expression to Lys.

1. Those CAN^{R} mutants which render *argO* expression independent of inhibition by Lys. These mutants include *argP*^{d}-S94L (pHYD926), L294F (pHYD930), andA68V (pHYD932). This class of mutants causes *argO* expression constitutive (or, nearly so) and higher than that with wild type *argP*$^{+}$. For one of the mutant (A68V), there is slight increase in *argO* expression in presence of Arg or CAN (from around 700 units to 900 units).

2. Those mutants in which *argO* expression is substantially inhibited by Lys. The representative class of this mutant is V144M (pHYD928). This mutant behaves like wild-type *argP*$^{+}$, pHYD915 in showing a decrease in *argO:lacZ* expession upon Lys addition. However, unlike *argP*$^{+}$, there is no effect of Arg or CAN on *argO* expression.

3. Those mutants in which *argO* expression is feebly affected by Lys. These mutants are P108S (pHYD927) and V144M (pHYD929). Both mutants are characterized by independence of Arg or CAN addition.

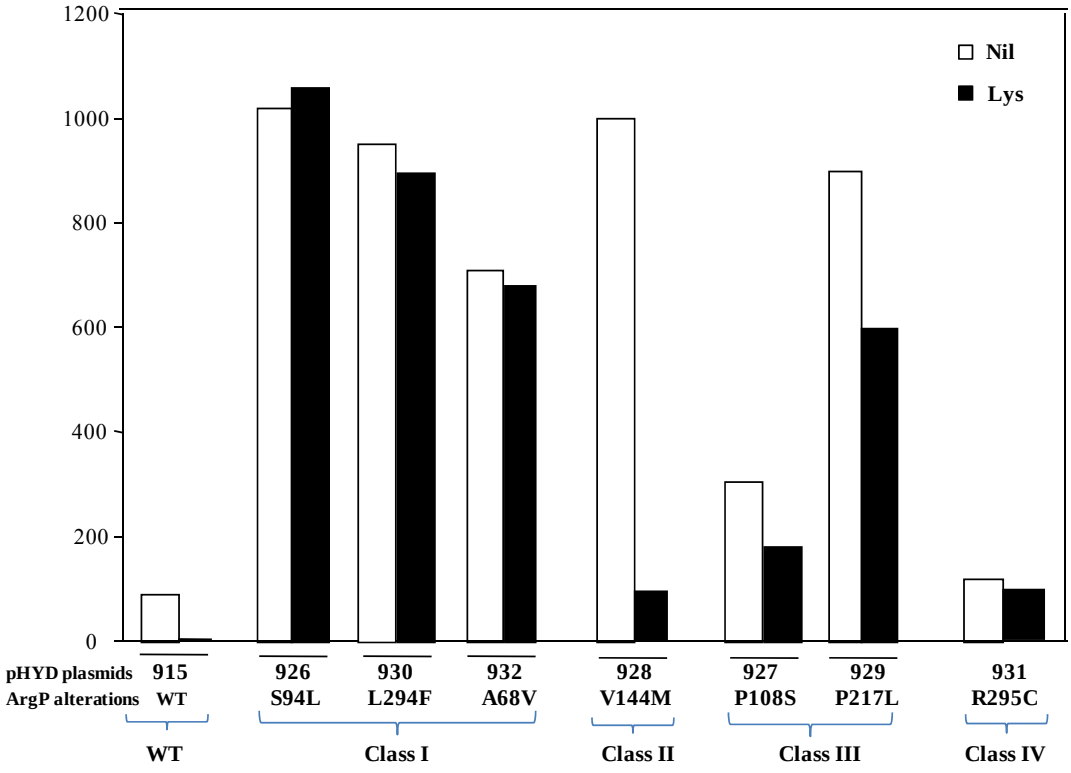


Figure 6.1. Different classes of CAN^R *argP* mutants based on the sensitivity to Lys for in vivo *argO* expression. Histograms represent the β-galactosidase specific activity of *argO:lacZ* measured from chromosomal *argP*$^-$ strain transformed with plasmids carrying wild-type (*argP*$^+$) or various CAN^R *argP* mutations as indicated, grown in absence or presence of Lys.

The remaining CAN^R *argP* allele present on pHYD931 (R295C), is recessive to wild-type and the expression of *argO* in presence of this plasmid was very different from that in any of the other mutants (Class IV in Fig. 6.1). In absence of co-effectors the level of *argO:lacZ* expression in presence of this plasmid was similar to that in the wild-type strain, but the expression was now rendered independent of any co-effector (Arg or Lys) addition. Thus the β-galactosidase specific activity was around 120 MU both in absence and in presence of the co-effectors.

6.2.2 Reconstitution of *argO* transcription in vitro in presence of ArgP^{d} (S94L):

A novel mechanism of *argO* transcriptional regulation was described in the previous Chapter, in which the open complex of RNAP at the *argO* promoter assembled in presence of ArgP and Lys was trapped or poised in an inactive ensemble, that is reversible to an active complex upon Arg addition. In an attempt to obtain additional mechanistic insights into this phenomenon, the mechanism of *argO* regulation by ArgP^{d} was sought to be investigated. For this purpose, one representative ArgP^{d} mutant (S94L), which causes constitutive expression of *argO,* was chosen for in vitro studies. In the following Sections of this Chapter, unless otherwise indicated, ArgP^{d} is used to refer to the S94L CAN^{R} trans-dominant *argP* mutant.

6.2.2.1 Preparation of *argP^{d}* construct for overexpression of ArgP^{d} protein:

For use in the in vitro experiments, the ArgP^{d} protein [with a C-terminal hexa-histidine (His_6) tag] was over-expressed and purified as described for ArgP protein (Chapter 3). First, the *argP^{d}* gene was amplified from the plasmid pHYD926 by PCR using JGARGP1r (5′- AGCAGACA ACA*CATATG*AAACGCCCGGA-3′) as forward primer and JGARGP3r (5′-ATTATTTGAT *CTCGAG*ATCCTGACGAAG-3′) as reverse primer, the PCR product was digested with *NdeI* and *XhoI* and cloned at *NdeI* and *XhoI* sites of pET21b (Novagen) expression vector to incorporate the hexa-histidine tag (His_6- tag) at the C-terminal end of the protein (the restriction sites are italicized in the oligonucleotide sequence in the parenthesis). The resultant plasmid was designated as pHYD1715. The cloned *argP^{d}* sequence was confirmed by sequencing. Complementation experiments established that the His_6-tag did not affect the function of the protein in conferring high and constitutive *argO-lac* expression in vivo (data not shown).

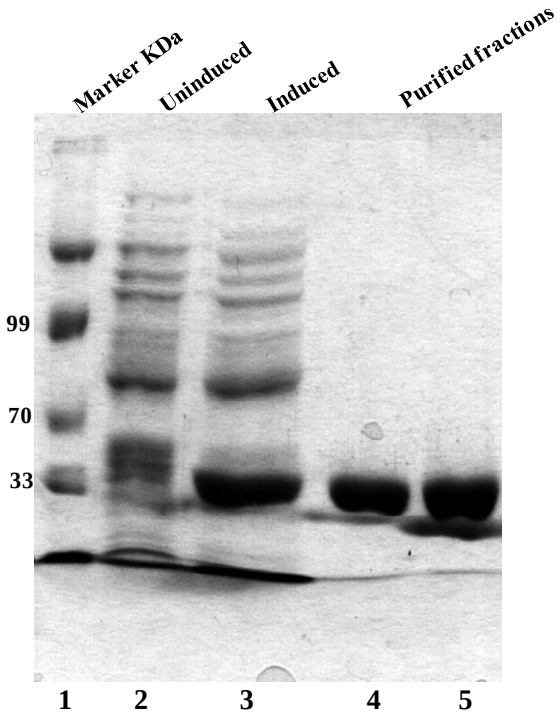


Figure 6.2. Overexpression and purification of His_6-$ArgP^d$, demonstrated by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis. Lanes 2 and 3 represent, respectively, extracts of induced and uninduced cultures of the strain carrying the plasmid construct for His_6-$ArgP^d$ overexpression. Lanes 4 and 5 represent purified His_6-ArgP fractions obtained by elution of proteins bound to Ni-NTA column. Lane 5, protein molecular-weight markers of sizes in kilodaltons (KDa) as indicated alongside.

6.2.2.2 Overexpression and purification of $ArgP^d$:

The plasmid construct of His_6-$ArgP^d$, pHYD1715, was transformed into strain BL21(DE3) and an ampicillin resistant (Amp^r) colony was inoculated in LB medium supplemented with 100 µg/ml Amp. The overnight grown primary culture was sub-cultured in 1:100 dilution and at an A_{600} of around 0.5, was induced with 1 mM isopropyl thio-β-D-galactoside (IPTG). The culture was harvested 3-hrs after the induction and processed for preparation of His_6-ArgP protein as follows. The harvested

cells of BL21(DE3)/pHYD1715 following IPTG induction were lysed, and the lysate was passed through a Ni-NTA affinity chromatography column in which the His-tagged protein was expected to be retained by non-covalent interaction (chelation) between Ni^{2+} in the column and the His-residues of the protein. The bound protein was eluted from the column with buffer containing 250 mM imidazole. Figure 6.2 shows the over-expression and purified fractions of $ArgP^d$ as determined by SDS-PAGE.

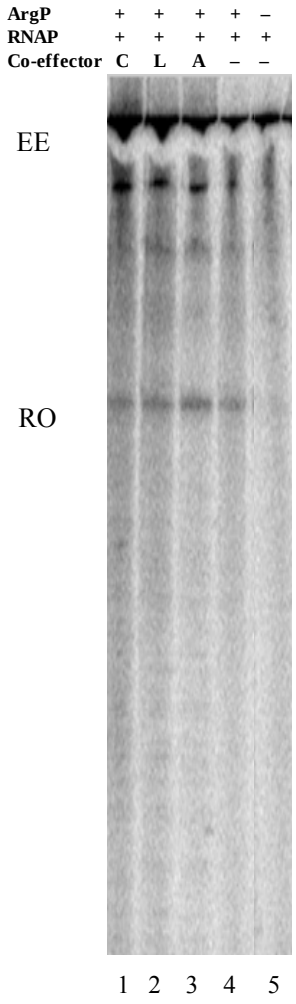


Figure 6.3. $ArgP^d$-mediated regulation of *argO* transcription. Radiolabelled in vitro transcription products obtained with the 427-bp *argO* template (with sequence from –293 to +109), in reaction mixes with various additives as indicated, and subjected to electrophoresis on a denaturing 10% polyacrylamide gel. RO, run-off transcript from *argO* promoter; EE, end-to-end transcription product; A, Arg; L, Lys; C, CAN.

6.2.3 In vitro *argO* transcription mediated by ArgP^{d}:

ArgP^{d}-mediated regulation of *argO* transcription was tested in a defined in vitro run-off transcription system using purified ArgP^{d} protein, σ^{70}-bearing RNAP holoenzyme, and a linear DNA template that encompasses the *argO* region from –293 to +109. As shown in Figure 6.3, bands representing run-off transcript of equal intensity were observed in all the lanes, except the lane where ArgP^{d} was not added. These data are consistent with those from the in vivo studies that ArgP^{d} renders *argO* transcription constitutive.

6.2.4 Interaction of Arg and Lys with ArgP^{d}:

As described in Chapter 3, circular dichroism (CD) and fluorescence spectroscopy studies had indicated that ArgP interacts with both co-effectors Arg and Lys. Similar studies were undertaken to investigate if ArgP^{d} protein interacts with Arg and Lys. CD experiments demonstrated no change in the spectral peaks of ArgP^{d} protein upon Arg or Lys addition (data not shown). Besides, gel filtration experiments in presence of Arg or Lys showed no change in the chromatogram (data not shown), indicating that there is no change in the oligomeric state (dimer) of this mutant protein and perhaps also that these co-effectors do not interact with the protein. Then, fluorescence spectrum of ArgP^{d} was measured in presence of increasing concentrations Arg or Lys. The ArgP^{d} protein was excited at a wavelength of 295 nm where only Trp residues will absorb. The emission spectra were recorded in the range of 300-500 nm and a scan speed of 50 nm/s. Protein used was at a concentration of 1 μM. There was slight but insignificant increase in the fluorescence spectra of ArgP^{d} upon addition of increasing concentration of the co-effectors (from 0 to 1 mM), Arg (Fig 6.4*A*) or Lys (Fig. 6.4*B*). These results

also suggest that ArgP^{d} might not directly interact with Arg or Lys, and can explain the finding that this protein acts independently of co-effectors for *argO* expression.

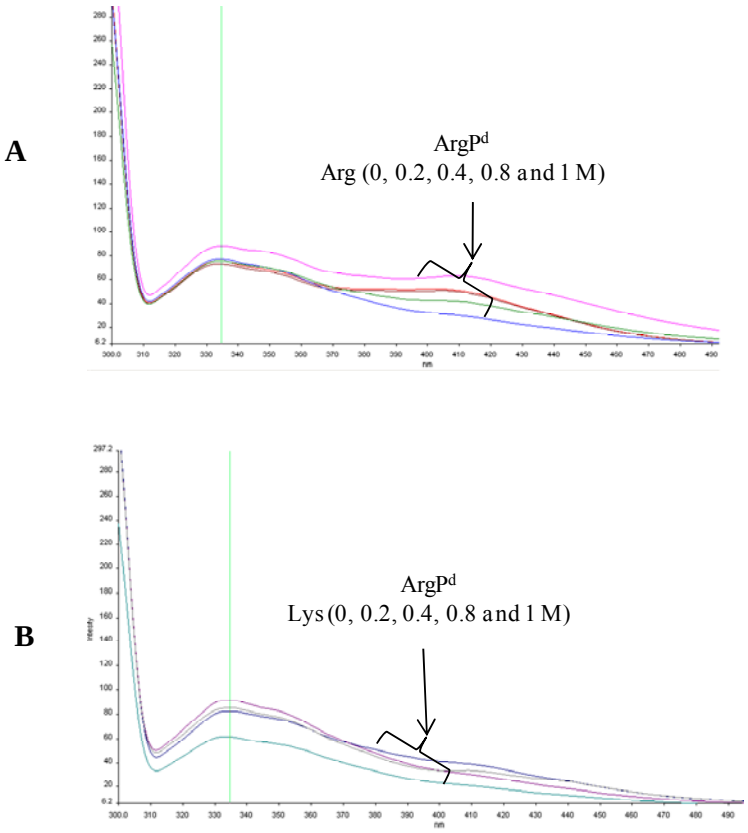


Figure 6.4. Fluorescence emission spectra of ArgP^{d} in presence of increasing concentrations of Arg (*A*) or Lys (*B*). The various peaks of ArgP^{d} in presence of increasing Arg or Lys concentrations (from 0 to 1 M) are indicated in the respective spectra.

6.2.5 ArgP^{d} interaction with *argO* regulatory region:

To investigate the mechanism of ArgP^{d}-mediated *argO* regulation, the purified ArgP^{d} protein [with C-terminal hexa-histidine (His_6) tag] was first tested for its ability to bind *argO* regulatory region by an EMSA experiment using the 427-bp fragment (end labelled) comprising the sequence from –293 to +109 of *argO*. The reaction products

were resolved by 5% non-denaturing polyacrylamide gel electrophoresis. Unexpectedly, as shown in the Figure 6.5 (lanes 1 to lane 4), no retardation by $ArgP^d$ of the *argO* radiolabelled DNA either in absence (lane 2) or presence of Arg (lane 3) or Lys (lane 4). This suggests that, although the protein renders the transcription of *argO* constitutive, it may not interact with the *argO* DNA in the absence of RNAP. This possibility was further examined in additional experiment as described below.

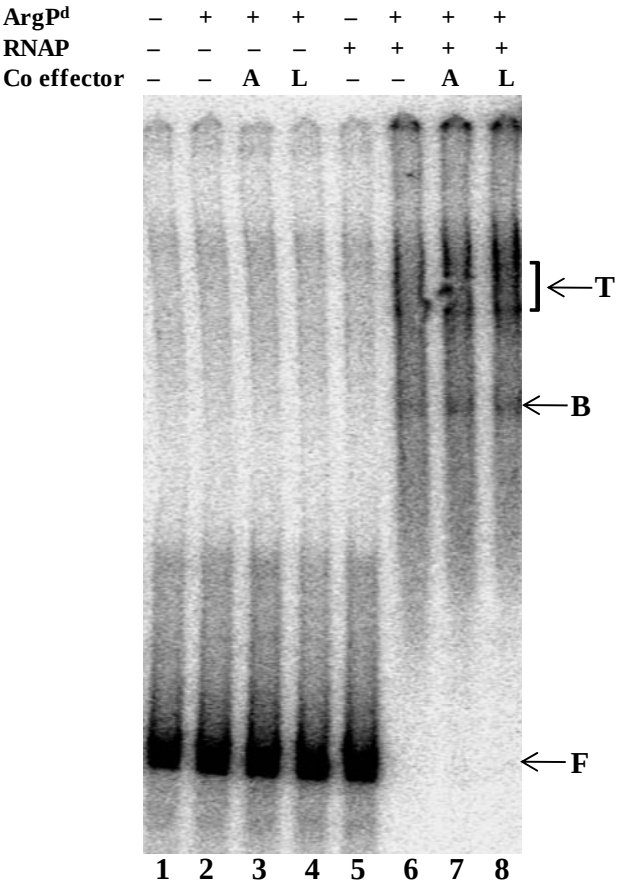


Figure 6.5. Binary interaction of $ArgP^d$ with *argO* regulatory region (lanes 1 to 4) and ternary interaction of $ArgP^d$ and RNAP with *argO* regulatory region (lanes 5 to 8). Electrophoretic mobility of radiolabeled *argO* fragment in absence or presence (as indicated) of 20 nM $ArgP^d$ (monomer concentration), 80 nM RNAP, and co-effectors Arg, Lys, or CAN at 0.1 mM. Bands corresponding to free DNA (F), DNA in binary complex, B (with $ArgP^d$ or RNAP), and that in ternary complex with $ArgP^d$ and RNAP (T), are marked. Here, $ArgP^d$ is the ArgP S94L mutant. A-Arg, L-Lys

6.2.6 Ternary complex formation at *argO* promoter in presence of ArgP^{d}:

To investigate if there is any recruitment of RNAP to the *argO* promoter in presence of ArgP^{d} with or without the addition of Arg or Lys, EMSA experiments were performed for ternary complex (RNAP-ArgP^{d}-*argO*) formation with both ArgP^{d} (20 nM), σ-factor bearing RNAP holoenzyme (80 nM) and 0.5 nM of *argO* DNA. Arg or Lys was added wherever necessary at a concentration of 0.1 mM. The binding reactions were incubated for 30-min before the products were resolved on a 4% native polyacrylamide gel (Fig. 6.5). Under the condition of this experiment when RNAP alone was added, all radioactivities remained in the unbound fraction (Fig. 6.5 lane 5). When both ArgP^{d} and RNAP were present, more than 90% of *argO* DNA was retarded in two bands that were both apparently unstable during electrophoresis: one which was taken to correspond to the ternary complex of RNAP-ArgP^{d}-*argO* DNA and the other (faint band), perhaps to a binary complex (either *argO*-RNAP or *argO*-ArgP^{d}) (Fig. 6.5 lanes 6 to 8). No difference was observed upon Arg or Lys addition (compare lanes 7 and 8 with lane 6). These results suggest that ArgP^{d} and RNAP bind to the *argO* regulatory DNA only when they are present together.

6.3 Discussion:

In this study, *argP* mutant alleles which confer CAN resistant phenotype, six trans-dominant and one recessive, were further characterized. It was found that the mutants exhibited heterogeneity in the levels of *argO* expression, and the differences in the dependency on co-effectors (Arg and Lys) was observed. On the basis of dependency on co-effectors for *argO* expression, the *argP* dominant mutants could be divided into following classes: 1. high and unaffected by Arg or Lys 2. slightly activated by Arg and substantially repressed by Lys, and 3. independent of Arg and feebly inhibited by Lys.

Besides, the lone *argP* recessive mutation conferring CAN^R was associated with low, constitutive expression of *argO*.

6.3.1 Mechanism of *argO* transcriptional regulation by $ArgP^d$-S94L:

As explained, approaches similar to those employed in the case of wild-type ArgP were used to study the mechanism of *argO* regulation by the mutant $ArgP^d$ protein. The various findings obtained can be summarised as follows: 1. $ArgP^d$-mediate regulation of *argO* is independent of co-effectors Arg or Lys, both in vivo and in vitro. 2. The regulation of *argO* transcription could be reconstituted in vitro using the purified His_6-$ArgP^d$ protein, and the RNAP holoenzyme. 3. Unlike the case with wild-type ArgP, the co-effector Arg and Lys might not interact directly with $ArgP^d$ protein. 4. Although $ArgP^d$ directly regulates the transcription of *argO*, it does not bind the regulatory region of *argO* as assessed by EMSA experiments, except in presence of RNAP.

Very few examples are available of systems where neither RNAP nor the activator could bind promoter DNA on their own but binding occur when both are added together. It is not clear at present whether $ArgP^d$ protein interacts with RNAP in solution to form a binary complex which then is able to bind to *argO* DNA, or whether all three ($ArgP^d$, RNAP, *argO*) molecules are simultaneously required to assemble together as a complex. Examples of activator proteins interact with free RNAP in solution before binding to promoter DNA are MarA and SoxS regulators (Griffith et al. 2002; Martin et al. 2002). As the activities of MarA and SoxS are controlled by their cellular concentration, this mechanism resembles to that of σ-factors, which reserve a certain number of RNAP molecules by binding to them. An example of the alternative case of simultaneous binding of several proteins to DNA in gene regulation is that of

the *P*s late promoter of phage P1, where three proteins (SspA, LpA and RNAP) are together needed to form a complex with the DNA (Hansen et al. 2003). In the case of $ArgP^{d}$, additional studies will be needed to elucidate the precise mechanism of its activation of *argO* transcription.

CHAPTER 7

Studies of ArgP interactions with other target genes

7.1 Introduction

In the previous Chapters, the ArgP- and Arg- or Lys-mediated regulation of *argO* transcription was studied in detail. It was shown that the activation of *argO* expression by Arg bound ArgP is by virtue of its ability to recruit RNAP at *argO* promoter and that its inhibition by Lys-bound ArgP is by trapping of RNAP at the promoter in a reversible, inactive complex after promoter opening but before promoter clearance. Besides, a possible mechanism was also postulated for the regulation of *argO* expression by CAN^R mutant $ArgP^d$-S94L protein.

As described in Chapter 1, the ArgP protein has previously also been studied as IciA, (that is, inhibitor of chromosomal replication initiation) which binds the 13-mer AT-rich regions in *oriC*. The IciA protein has also been reported to bind the regulatory regions of *dnaA* and *nrdA* and to activate transcription from these promoters. However, these studies had been performed prior to the knowledge that ArgP is involved in regulating Arg export through ArgO and that Arg and Lys are the co-effectors of the protein.

Besides, a study from this laboratory had observed that *argP* null mutant in *gltBD*$^-$ background is osmosensitive and crippled for NH_4^+ assimilation (described in Chapter 1), suggesting a possible role of ArgP in the regulation of *gdhA* (encoding glutamate dehydrogenase, which is involved in Glu-biosynthesis and NH_4^+ assimilation in *E. coli*).

Therefore, in this study, to further investigate, the GDH activities and Glu and potassium levels were measured from *argP gltBD* and *gltBD* mutant strains. Besides,

the promoter-*lac* fusions were constructed for the promoters of *dnaA*, *nrdA* and *gdhA*, and *lacZ* expression was measured in *argP*$^{-}$, *argP*$^{+}$ and *argP*^{d} backgrounds to compare with that of the *lac* fusion with the promoter of *argO*. This Chapter also describes the measurements of binding affinities of ArgP to DNA fragments bearing the regulatory regions of *dnaA*, *nrdA*, *gdhA* and the intergenic region of *argP-yqfE* (which is also a potential target region of ArgP as described in Chapter 1), as well as to *oriC*, and these values have been compared with that of ArgP binding to *argO* regulatory DNA.

7.2 Results:

7.2.1 GDH activities and potassium and Glu levels of *argP* and *gltBD* mutant strains:

Intracellular potassium and Glu accumulation are known to be important component of the osmoregulatory response in *E. coli* (Csonka and Epstein 1996). It was hypothesized that the osmosensitivity of *argP gltBD* mutants is because they are limited for Glu accumulation as a compatible solute and as a counterion for potassium, in media of elevated osmolarity, and that as a consequence, enough potassium is also not accumulated during hyperosmotic upshift (Csonka and Epstein 1996). To check this, the potassium and Glu levels and GDH activities were measured for these strains by methods described earlier for Glu (Kustu et al. 1994; Csonka et al. 1994), GDH activities (Gowrishankar and Pittard 1982; Meers et al. 1970) and potassium (Jennifer and Russel 2002). The values obtained are presented in Table 7.1.

Table 7.1 The levels of Glu and potassium and activity of Glu dehydrogenase (GDH) enzyme in *gltBD* and *argP gltBD* strains.

Strain number (genotype)	GDH sp. act (mU/mg of protein)		Glu level (nmol/mg dry wt. of cells)		Potassium level (nmol/mg dry wt. of cells)	
	Low[a]	High[a]	Low	High	Low	High
MC4100 (wild-type)	90	105	36	212	285	720
GJ4645 (*gltBD*)	111	224	26	117	252	543
GJ4654 (*argP gltBD*)	54	46	18	50	243	373

[a] – The indicated strains were grown to mid-exponential phase in the media of either low osmolarity (glucose minimal A supplemented with 40 μg/ml glycine betaine; Low) or high osmolarity (glucose minimal A medium supplemented with 0.6 mM NaCl along with 40 μg/ml glycine betaine; High) before the measurement were made as described in Chapter 2, Sections 2.2.4.17, 2.2.4.18 and 2.2.4.19.

As depicted in Table 7.1, growth under hyperosmotic stress was associated with a two-fold increase in GDH specific activity in GJ4652 (*gltBD*) but not in MC4100 (wild-type), reflecting the existence of redundant pathways for increased Glu synthesis for osmoregulation in the latter strain. On the other hand, for the *argP gltBD* strain (GJ4654), the GDH activity in the low-osmolarity culture was only half of the *gltBD* strain and there was no increase in the high osmolar condition. Concomitantly, the magnitude of accumulation of Glu and of K^+ in cells of wild-type strain grown at the

elevated osmolarity was substantial, but such accumulation was significantly less in *gltBD* strain and the reduction was even more pronounced (around 80%) for the *argP gltBD* mutant. These results fairly agree with the hypothesis as to how ArgP is involved in osmotolerance. Thus, it can be suggested that the degree of compromise in the GDH catalyzed pathway of Glu synthesis (and of NH_4^+ assimilation) in *argP gltBD* strain is such that the Glu levels are sufficient for growth in the low osmolarity media but not for growth in the high osmolarity media.

The mechanism by which a null mutation in *argP* reduces GDH activity and consequently, osmotolerance (and NH_4^+ assimilation) in the *gltBD* mutant is not known. The results described here for *argP* mutation in *gltBD* mutant strain is most simply explained by the hypothesis that there is a second gene whose regulation mirrors that of *argO*, which is involved in modulating the GDH activities in *gltBD* mutant strain. That this second gene is perhaps *gdhA* itself is suggested by the following. Janes et al. (2001) and Goss et al. (2002) have shown that the transcription of *gdhA* in *Kleibsiella aerogenes* is repressed about three-fold upon Lys supplementation (similar to ArgP- and Lys-mediated inhibition of *argO*). To explain this, the authors postulated that an unidentified regulator protein which is Lys sensitive activates *gdhA* in this strain. Based on results of the studies described herein, it is likely that this regulator protein is an ortholog of *E. coli* ArgP.

7.2.2 Studies of various target DNA sequences of ArgP:

Most of the evidence reported for ArgP regulation of genes like *dnaA* or *nrdA* or for inhibition of replication at *oriC* has come from in vitro studies, and the investigators had not examined the role of co-effectors such as Arg or Lys in their work. Therefore, in the present study, the DNA fragments of *dnaA*, *nrdA*, *gdhA*, and *argP/yqfE* encompassing their *cis* regulatory elements, and of *oriC* comprising the three 13-mer

sequences previously reported to bind IciA were amplified for use in in vitro binding studies and/or measurement of *lac* fusion expression levels in vivo (*dnaA* and *nrdA*).

7.2.2.1 Amplification of regulatory regions of *dnaA, nrdA, gdhA, argP/yqfE* and construction of promoter-*lacZ* fusions:

A fragment of *dnaA* from –400 to +40 with respect to translation start site was amplified with a pair of primers JGADNAf (5′-GTAAAGCGAAGGATCGTCCT-3′) forward and JGADNAr (5′-TCTGTGGCTGGTAACTCATCCT-3′) reverse and cloned in TA-cloning vector (pCR2.1, Invitrogen). One of the clones (where the *dnaA* promoters P1 and P2 in the fragment are in similar orientation to that of *lacZ* promoter ahead of multiple cloning site of the vector) was designated as pHYD1742. The fragment was digested with *HindIII* and *XbaI* and sub-cloned in the same orientation to the respective sites of the *lac* fusion vector pMU575 to generate the plasmid pHYD1746.

In the similar way, a 420-bp *nrdA* fragment extending from –360 to +60 with respect to translation start site encompassing the entire regulatory region was amplified using a pair of primers JGANRDf (5′-AAGTCATGAATAATTTTCTTATAA-3′) forward and JGANRDr (5′-GTATGTTTGGGATTCACTGCA-3′) reverse, and cloned in the similar orientation to that of *dnaA* in TA-cloning vector (pCR2.1, Invitrogen) to generate plasmid pHYD1744. It was further sub-cloned into *HindIII* and *XbaI* sites of pMU575 to generate plasmid pHYD1747.

For *gdhA* promoter region, a 462-bp fragment of *gdhA* regulatory region extending from –400 to +62 with respect to translation start site of the structural gene was amplified by a PCR reaction using a pair of primer JGAGDHf (5′-ATTTTGATCCTG CAGAACGCAGCACTG-3′) forward and JGAGDHr (5′-GTTTGATTCGGATCCCG

CTTTTGGACATG-3′) reverse and this fragment was used for further EMSA experiments. For in vivo reporter assay to measure the expression level of *gdhA*, a chromosomal *gdhA-lacZ* fusion previously obtained in this laboratory was employed following strain construction described in the following Section.

Similarly, the common sequence of *yqfE* and *argP* regulatory region (365-bp long extending from –259 to +103 with respect to *yqfE* translation start site which also represents the stretch from –275 to +90 with respect to translation start site of the adjacent divergently transcribed *argP* structural gene) was amplified by PCR using a pair of primers JGJYQFE-Of (5′-GGCGC ACCAGTCGACCGTTCGCAA-3′) forward and JGJYQFE-Or (5′-TAATGCGCTGGT CGACG GCTGATT-3′) reverse to be used for EMSA experiments.

For o*riC* DNA, a fragment of 85-bp encompassing the three 13-mer sequences previously reported to bind IciA from the region of *oriC* in *E. coli* chromosome was amplified by PCR using a pair of primers JGAORICf (5′-GGATCCGGATAAAACA TGGTGA-3′) forward and JGAORICr (5′-ATCCTTGTTATCCACAGGGCA-3′) reverse to be used for ArgP binding studies.

7.2.2.2 Construction of chromosomal *gdhA:lacZ* fusion strain:

The strain GJ980 (Saroja and Gowrishankar 1996) carries *gdhA::lacZ* Kan (from λMu55 prophage) in its chromosome. It was transferred to MC4100 as follows. A phage P1 lysate prepared on GJ980 was used to transduce in MC4100 to Kan^R. The Kan^R colonies were then screened for Lac^+ phenotype and a lac^+ Kan^r colony was designated as GJ5318 for use in vivo β-galactosidase assays. An $argP^-$ strain with *gdhA::lacZ* fusion was constructed by transferring *argP*::Tet^r (tetracycline resistance) locus from the strain GJ4892 to GJ5318. The transductants were selected for Tet^r and

confirmed for *argP* mutation by testing for their CAN^{SS} phenotype on minimal A glucose agar plates supplemented with 40 μg/ml CAN. One such colony was designated as GJ5319.

7.2.2.3 In vivo expression studies using *lacZ* fusions with promoters of *dnaA, nrdA* and *gdhA*:

The pMU575 constructs of *dnaA* and *nrdA* promoters (pHYD1746 and pHYD1747) along with the control *argO* promoter derivative (pHYD1723) were transformed into $argP^+$ (GJ4892 transformed with pHYD915), $argP^-$ (GJ4892 transformed with pCL1920) and $argP^d$ (GJ4892 transformed with pHYD926) strains. β-Galactosidase assays were then performed with cultures of the strain derivatives grown in presence and absence of Arg or Lys. For *gdhA* expression, the β-galactosidase assays were performed using the strains GJ5318 and GJ5319 grown in presence and absence of Arg or Lys. The values of β-galactosidase specific activities (in Miller unit, MU) of the various strains are given in Table 7.2.

For the control *argO-lac* fusion strain, the data obtained were consistent with the previous observation (that is, ArgP-dependent activation by Arg and inactivation by Lys, and constitutive expression in presence of $ArgP^d$). On the other hand, there was no difference in the values of β-galactosidase specific activities for $argP^-$ strain from that of $argP^+$ or in presence of transdominant allele ($argP^d$) for *dnaA-lac* and *nrdA-lac* fusions. The addition of Arg or Lys also did not cause any change to the expression level from the respective promoters (the expression levels averaged at around 120 Miller units). This perhaps suggests that ArgP is not involved in transcriptional regulation of either *dnaA* or *nrdA*, contrary to the conclusions drawn from previous

reports that ArgP activates the transcription from promoter P1 of *dnaA* in vitro and also from the *nrd* promoter (Lee et al. 1996, 1997; Han et al. 1998).

On the other hand, the β-galactosidase specific activity in case of *gdh:lacZ* fusion was observed to be lower in the *argP* mutant (around 60 MU) compared to that in the *argP*$^+$ strain (around 350 MU). This is in agreement with the observation above that there was more GDH activity in cultures of *argP*$^+$ than that of *argP*$^-$ strains. This indicates that ArgP positively regulates the expression of *gdhA* in vivo, either directly or indirectly. However, the effects of co-effectors of ArgP (Arg and Lys) on the regulation of *gdhA* were not very clear. There was no change in the β-galactosidase specific activity upon Arg addition, but a moderate reduction in the activity was observed (to around 230 MU) for the *argP*$^+$ cultures grown in presence of Lys.

Table 7.2 β-galactosidase specific activities of various genes measured from the *lacZ* fusion with the respective promoters.

Strain ⟶	*argP*$^-$			*argP*$^+$			*argP*^{d}		
lacZ fusion to promoter of	Nil	Arg	Lys	Nil	Arg	Lys	Nil	Arg	Lys
argO	10	5	5	100	450	8	1050	980	1100
dnaA	118	130	115	140	120	125	75	80	90
nrdA	180	210	225	130	155	140	108	120	130
gdhA	70	60	75	350	380	230	nd	nd	nd

nd – Not determined.

[#] *argP*$^-$, *argP*$^+$ and *argP*^{d} strains were respectively, GJ4892 transformed with pCL1920, pHYD915 and pHYD926. For *gdhA* expression studies, *argP*$^-$, *argP*$^+$ are respectively, GJ5319 and GJ5318.

7.2.3 Binding studies of ArgP to various target DNA fragments:

7.2.3.1 Evidence of ArgP binding to the regulatory regions of *dnaA, nrdA, gdhA* and the DNA sequence *oriC*:

To investigate the binding of ArgP to some of the previously reported DNA sequences, the PCR amplified DNA fragments of *dnaA*, *oriC*, *nrdA*, *gdhA* and common regulatory sequence of *yqfE* and *argP* (*argP/yqfE*) were radiolabelled at the 5′-ends using polynucleotide kinase and ^{32}P-γ-ATP, and EMSA experiments were carried out using the His_6-ArgP protein. The 95-bp *argO* DNA fragment was used as a control in these experiments. Around 0.5 nM of DNA fragments were incubated with 20 nM ArgP (monomer concentration) in the binding buffer with or without Arg, Lys and His, incubated at room temperature for 30-min, and resolved by electrophoresis on a 5% non denaturing polyacrylamide gel.

As shown in Figure 7.1, in absence of co-effectors, whereas there was almost 100% retardation of the labelled *argO* fragment by ArgP (lanes 20 to 22), *nrdA* (lanes 6 to 10) and *dnaA* (lanes 1 to 5) exhibited much lower ratios of labelled DNA bound by ArgP (retarded band), suggesting a weaker, if not negligible, interaction of these fragments with ArgP. However, in case of *oriC* (lanes 11 to 15), a good binding by ArgP (but still much less than that for *argO*) was observed. Likewise, ArgP also retarded the *gdhA* regulatory fragment (lanes 16 to 19), again with less affinity than that for *argO* fragment.

Arg or His addition did not affect the binding of ArgP to any of the fragments (Fig 7.1 compare lanes 3 and 5 with 2; 8 and 10 with 7; 13 and 15 with 12; 18 with 17). However, Lys addition appeared to increase the affinity of binding of ArgP to the respective DNA fragments (compare lanes 2 with 4, 7 with 9, 12 with 14, 17 with 19).

Besides, Lys addition also resulted in supershifted bands in case of *dnaA*, *nrdA* and *gdhA* (Fig. 7.1. lanes 4, 9 and 19), these findings have not been investigated further. In case of *gdhA*, the observation may perhaps be correlated with the in vivo regulation data that Lys addition causes reduction in the β-galactosidase specific activity of *gdh:lacZ* fusion strain.

In case of *argP-yqfE* fragment, an insignificantly weak binding of ArgP was observed which may suggest the absence of ArgP regulation over the expression of these genes (refer to Fig. 7.4 lanes 7 to 9).

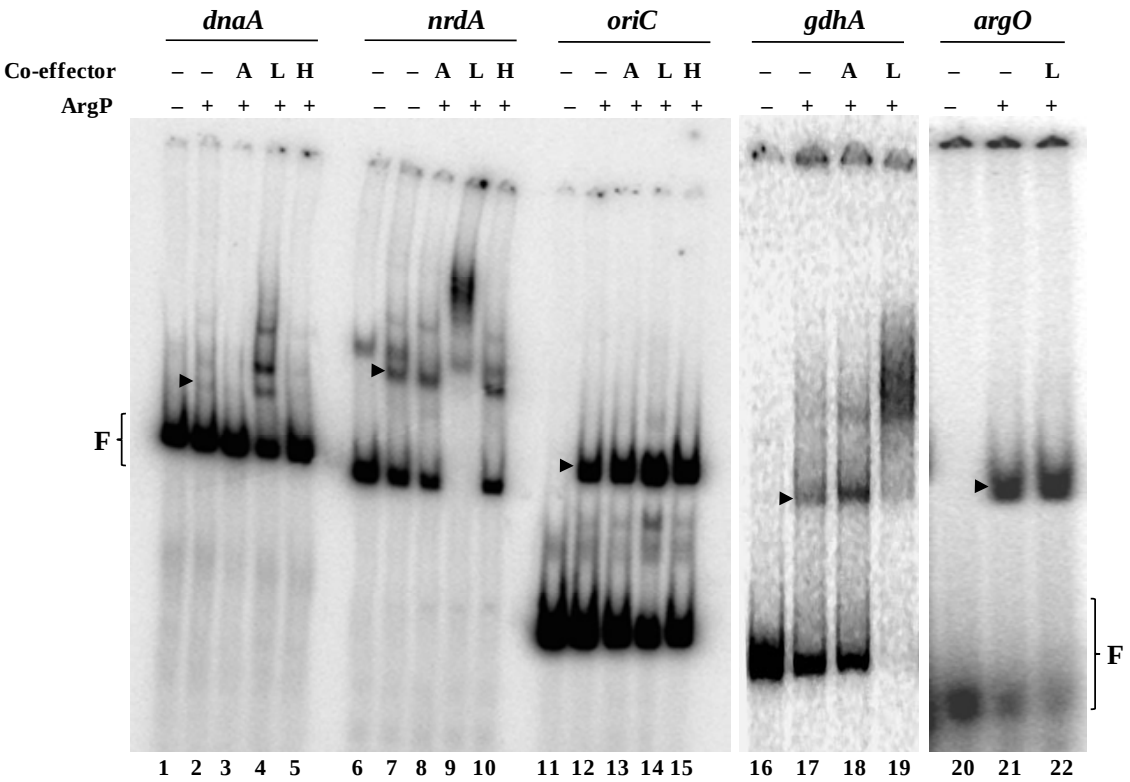


Figure 7.1. EMSA experiment of ArgP binding to fragments of *dnaA*, *nrdA*, *oriC* and *gdhA* in presence and absence of Arg, Lys or His. The free DNA bands (F) and the binary complexes with ArgP are indicated by arrowheads in the lanes without co-effector addition. A-Arg, L-Lys, H-His.

7.2.3.2 Heterologous competition experiments of *argO* regulatory DNA with various DNA fragments (*dnaA, nrdA* and *oriC*):

The EMSA experiment using various DNA fragments described above revealed that ArgP binding exhibits different affinities to different DNA sequences. All of the other DNA fragments studied showed weaker binding to ArgP than that of *argO* regulatory DNA. Therefore, the difference in the binding affinities of ArgP to *argO* regulatory DNA and rest of the fragments was investigated by heterologous competitions with unlabelled DNA.

EMSA experiments were set up with ArgP and the labelled DNA fragments (*dnaA, nrdA, oriC*) in the absence or presence of equivalent or 100-fold excess of unlabelled *argO* fragment. In the similar way, the binding reaction of *argO* fragment and ArgP was also competed with equivalent and 100-fold excess of unlabelled fragments of *dnaA*, *nrdA* and *oriC*. The data from these two sets of competition experiments are shown in Figures 7.2 and 7.3, respectively.

In the case of *dnaA* (Fig. 7.2, lanes 1 to 4), there was insignificant binding by ArgP even in absence of *argO* (consistent with the data of Fig. 7.1), and there was no alteration upon addition of *argO*. For *nrdA* (Fig. 7.2, lanes 5 to 8) and *oriC* (Fig. 7.2, lanes 9 to 12), the bound fractions were decreased in presence of the equivalent concentration of unlabelled *argO* fragment and were almost completely abolished when 100-fold excess of *argO* was added. This suggested that the *argO* fragment could out-compete *dnaA*, *nrdA* and *oriC* for ArgP binding. When the competition was carried in the reverse way, that is, the binding reaction of ArgP with *argO* was tested in presence of unlabelled *dnaA*, *nrdA*, or *oriC* fragments, none of the fragments could out-compete

argO for ArgP binding even in 100-fold excess (Fig. 7.3). This strongly suggests that *argO* has higher binding affinity for ArgP than that of regulatory regions of *dnaA*, *nrdA* or *oriC* DNA fragment.

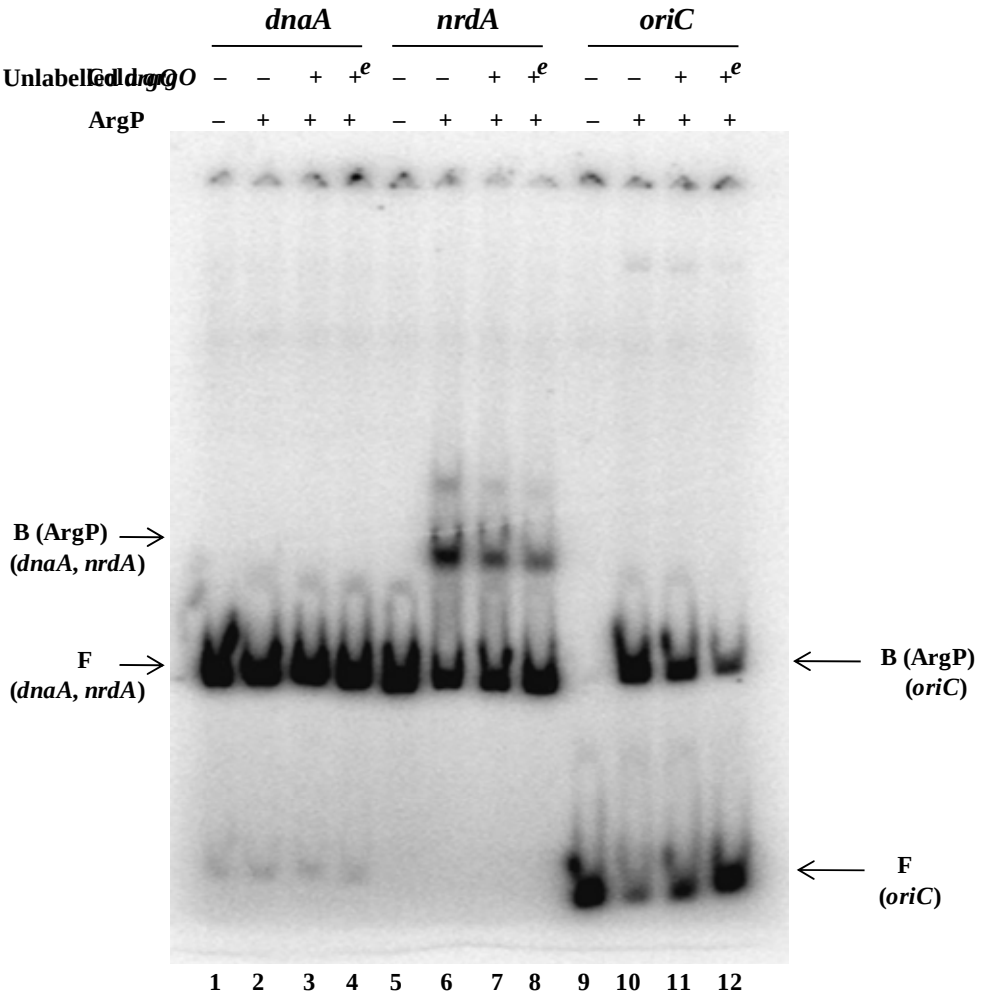


Figure 7.2. Competition with heterologous unlabelled *argO* fragment in the EMSA reactions of ArgP binding to *dnaA*, *nrdA* and *oriC,* as indicated. The free DNA (F) of respective DNA fragments and the binary complex with ArgP (B) are indicated. For unlabelled *argO*, + indicates the addition of equivalent concentration to that of labeled heterologous DNA fragment; $+^e$ indicates the addition in 100-fold excess.

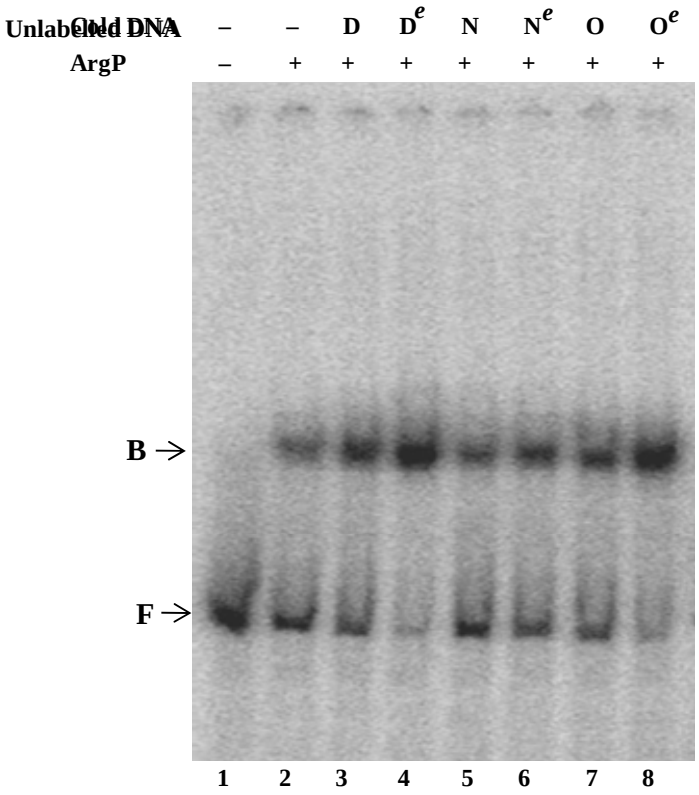


Figure 7.3. Competition with heterologous unlabelled DNA fragments (*dnaA, nrdA* and *oriC*) in the EMSA reactions of ArgP binding to *argO* regulatory region. The free DNA (F) of respective DNA fragments and the binary complex with ArgP (B) are marked. D, N, and C indicates respectively the addition of equivalent concentration of unlabelled *dnaA, nrdA* and *oriC* to that of labelled heterologous *argO* fragment; ^{e} indicates the addition in 100-fold excess of the respective fragments.

7.2.3.3 EMSA experiment with *argO* mixed separately with *dnaA, nrdA, yqfE* or *gdhA*:

For further understanding, EMSA experiment was carried out with two labelled DNA fragments in the same reaction mix, one being the 95-bp fragment of *argO*, mixed which extends from –90 to +5 (described in Chapter 4) and which contains the complete ArgP binding region on *argO* regulatory DNA, and the other being *dnaA*, *nrdA*, *yqfE* or *gdhA*. As shown in the Figure 7.4, at the concentration of ArgP used (20

nM), when nearly complete retardation was observed for *argO* as determine by the reduction in the intensity of band corresponding to free *argO* DNA (lanes 2, 3; 5, 6; 8, 9; 11, 12), there was little if any retardation of the bands for *dnaA* (lanes 2, 3), *nrdA* (lanes 5, 6), *gdhA* (11, 12) and *yqfE* (lanes 8, 9). (Additional bands which can be from contaminating DNA are seen in the lane 4 and 5 and very faintly in the *dnaA* lanes). Once again, the *nrdA* fragment exhibited retarded mobility in presence of ArgP and Lys (lane 6). This experiment also independently supports the observation that *argO* has higher affinity of ArgP binding than any of the other DNA fragments under question.

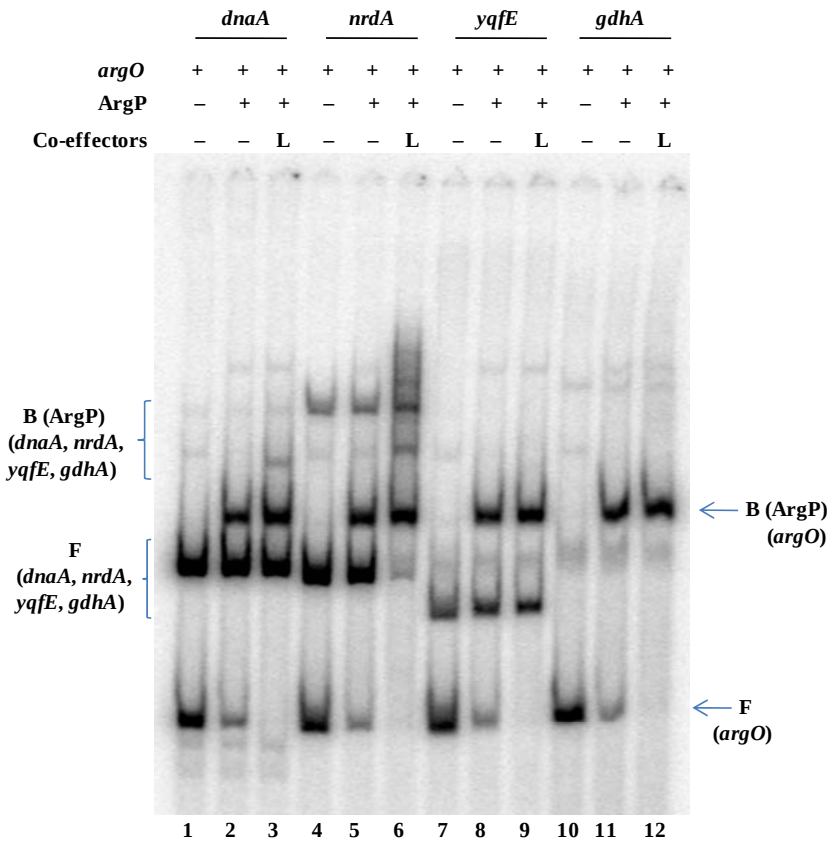


Figure 7.4. EMSA experiment of labelled *argO* mixed separately with labelled *dnaA* (lanes 1 to 3), labelled *nrdA* (lanes 4 to 6), labelled *yqfE* (lanes 7 to 9) or labelled *gdhA* (lanes 10 to 12) for ArgP binding in presence and absence of Lys. The respective free DNA bands (F) and the binary complexes with ArgP (B) are indicated. L-Lys.

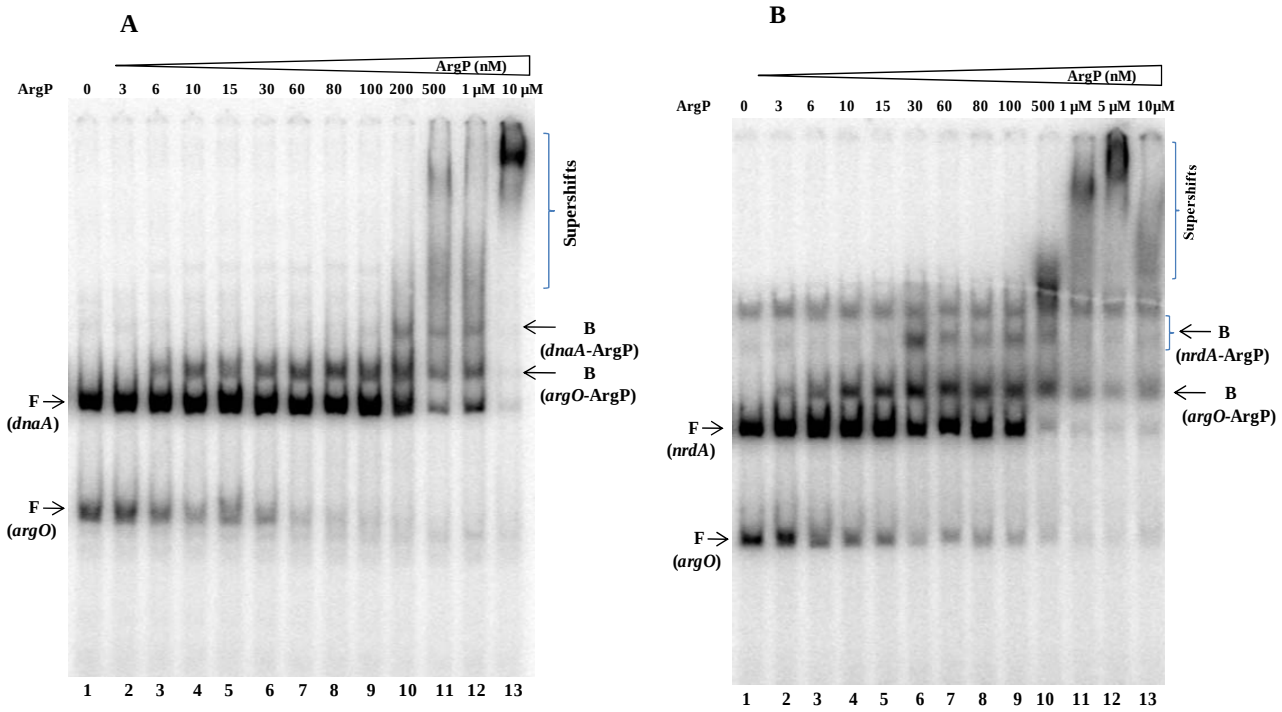


Figure 7.5. Comparison of binding affinity of ArgP to *argO* fragment with that of *dnaA* and *nrdA* fragments. EMSA experiments in presence of increasing concentrations of ArgP as indicated with a mixture of labelled *argO* DNA with labelled *dnaA* (*A*) and labelled *nrdA* (*B*). Unless otherwise indicated, ArgP concentration is in nM. The respective binary complex with ArgP (B) and free DNA (F) are also indicated. K_d was calculated for each DNA-ArgP interaction gels *A* and *B*.

7.2.3.4 Comparison of affinities of ArgP binding to *argO* and that of *dnaA, nrdA, yqfE, oriC* or *gdhA* :

The binding affinity of ArgP to the fragments (*dnaA*, *nrdA*, *oriC*, *yqfE/argP*, *gdhA*) was compared with that of ArgP to *argO* by measurement of the K_d of the respective binding reactions. For this purpose, the equivalent amounts of radiolabelled fragments of *argO* was mixed separately with labelled DNA fragments (*dnaA*, *nrdA*, *oriC*, *yqfE/argP* or *gdhA*) as described in the previous Section. EMSA experiments were carried out independently using the mixtures of template with increasing concentrations of ArgP from 0 to 10 µM (0, 0.003, 0.006, 0.01, 0.015, 0.03, 0.1, 0.2, 0.5, 1.0, 2.0 and

10 μM). From each experiment, the increase in the bound fractions of various DNA fragments and *argO* upon addition of increasing amount of ArgP was quantitated and K_d was calculated as described in Chapter 3, independently for *argO* and for the other fragment in each of the dual-label mixures (*dnaA*, *nrdA*, *oriC*, *yqfE/argP* or *gdhA*). Representative autoradiograms from the EMSA experiments with *dnaA* and *nrdA* are shown in Fig. 7.5, and the K_d values calculated for all the fragments are given in Table 7.3. The various K_d suggests that there is 10- to 100-fold difference between the binding affinity of ArgP to *argO* and that to other DNA fragments studied. Fragments like *gdhA* and *oriC* exhibit 10-fold more K_d and *nrdA* exhibits around 20-fold more K_d than *argO*. However, *dnaA* or *yqfE* exhibit 100-fold or more K_d than *argO*.

Table 7.3 Comparison of K_d values of ArgP binding to various target DNA fragments with that of *argO* regulatory DNA. The EMSA experiment was performed with a mixure of the two labelled DNAs (one of which was *argO* and the other was *dnaA, nrdA, oriC, gdhA,* or *yqfE*), and K_d was calculated for both the fragments from the same experiment.

Labelled DNA fragments used for EMSA (along with 95-bp labelled *argO*)	Length of the fragment	K_d of ArgP binding to the respective fragments (in nM)	K_d of ArgP binding to *argO* calculated from the same gel (nM)
dnaA	420-bp	~700	~8
nrdA	440-bp	~150	~7
oriC	85-bp	~90	~8
gdhA	460-bp	~90	~7
yqfE	360-bp	~800	~8
argO	95-bp	~6	–

7.3 Conclusion:

ArgP (IciA) was reported to activate the transcription from *dnaA* and *nrdA* promoters (Lee et al. 1996, 1997; Han et al. 1998) and bind 13-mer AT rich sequences in the *oriC* to inhibit the initiation of replication in vitro (Hwang and Kornberg 1990, 1992; Thony et al. 1991; Hwang et al. 1992). However, no associated phenotypes were reported, and indeed *argP* (*iciA*) null mutants did not exhibit any replication-related phenotype (Thony et al. 1991; Lee et al. 1996; Celis 1999; Nandineni and Gowrishankar 2004). In the present study, the binding characteristics of ArgP to *argO* regulatory region was compared to that of the previously reported binding sequences and the genes controlled by ArgP. It can be concluded from the results described in this Chapter that the binding strength and the affinity of ArgP binding is much higher for *argO* than that for *dnaA*, *nrdA*, *oriC*, *argP/yqfE* or *gdhA*, with the difference in the K_d of ArgP binding being 10- to 100-fold. Furthermore, in vivo studies with promoter *lac* fusions also indicated *dnaA* and *nrdA* are not regulated by ArgP. However, these observations argue against the earlier reports (Lee et al. 1996, 1997; Han et al. 1998) of the regulation of *dnaA* and *nrdA* by ArgP. On the other hand, *gdhA* transcription in vivo appears to be under direct or indirect control of ArgP, and this needs to be further investigated.

In conclusion, it can be proposed that ArgP may have many more targets than *argO* (ArgP regulon) and that *argO* regulation could be considered as a prototype or reference example in the study of ArgP-mediated gene regulation.

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