IDENTIFICATION OF RpoS REGULATED GENES AND THEIR FUNCTIONS IN ESCHERICHIA COLI

BY

S.R.V. VIJAYAKUMAR, M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

IDENTIFICATION OF RpoS REGULATED GENES AND THEIR FUNCTIONS IN ESCHERICHIA COLI

BY

S.R.V. VIJAYAKUMAR, M.Sc.

MASTER OF SCIENCE (2002) McMaster University

(Biology) Hamilton, Ontario

TITLE: Identification of RpoS regulated Genes and Their Function in *Escherichia coli*

Author: S.R.V. Vijayakumar, M.Sc. (M.S. University, Tamil Nadu, India)

Supervisor: Dr. H. E. Schellhorn

Number of Pages: i – x, pp 1-132

Abstract

E. coli expresses an alternative sigma factor, RpoS, in response to starvation and environmental stresses. RpoS is a global regulator and it controls numerous genes, which aids in counteracting these stresses. The RpoS regulon is large but is not completely characterized. We have previously identified over one hundred RpoS-dependent fusions in a genetic screen based on the differential expression of an operon-*lacZ* fusion bank in *rpoS* mutant and wild type backgrounds. Forty-eight independent gene fusions were identified including several in well-characterized RpoS-regulated genes such as *osmY*, *katE* and *otsA*. Many of the fusions mapped to genes of unknown function or to genes that were not previously known to be under RpoS control. Based on the homology to other known bacterial genes, some of the RpoS regulated genes with unknown functions may be important for nutrient scavenging. To gain a better insight into the functions of these poorly characterized genes, we tested the ability of the fusion mutants to utilize various carbon sources and to utilize individual amino acids as carbon and nitrogen sources. The results indicate that most of the strains in *rpoS*$^{-}$ backgrounds exhibited better growth in succinate and fumarate and in several amino acids than did the corresponding strains in wild-type backgrounds.

ACKNOWLEDGEMENTS

First and foremost I like to express my deep sense of gratitude and indebtness to my supervisor Dr. H. E. Schellhorn for giving me the opportunity to come to this country and pursue graduate studies in his lab. His constant criticism, valuable comments and timely advice helped me to overcome the obstacles I faced during these two years. I also thank Dr. Suleiman Igdoura for giving valuble comments and suggestions after each committee meeting.

Next I like to thank my labmates past and present whose constant encouragement and help made my stay here enjoyable. I like to thank Sabine for being my "coffee date" and for the words of encouragement when everything seemed bleak. Cheryl Patten, for patiently listening to my hypotheses and for providing valuble comments for my thesis, Galen Chen for helping me with computer related stuff, Primo for being my very good friend and all the good times we had in and out of the lab. Mike, for helping me with my project when I needed it and for being a great company in Orlando and Saima, for keeping the lab well stocked. I also thank the under-grads (Kelly, Jonathon and Jana) for making the lab lively and for their help with glasswares and media.

Last but not the least I thank santosh for being a great friend and for being the lead guitarist in the crazy parties we had. Finally I'll be failing in my duty if I don't thank my beloved parents whose unwavering faith in me has taken me where I am now.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 INTRODUCTION

Bacteria reproduce by binary fission and grow exponentially under suitable conditions. Bacterial growth is divided into four phases depending on the stage of growth. When a bacterial culture is freshly inoculated into a growth medium, its growth does not begin immediately but the bacteria acclimatize to the growth conditions and produces enzymes and macromolecules necessary to initiate growth. This stage is known as the lag phase. This is followed by rapid proliferation, where bacteria double exponentiall y until most of the nutrients are utilized. This stage of growth is known as the exponential phase or log phase. At the end of exponential phase, the rapidly growing cells utilize most of the nutrients and the media contains excreted metabolic byproducts. The lack of nutrients and accumulation of metabolic by-products lead to stress. At this point the bacteria ceases to reproduce and the growth is minimal. This stage is known as the stationary phase. The final stage is the death phase where bacterial cells start to die off. This type of growth is typical for a laboratory batch culture where there is an ample supply of nutrients. However, in most natural environmental conditions bacteria is under stress due to sub-optimal growth conditions such as extremes of temperature, pH, osmolarity and nutrient limitation. Under these circumstances many sporulating bacteria form recalcitrant spores that germinate

again under optimal conditions (Vulic and Kolter, 2001). But non-sporulating bacteria such as *E. coli* enters stationary phase to avoid unfavorable growth conditions. Stationary phase of non-spore formers can be compared with dormant spores of the spore-former (Vulic and Kolter, 2001). The major difference is that the spores are metabolically inactive structures while stationary phase cells maintain a low metabolic rate (Zambrano and Kolter, 1996). Upon entry into stationary phase *E. coli* induces an alternative sigma factor known as RpoS or σ-^{38}. RpoS in turn controls a number of genes that help *E. coli* to counteract stresses.

1.1.0 rpoS and its regulation.

The *rpoS* gene forms an operon with *nlpD*, which codes for a lipoprotein of unknown function (Lange and Hengge-Aronis, 1994). There are three promoters for *rpoS*, two (*nlpD*p1 and *nlpD*p2) lie upstream of *nlpD* and makes a polycistronic mRNA transcript (Lange *et al.*, 1995). These two are weak promoters and are responsible for the low levels of σ^s transcript during exponential phase (Lange *et al.*, 1995) and the main promoter, which lies within the *nlpD* gene, produces a monocistronic transcript (Lange *et al.*, 1995). This promoter is induced on entry into stationary phase and contributes to the high levels of *rpoS* mRNA in stationary phase (Lange *et al.*, 1995). The regulatory network involving *rpoS* is complex and requires a number of factors, which act at the level of transcription, translation and proteolysis (Hengge-Aronis, 2000).

1.1.3 Transcriptional regulation of *rpoS*.

Transcriptional regulation of *rpoS* is still far from being well understood. Several factors such as guanosine 3',5'-bispyrophosphate (ppGpp), polyphosphate, and cyclic AMP (cAMP) receptor protein (CRP) (cAMP-CRP) have been implicated in the transcriptional regulation of *rpoS* (Hengge-Aronis, 2002). ppGpp accumulates under amino acid and carbon starvation conditions and is synthesized by ppGpp synthase I encoded by *relA* and also by ppGpp synthase II encoded by *spoT* (Barker *et al.*, 2001). ppGpp has been previously demonstrated to positively regulate *rpoS* expression (Gentry *et al.*, 1993). The mechanism by which ppGpp upregulates *rpoS* transcription is not clear, but it has been suggested that ppGpp may affect transcript stability or transcript elongation rather than transcriptional initiation (Lange *et al.*, 1995). Recent studies on *rpoS-lacZ* fusions in a ppGpp mutant background have revealed that ppGpp does not affect the regulatory expression of *rpoS*, but has an effect on its basal expression (Hirsch and Elliott, 2002). ppGpp also positively affects the expression of genes required for polyphosphate synthesis (Cashel *et al.*, 1996). Polyphosphates regulates gene expression in response to nutrient limitation and stresses (Rao and Kornberg, 1996). Polyphosphates also positively affect *rpoS* transcription (Shiba *et al.*, 1997). It has been proposed that polyphosphates form a complex with RNA polymerase and thereby initiate transcription of genes under its control including *rpoS* (Shiba *et al.*, 1997). cAMP-CRP negatively regulate *rpoS* transcription (Lange and Hengge-Aronis, 1994). In *crp* (encoding cAMP-CRP

protein) and *cya* (encoding adenylate cyclase) mutants, RpoS levels increase even in exponential phase indicating that cAMP-CRP acts as a negative regulator of *rpoS* transcription (Lange and Hengge-Aronis, 1994). Moreover, the presence of two putative cAMP-CRP binding sites upstream and downstream of *rpoS*p indicates that cAMP-CRP may have a regulatory effect on *rpoS* transcription, but the exact role of these binding sites in transcriptional attenuation of *rpoS* is yet to be proved (Hengge-Aronis, 2002).

1.1.3 Post-transcriptional regulation of *rpoS*.

The *rpoS*-mRNA at its 5' end has a long leader sequence, which is important for translational regulation (Lange *et al.*, 1995). This 5' region is approximately 340 nucleotides (nt) long (Hengge-Aronis, 2002) and can form a stable hairpin stem-loop structure (Brown and Elliott, 1997). The downstream region of this initial step-loop structure is the "translational initiation region" (TIR), which has the potential to form two structures, one involving the Shine-Dalgarno region and the other, in the region around the Shine-Dalgarno region that forms the "internal upstream antisense" (Hengge-Aronis, 2002). But, the exact location of this "internal upstream antisense" is speculative (Hengge-Aronis, 2002). Mutation of the hairpin stem-loop structure leads to the constitutive expression of *rpoS* indicating the inhibitory role of this structure in *rpoS* translation (Brown and Elliott, 1997). There are several small proteins and regulatory RNA's that modulate translation of *rpoS*-mRNA (Hengge-Aronis, 2002). The three well-known regulatory RNA's that affects *rpoS* translation includes DsrA (Sledjeski *et*

al., 1996), RprA (Majdalani *et al.*, 2002) and OxyS (Altuvia *et al.*, 1997). DsrA and RprA are positive regulators (Majdalani *et al.*, 2002; Sledjeski *et al.*, 1996) while OxyS is a negative regulator of *rpoS* translation (Altuvia *et al.*, 1997). The *dsrA* gene codes for an 87-nt untranslated RNA, which folds into a three-stem loop structure (Majdalani *et al.*, 1998). The RprA RNA is 106-nt long and is encoded by the *rprA* gene (Majdalani *et al.*, 2001). Like DsrA, RprA also forms a three-stem loop structure (Majdalani *et al.*, 2001). These two RNA have complementary regions in their stem-loop structures, which can bind to the 5' unpaired region of the *rpoS*-mRNA and to another region near TIR (Majdalani *et al.*, 2001). This binding opens up the TIR of the *rpoS*-mRNA for ribosome binding leading to subsequent translation of the *rpoS*-mRNA (Majdalani *et al.*, 2001). The OxyS RNA is 109-nt long and also form hairpin stem-loops similar to DsrA (Altuvia *et al.*, 1997). OxyS is a member of the OxyR regulon and is induced in response to oxidative stress (Altuvia *et al.*, 1997). The negative regulation of *rpoS* by OxyR is achieved by the binding of a long A – rich single-stranded region between stem-loops 2 and 3 to the *rpoS*-mRNA (Zhang *et al.*, 1998). Several small proteins are also implicated in the modulation of *rpoS* translation; these proteins binds to the *rpoS*-mRNA directly or form a complex with regulatory RNA there by modulating the translation of *rpoS* (Hengge-Aronis, 2002). Host factor Q (Hfq) was first identified as a protein required for replication of the RNA phage Qβ (Franze de Fernandez *et al.*, 1972). Hfq can directly bind to the 5' leader sequence of the *rpoS*-mRNA (Muffler *et al.*, 1996); but the exact mechanism by

which it regulates *rpoS*-mRNA translation is unknown (Hengge-Aronis, 2002). Hfq also binds to DsrA in vitro (Sledjeski *et al.*, 2001). This suggests that Hfq and DsrA might work in concert in regulating *rpoS* translation (Lease and Belfort, 2000), which may be accomplished by altering DsrA structure or by forming an RNA-protein complex (Sledjeski *et al.*, 2001). Nucleoid-associated histone-like proteins such as HU (heat-unstable nucleoid protein), H-NS (histone-like nucleoid structuring protein) and StpA (suppressor of *td* mutant phenotype) also have been reported to regulate *rpoS* translation (Hengge-Aronis, 2002). HU is a heterodimeric and abundant nucleoid-associated protein encoded by two genes *hupA* and *hupB* (Kano *et al.*, 1986). The HU protein functions in nucleoid organization and gene regulation (Schmid, 1990). Moreover, HU is also important in long-term survival during starvation (Claret and Rouviere-Yaniv, 1997). In vitro studies have revealed that HU proteins bind to *rpoS*-mRNA with high affinity (Balandina *et al.*, 2001). It has been hypothesized that HU protein alters the *rpoS*-mRNA secondary structure and thereby initiating translation (Hengge-Aronis, 2002). H-NS and StpA are also abundant nucleoid-associated proteins, which are required for nucleoid organization and gene regulation (Atlung and Ingmer, 1997). StpA is another nucleoid-associated protein, which may function as a positive regulator of *rpoS* translation (Hengge-Aronis, 2000). The mechanism of action of this protein on *rpoS* translation is also unknown. H-NS is encoded by *hns* and like HU forms heterodimers (Atlung and Ingmer, 1997). Mutations in *hns* increases cellular levels of RpoS even in exponential phase and

also reduces proteolysis of RpoS indicating that H-NS acts as a negative regulator of RpoS (Yamashino *et al.*, 1995). It still remains to be elucidated how H-NS brings about negative regulation of *rpoS*-mRNA. UDP-glucose is another known negative regulator of *rpoS* translation (Bohringer *et al.*, 1995). Mutations in *pgi*, *pgm* and *galU* encoding phosphoglucoisomerase, phosphoglucomutase and UDP-glucose pyrophosphorylase all of which is required for UDP-glucose formation shows increased levels of RpoS (Bohringer *et al.*, 1995). This indicates that UDP-glucose may act as a negative regulator of *rpoS*. But, the mechanism by which UDP-glucose controls post-transcriptional expression of *rpoS* is unknown (Bohringer *et al.*, 1995).

1.1.4 Regulation of RpoS by proteolysis.

It is well known that RpoS levels peaks in stationary phase, but a basal level of *rpoS* expression is maintained even in exponential phase (Schellhorn *et al.*, 1998). The relatively short half-life of RpoS in exponential phase is due to the rapid proteolysis of the RpoS protein. This is brought about by an ATP-dependent hydrolyzing protease, ClpPX encoded by the genes of the *clpPX* operon (Maurizi *et al.*, 1990). Mutations in either of the ClpPX encoding genes increase the cellular levels of RpoS indicating that ClpPX regulates RpoS (Schweder *et al.*, 1996). ClpPX cannot recognize RpoS by itself, this recognition is brought about by a protein of the two-component response regulatory family known as RssB (Pratt and Silhavy, 1996; Zhou and Gottesman, 1998). In its phosphorylated form RssB recognizes RpoS and binds to region 2.5 of the RpoS

protein (Klauck *et al.*, 2001). The protein:protein interaction of RssB with RpoS occurs via both the C and N-terminal regions of RssB with region 2.5 of RpoS (Klauck *et al.*, 2001). This complex is recognized by ClpPX protease, which degrades RpoS and releases RssB thereby making this a cyclic event (Klauck *et al.*, 2001). The exact process by which RpoS becomes stable in stationary phase or during other stresses is not clearly understood (Hengge-Aronis, 2002). There are several postulates regarding this process, one which states that RssB becomes dephosphorylated by a specific RssB phosphotases thereby inhibiting the binding of RssB to RpoS (Hengge-Aronis, 2002). Another possibility for increase RpoS stability is due to the increase cellular concentration of RpoS, which in turn titers out RssB thereby increasing RpoS stability (Hengge-Aronis, 2002).

1.2.0 Changes in Stationary phase (At gene expression level).

RpoS by itself does not contribute to any of the stress protective role during starvation or stress. This is mainly due to the induction of numerous genes that are targets for RpoS-dependent activation. To date nearly 100 genes have been identified to be under the positive control of RpoS (Ishihama, 2000). However, RpoS is not the only player in stationary phase, there are several other regulatory genes that play an important role during this crucial period. The stationary phase response in *E. coli* can be broadly classified into (i) RpoS independent changes and (ii) RpoS dependent changes.

1.2.1 RpoS independent changes.

Apart from RpoS there are several other global regulators that contribute to the stationary phase specific changes in *E. coli*. This includes ArcBA, nucleoid-associated proteins, ppGpp, and cAMP-CRP to name a few.

ArcBA.

ArcA belongs to the family of regulatory proteins with a receiver domain and a helix-turn-helix motif, while ArcB unlike other sensor kinases has both transmitter and a receiver domain (Iuchi, 1993). Both ArcB and ArcA form a typical two-component regulator (TCR) system encoded by the *arcBA* operon (Iuchi and Lin, 1988). ArcA gets phosphorylated via ArcB under anaerobic conditions (Iuchi and Lin, 1988). Apart from anaerobiosis, several other signals have been proposed to induce ArcBA, this includes intermediates of electron transport chain, Fe^{2+} complex (Iuchi and Lin, 1988; Iuchi, 1993), metabolites such as pyruvate, acetate or NADH (Iuchi, 1993). The phosphorylated ArcA acts as both repressor and activator of several genes in *E. coli* (Iuchi and Lin, 1988). ArcA represses enzymes of TCA cycle such as *icd* (isocitrate dehydrogenase, *lpd* (lipoamide dehydrogenase E3), *sdhA* (succinate dehydrogenase) and *mdh* (malate dehydrogenase) but activates the expression of *pfl* (pyruvate formate lyase) and *cyd* (cytochrome *d* complex) under microerobic conditions (Iuchi and Lin, 1988). ArcBA TCR also functions in modulating gene expression under aerobic carbon starvation conditions (Nystrom *et al.*, 1996). Recently in *Salmonella typhimurium*, ArcA along with RpoS has been shown to be required

for transition into stationary phase under microaerobic conditions (Sevcik *et al.*, 2001).

Nucleoid-associated proteins.

To date 10 DNA binding structural proteins has been identified in *E. coli* (Ali *et al.*, 1999). Of these, HU, H-NS, integration host factor (IHF), and DNA binding protein from starved cells (Dps) are classified as nucleoid – associated proteins since all these proteins bind to the DNA without any sequence specificity with exception of IHF (Schmid, 1990). The main function of these proteins is to maintain structural organization of the nucleoid (Ali *et al.*, 1999). Recently the role of these proteins in DNA replication, recombination and transcription has been revealed (Almiron *et al.*, 1992; Atlung and Ingmer, 1997; Finkel and Johnson, 1992). Dps is one of the most abundant proteins during stationary phase (Ali *et al.*, 1999). The cellular level of Dps is growth phase regulated and it increases substantially during late stationary phase (Ali *et al.*, 1999). Dps is also induced by oxidative (Altuvia *et al.*, 1994) and nutritional stresses (Almiron *et al.*, 1992). Protein expression profiles of *dps* mutants show high pleiotropy during prolonged starvation, moreover *dps* mutants are sensitive to oxidative stress (Almiron *et al.*, 1992). It was previously proposed that oxidative stress sensitivity of *dps* mutants might be due to the regulation of DNA repair enzymes and catalases by Dps (Almiron *et al.*, 1992). Recent evidence indicate that Dps directly protect DNA by forming ferritin-like structures with DNA thereby protecting it from oxidative stress (Wolf *et al.*, 1999). Integration host factor (IHF)

encoded by *ihfA* and *ihfB* is a sequence specific DNA-binding protein, which is a host factor for integrative recombination of λ phage (Craig and Nash, 1984). IHF binds to the promoter region of genes and destabilize the DNA thereby forming open complexes in the promoter region, which increases the rate of transcription (Parekh and Hatfield, 1996). Microarray analysis of *E. coli* strains in *Ihf*$^+$ and *ihf*$^-$ background has revealed that IHF acts as a positive regulator of a number of genes, including those required for oxidative stress protection (*sodA*, *sodB* and *dps*), global gene regulation (*arcA*) and a number of genes with uncharacterized functions (Arfin *et al.*, 2000). IHF also regulates genes of the Pho regulon, *pstSCAB*, encoding a phosphate transporter and *phoA*, encoding alkaline phosphatase (Spira and Yagil, 1999). H-NS is a nucleoid-associated histone like protein which binds with high affinity to curved DNA (Atlung and Ingmer, 1997). H-NS compacts DNA (Dame *et al.*, 2000) and also functions as a global repressor of gene expression (Atlung and Ingmer, 1997). Nearly 35 genes have been identified to be under the negative control of H-NS many of which are also controlled by RpoS (Atlung and Ingmer, 1997). The *rpoS* gene itself is negatively regulated by H-NS, which explains why many of the RpoS regulated genes are down regulated by H-NS (Barth *et al.*, 1995). HU is another histone like protein encoded by two genes, *hupA* and *hupB* (Kano *et al.*, 1986). The *hupB* gene codes for HU1 and *hupA* codes for HU2 component of HU (Kano *et al.*, 1986). These two components bind together to form the HU protein (Kano *et al.*, 1986). The level of HU protein is high during early stationary phase and decreases in

the late stationary phase (Ali *et al.*, 1999). HU proteins functions in DNA inversion, replication and transposition (Drlica and Rouviere-Yaniv, 1987). Mutations in *hupA* and *hupB* results in poor growth, formation of anucleate cells and also affects proper septum placement (Huisman *et al.*, 1989). This phenotype observed in *hupAB* mutants is due to the mutations in *minCDE* operon required for proper septum placement (Rothfield *et al.*, 2001) and *mukFEB* operon required for proper chromosome partitioning. This suggests that *hupAB* has a role in proper chromosome partitioning and septum placement by controlling *minCDE* and *mukFEB* operons (Jaffe *et al.*, 1997). Moreover, HU also controls translation of *rpoS* by directly binding to the *rpoS*-mRNA (Balandina *et al.*, 2001).

The stringent response.

When starved for amino acids, *E. coli* responds by producing an alarmone, ppGpp (Cashel *et al.*, 1996) which down regulates the transcription of ribosomal RNA operons and upregulate some of the amino acid biosynthetic operons especially those required for histidine and arginine biosynthesis (Barker *et al.*, 2001; Chang *et al.*, 2002). This type of regulation is important during starvation since down regulation of ribosomal RNA operons leads to a decrease in protein synthesis thereby conserving energy for survival related cellular processes (Barker *et al.*, 2001; Chang *et al.*, 2002). The MazEF chromosomal addiction module is also under the control of ppGpp (Aizenman *et al.*, 1996). This addiction module encoded by the *mazEF* gene is upregulated by ppGpp and is responsible

for the programmed cell death of *E. coli* (Aizenman *et al.*, 1996). Programmed cell death has been implicated as a physiological response to overcome nutrient starvation (Aizenman *et al.*, 1996; Bishop *et al.*, 1998). Mutation in genes encoding ppGpp synthesis leads to pleiotropic effect and also negatively affects RpoS levels in *E. coli* suggesting that *rpoS* is also under the positive control of ppGpp (Gentry *et al.*, 1993).

cAMP/CRP complex.

Carbon catabolite repression is a well-known phenomenon in prokaryotes as well as in eukaryotes where the availability of a readily utilizable carbon source such as glucose represses the uptake of other carbon sources. In *E. coli*, cAMP/CRP complex is encoded by *cya* and *crp* genes respectively induces genes required for the utilization of alternative carbon sources. The cAMP/CRP complex brings about transcriptional regulation by binding to cAMP/CRP binding sites in the promoter and bends the DNA thereby facilitating open complex formation in the TIR (Saier, 1998). The cAMP/CRP complex modulates expression of numerous genes both positively and negatively. Strains carrying mutations in *cya* and *crp* genes grow slowly in many carbon sources and also exhibit abnormal cell morphology and diminished cell size (D'Ari *et al.*, 1988).

1.2.2 RpoS-dependent changes.

RpoS has always been implicated with stress related functions but in recent years, the role of RpoS in other cellular and survival related functions have been revealed. In many pathogenic enteric bacteria RpoS has been shown to control

virulence and related functions. In *S. typhimurium* RpoS controls *spvR-ABCD* plasmid, which codes for virulence and intracellular growth (Fang *et al.*, 1992). In fact, the attenuated vaccine strain *S. typhi* Ty21a, is an RpoS mutant (Coynault *et al.*, 1996). In enterohaemorraghic *E. coli*, the *esp* operon encoding the type III secretion system required for adhesion to epithelial cells is under RpoS control (Beltrametti *et al.*, 1999). Fimbriae or pili are required for the proper attachment of bacteria to its host. In *E. coli* the curli fimbriae encoded by the *csg* operon (*csgAB* and *csgDEFG*) is under the control of RpoS (Hammar *et al.*, 1995; Olsen *et al.*, 1993). Recent evidences indicate that curli fimbriae of *E. coli* form amyloid fibers (Chapman *et al.*, 2002). The amyloid fibers are usually formed by misguided protein folding pathway and are attributed to many human neuro-degenerative disease such as Alzheimer's and prion diseases in humans (Chapman *et al.*, 2002). It has been proposed that bacterial amyloids may play a role in certain human neuro-degenerative diseases (Chapman *et al.*, 2002). RT-PCR analysis of *P. aeruginosa* strains from patients suffering from chronic cystic fibrosis showed high levels of *rpoS*-mRNA indicating that RpoS may have a possible role in chronic bacterial infections (Foley *et al.*, 1999). Being an intestinal commensal, *E. coli* has evolved mechanisms that can resist high acidity. Moreover, the virulence of *E. coli* O157:H7 is also attributed to its ability to resist acidic conditions. The genes of the *gad* operon encoding two glutamate decarboxylase (*gadA* and *gadB*) and a putative glutamate:γ-aminobutyric acid antiporter (*gadC*) are required for acid resistance in *E. coli* (Castanie-Cornet *et*

al., 1999). These genes are under the positive control of RpoS in stationary phase (Castanie-Cornet *et al.*, 1999). Other cellular functions controlled by RpoS include cell morphology and cell division. The *bolA* gene product, by controlling penicillin-binding proteins (PBPs) confers round morphology to the stationary phase cells (Santos *et al.*, 2002). Moreover, the proteins of the cell division machinery, FtsQ and FtsA are also under RpoS control (Sitnikov *et al.*, 1996). These proteins are encoded by the *ftsQAZ* operon. This operon exhibits two promoters P1 and P2 in the upstream region, the promoter P2 is regulated by SdiA, while the P2 promoter belongs to the family of "gear-box" promoters, which is regulated by growth phase and RpoS (Sitnikov *et al.*, 1996). The regulation of FtsQ and FtsA proteins indicates that RpoS may play a part in cell division in *E. coli* (Sitnikov *et al.*, 1996). In stationary phase, RpoS mediates a part of the changes that occur in metabolic pathways and this is brought about by regulating the genes that are involved in these pathways. Some of the well-known examples of genes of the metabolic pathway regulated by RpoS include *acs*, which codes for acetyl-coA synthase, *poxB*, encoded by pyruvate oxidase, *glgS*, encoding enzyme required for glycogen synthesis, *talA*, encoding transaldolase A of the pentose phosphate pathway (Huisman *et al.*, 1996).

1.3.0 Changes in stationary phase (At cellular level).

All these changes brought about by RpoS and other regulators at the genetic level are exemplified in the gross cell morphology and physiology. Stationary phase cells differ from their exponential phase counterpart in many

aspect including cell size, shape, intracellular composition and metabolism (Fig. 1). Many of these changes are important for survival of *E. coli* in stationary phase. The changes that occur at cellular level can be broadly classified into two types: (a) Morphological changes and (b) Physiological changes.

1.3.1 Morphological changes.

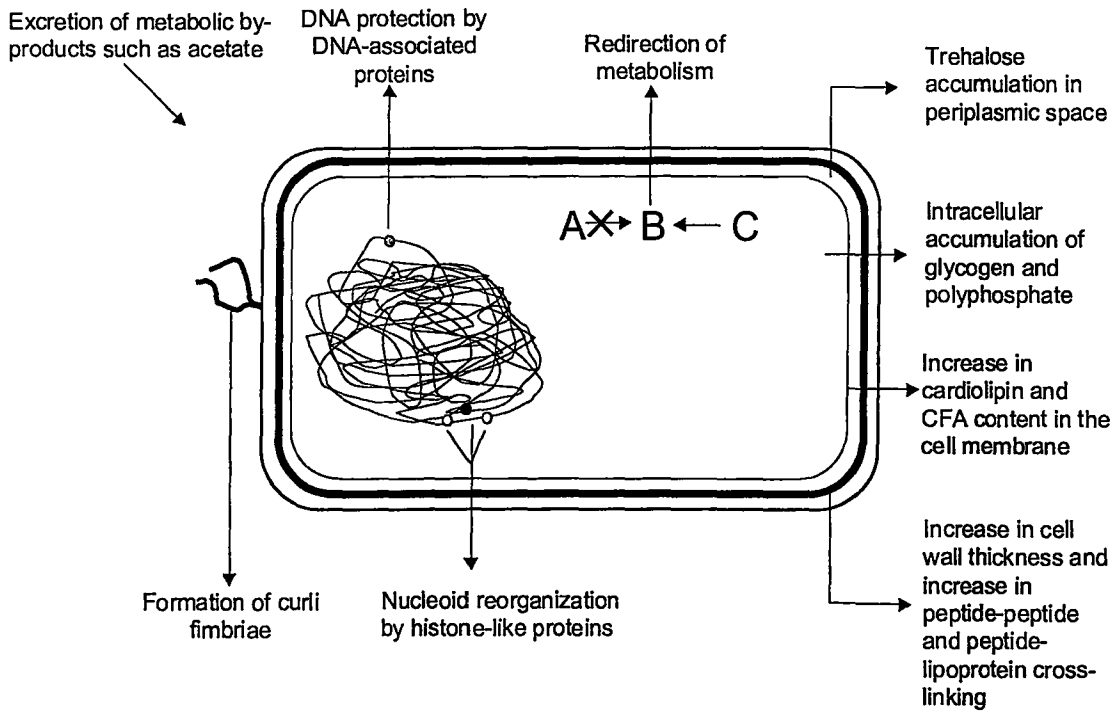
It is well known that *E. coli* undergoes changes in cell shape in stationary phase. These morphological changes can be either direct due to the change in gross cell shape or may be mediated indirectly due to changes in cell wall size or cell membrane composition (Huisman *et al.*, 1996).

Changes in cell morphology.

Under normal exponential growth conditions *E. coli* cells are rod shaped, as they enter the stationary phase they attain a round morphology. This change in cell shape is mediated by a morphogene *bolA* (Aldea *et al.*, 1988). The *bolA* gene has two promoters *bolA2p*, which is constitutively activated by RpoD but the expression from this promoter is very low (Lange and Hengge-Aronis, 1991) and the second promoter *bolA1p* is the main promoter activated by RpoS in stationary phase (Lange and Hengge-Aronis, 1991) and also by stress (Santos *et al.*, 2002). The *bolA* gene product, BolA is a transcriptional regulator with a helix-turn-helix DNA binding motif (Aldea *et al.*, 1988). Recently, BolA has been shown to control two D,D carboyxpeptidases, pencillin-binding protein 5 (PBP 5) encoded by *dacA* and pencillin-binding protein 6 (PBP 6) encoded by *dacC* (Santos *et al.*, 2002). D,D-carboxypeptidases play an important role in murien

turnover in *E. coli* (Nelson *et al.*, 2002). The change in cell shape during stationary phase reduces the surface to volume ratio of the cell and may limit the area exposed to the damaging effects encountered under these conditions (Santos *et al.*, 2002). Another cell division related operon under the control of RpoS is *ftsQAZ* (Sitnikov *et al.*, 1996). This operon encodes for proteins that are important in cell septum formation. Cytokinesis in bacteria requires coordinated action of nine different proteins including FtsQ, FtsA and FtsZ (Chen and Beckwith, 2001). FtsZ is one of the well-characterized proteins, which localizes at the septal rings and constricts it during cytokinesis (Addinall *et al.*, 1996). FtsA, is an actin homolog while the function of FtsQ is unknown (Chen and Beckwith, 2001). These proteins along with other Fts proteins have been localized in the septal rings indicating their role in cytokinesis (Chen and Beckwith, 2001). The *bolA* gene has been previously reported to be regulated by FtsZ (Aldea *et al.*, 1988). These findings reveal the complexity of transcriptional regulation during stationary phase and the possible role of RpoS in cytokinesis related functions.

Figure 1. Morphological and physiological changes associated with stationary phase. *E. coli* in stationary phase undergoes drastic changes in overall cell shape, nucleoid organization, membrane composition and metabolism.



Adapted from Husiman et al, 1996

Changes in cell membrane and cell wall composition.

In stationary phase, the cell membrane of *E. coli* undergoes changes in its chemical composition. The membrane of *E. coli* is mainly composed of monounsaturated fatty acids such as palmitoleic acid and oleic acid (Huisman *et al.*, 1996). Upon entry into stationary phase there is a 10-fold reduction in monounsaturated fatty acid content and concomitant increase in the levels of cyclopropane fatty acids (Huisman *et al.*, 1996). Cyclopropane fatty biosynthesis is dependent on the enzyme cyclopropane fatty acid synthase encoded by the *cfa* gene (Grogan and Cronan, Jr., 1997). This gene is under the control of RpoS (Chang and Cronan, Jr., 1999). Strains carrying null mutations in *cfa* gene exhibit abnormal sensitivity to increase in acidity (Chang and Cronan, Jr., 1999). Mutations in *cfa* and *rpoS* double mutants render the cell more sensitive to acidic conditions compared to the strains with mutations in either of these genes (Chang and Cronan, Jr., 1999). Changes also occur in the cell wall thickness during stationary phase (Huisman *et al.*, 1996). Under normal growth conditions *E. coli* cell wall consists of two to three layers of peptidoglycan this is increased to about five layers during stationary phase (Mengin-Lecreulx and van Heijenoort, 1985). The increase in the cell wall thickness may in turn increase the mechanical strength of the cell walls.

Changes in nucleoid organization.

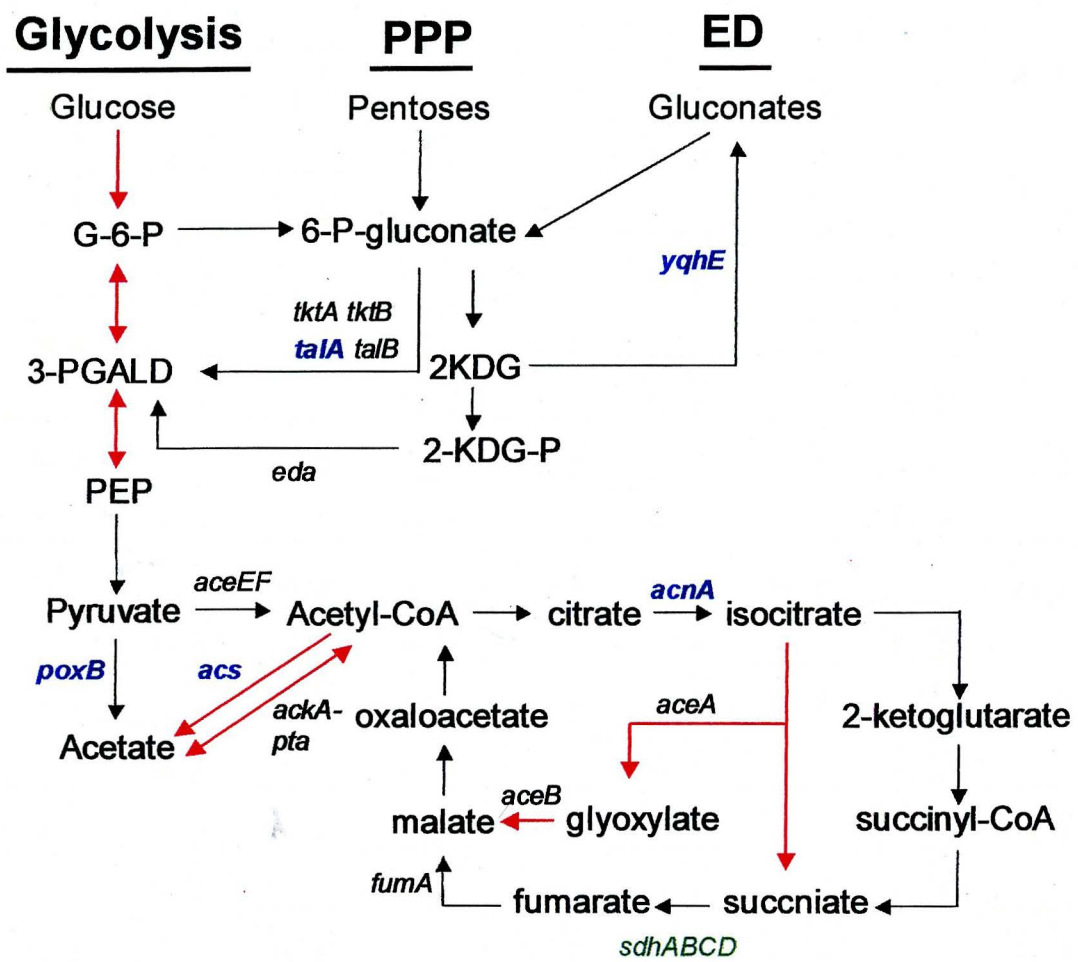
The nucleoid of *E. coli* is so large that it has to be compacted 1000 fold to fit the dimensions of the cell (Schmid, 1990). This is accomplished by a family of

DNA-binding proteins known as nucleoid-associated histone-like proteins (Schmid, 1990). So far, four different histone-like proteins have been identified in *E. coli*, these include H-NS, HU, IHF and Fis (Schmid, 1990). These proteins apart from compacting DNA, have protective and regulatory roles (Schmid, 1990). Atomic force microscopy has revealed that H-NS compacts DNA effectively to a dumble-shaped form (Dame *et al.*, 2000). *E. coli* nucleoid is more compact in stationary phase than in exponential phase (Huisman *et al.*, 1996). By compacting DNA the nucleoid associated proteins make it inaccessible to unnecessary transcription thereby conserving energy (Huisman *et al.*, 1996).

1.3.2 Physiological changes.

In stationary phase significant changes occur in the metabolic pathways (Fig 2), which include redirection of some metabolic functions in the tricarboxylic acid (TCA) cycle, formation and release of metabolic byproducts such as acetate, formate and D-lactate and accumulation of storage products such as glycogen and polyphosphate (Huisman *et al.*, 1996). These changes are important for the survival of *E. coli* in stationary phase. *E. coli* growing on excess glucose excretes acetate and low levels of D-lactate as metabolic by products. This seems like a wasteful and inefficient use of an energy source. But, in fact, this is one of the strategies employed by *E. coli* to survive nutrient limited stationary phase conditions. When levels of glucose drops down as the cells reach stationary phase they start utilizing acetate and D-lactate as carbon sources.

Figure 2. Metabolic changes during stationary phase. The pathways shown in red are upregulated in response to stationary phase. The genes shown in bold italics are upregulated by RpoS, while the genes shown in green are down-regulated by RpoS and the genes shown in blue are upregulated by ArcBA. (PPP refers to pentose phosphate pathway and ED refers to Entner-Doudorff pathway)


Glycolysis
PPP
ED
Glucose
Pentoses
Gluconates
G-6-P
6-P-gluconate
yqhE
tktA tktB
talA talB
3-PGALD
2KDG
2-KDG-P
eda
PEP
Pyruvate
aceEF
Acetyl-CoA
citrate
acnA
isocitrate
poxB
acs
ackA-
pta
Acetate
oxaloacetate
aceA
2-ketoglutarate
aceB
malate
glyoxylate
succinyl-CoA
fumA
fumarate
succniate
sdhABCD

Acetate is formed from pyruvate via acetyl-CoA. Conversion of acetyl-coA to acetate occurs via two pathways, the acetate kinase-acetyl phosphotransferase (AckA-Pta) pathway encoded by *ackA* and *pta* genes and the acetyl-CoA synthase (Acs) pathway, encoded by the *acs* gene (Shin *et al.*, 1997). The AckA-Pta pathway converts acetyl-CoA to acetate via acetyl phosphate and *acs* pathway converts acetyl-CoA directly to acetate. The AckA-Pta pathway is constitutively expressed while the Acs pathway is under the positive control of RpoS (Shin *et al.*, 1997). Pyruvate can also be converted directly to acetate without the formation of acetyl-CoA (Chang *et al.*, 2002). This is brought by pyruvate oxidase (PoxB) encoded by *poxB* (Chang *et al.*, 2002). The *poxB* gene is induced by RpoS and has been suggested to play an important role in survival of *E. coli* cells during the transition from exponential to stationary phase (Chang *et al.*, 2002). Growth on acetate also requires the upregulation of the glyoxylate bypass since this is the only pathway by which acetate can be efficiently utilized (Cronan, Jr. and LaPorte, 1996). The glyoxylate bypass is upregulated with the concomitant down regulation of TCA cycle enzymes (Nystrom *et al.*, 1996). Down-regulation of TCA cycle enzymes is important because increased TCA cycle activity produces oxygen radicals that are detrimental to the cells (Nystrom *et al.*, 1996). ArcAB rather than RpoS bring about these changes, but RpoS have been implicated to control some of these adjustments (Hengge-Aronis, 2000). Another important physiological change in carbon starved *E. coli* cells is the accumulation of glycogen (Romeo and Preiss, 1989). Glycogen is synthesized

from ADP-glucose by ADP-glucose pyrophosphorylase and glycogen synthase encoded by *glgCAP* operon (Preiss, 1996). These genes are under the positive control of cAMP-CRP (Preiss, 1996). Glycogen synthase encoded by *glgS*, is another gene involved in glycogen biosynthesis and is under the positive control of RpoS (Hengge-Aronis and Fischer, 1992). Glycogen is an important storage product since it can serve as an intracellular carbon reserve when the cells are starved for nutrients (Preiss, 1996). In stationary phase *E. coli* also accumulates treholase in the periplasmic space (Strom and Kaasen, 1993). Treholase accumulation serves two functions, in the absence of other compatible solutes trehalose serves as an osmo-protectant but in the presence of compatible solutes trehalose can be converted to glucose and can be utilized at a carbon source (Strom and Kaasen, 1993). Trehalose biosynthesis is under the control of two genes *otsA* and *otsB* (Kaasen *et al.*, 1992), while trehalose catabolism is dependent on *treA* (Strom and Kaasen, 1993). These two functions in *E. coli* are under the positive control of RpoS (Hengge-Aronis *et al.*, 1991).

1.4.0 Maintenance of Stationary phase.

Most of the above mentioned changes occur during the early stationary phase and the cells will again resume normal growth when they encounter optimal conditions. But, what happens if they don't encounter optimal growing conditions? What are the strategies that *E. coli* employ to survive stationary phase when all the carbon reserves and released metabolic by products such as acetate is exhausted? Macromolecular degradation is one of the mechanisms by

which *E. coli* cells survive long term starvation (Matin *et al.*, 1989). The important macromolecules that are degraded by starving *E.coli* include RNA (Kaplan and Apirion, 1975), proteins (Miller, 1996) and lipids (DiRusso and Nystrom, 1998). The RNase activity in *E. coli* under starvation conditions increases from to two to eight fold (Kaplan and Apirion, 1975). Ribosomal RNA is preferentially degraded to nucleotides and proteins, and subsequently used by the cells as nutrient sources and for other cellular functions (Kaplan and Apirion, 1975). Enhanced proteolysis has been reported in starving *E. coli* (Miller, 1996). This increase in proteolysis provides the starving cells with vital amino acids and possibly eliminates abnormal proteins synthesized during starvation and stress (Miller, 1996). Protein degradation is brought about by Lon and ClpPX proteases (Kuroda *et al.*, 2001). Polyphosphates also play a major role in starvation-induced proteolysis (Kuroda *et al.*, 1999; Kuroda *et al.*, 2001). It has been proposed that polyphosphate complexes with ribosomes thereby making it available to proteolytic degradation by Lon and ClpPX (Kuroda *et al.*, 2001). Starving *E. coli* also obtain vital carbon and energy source by degrading membrane lipids (DiRusso and Nystrom, 1998). Though the exact mechanism how this degradation is brought about is unknown, it has been suggested that phospholipases may play an important role (DiRusso and Nystrom, 1998). Several authors have reported that nutrients released by a population of lysing cells in stationary phase may provide nutrients to the surviving cells (Aizenman *et al.*, 1996; Bishop *et al.*, 1998; Zinser and Kolter, 1999). Identification of

chromosomal addiction modules such as MazEF and EcnBA suggests that they may have a role in programmed cell death in bacteria (Aizenman *et al.*, 1996; Bishop *et al.*, 1998). Moreover, high degree of DNA damage due to oxidative stress in stationary phase may also lead to cell lysis mediated through autolysins such as membrane bound lytic transglycosylase (MltB) (Lewis, 2000). But what determines which population of cells undergoes lysis? It is hypothesized that during the onset of starvation all the cells of the parental genotype have an equal chance for competing for the available nutrient and as the nutrients are exhausted the cells start to die off however, rare mutations in *rpoS* result in GASP (Growth Advantage Stationary Phase) mutants that have a competitive advantage over their parental strains owing to the enhanced catabolic capabilities. These GASP mutants grow and divide by utilizing the nutrients released by the lysing parental cells (Zinser and Kolter, 1999). Moreover, *rpoS* mutants have enhanced nutrient scavenging capability than the parental strains (Zinser and Kolter, 1999).

E. coli also express high affinity uptake systems under nutrient limited conditions these include MalT, LamB for carbon sources (Death and Ferenci, 1994) and LIV-I, ProU for amino acids (Ferro-Luzzi Ames, 1986), which help scavenge the scarce nutrients efficiently. These high affinity transport system has evolved to recapture the nutrients such as amino acids that are lost due to the leakage during biosynthesis (Ferro-Luzzi Ames, 1986). This recapture mechanism is very important to the cell because amino acid biosynthesis is an

energy expensive process and recapturing of the leaking amino acids is very economical (Kjelleberg *et al.*, 1987). During the stationary phase nutrients are not completely exhausted because cells entering stationary phase excrete metabolic byproducts and nutrients are also released from the lysing cells. Under glucose limited continuous culture *E. coli* induces high affinity glucose uptake systems as well as *rpoS* (Notley and Ferenci, 1996). This raises the question whether RpoS regulated genes have any role in nutrient scavenging and metabolism in stationary phase?

1.5.0 Identification of RpoS regulated genes.

Studies by McCann *et al.*, (1991) have revealed that RpoS regulon is large and recently Ishihama, (2000) have reported that RpoS controls nearly 100 genes. This list is still growing and many genes under RpoS control are being identified. Since RpoS regulon is very large complete characterization of genes under the control of RpoS has been slow (Schellhorn *et al.*, 1998). One of the methods to identify the genes of a regulon is by using genetic screening technique. In this method a mutant bank containing random *lacZ* fusion is created using lamda p*lac*Mu and *rpoS* null allele is introduced into these strains by transduction. The strains are then selected for RpoS dependence based on the expression of *lacZ* (Schellhorn *et al.*, 1998). The main advantage of this method is that lambda p*lac*Mu integrates with Mu S and Mu C sites flanking the gene of interest (Bremer *et al.*, 1984). The integrated prophage is then excised by inducing it with UV light and the phages thus produced carry a part of adjacent

host DNA. The hosts DNA can then be sequenced from Mu C end (Roy *et al.*, 1995). The DNA thus sequenced can then be identified using homology searches.

1.6.0 Purpose of this study.

Genomes of many organisms have been completely sequenced in recent years and 90% of the genomes that have been completely sequenced are of microbial origin (O'Connor *et al.*, 2000). The genomic data amassed have yet to make a significant impact in biology as many of the genes identified have not been assigned any function. For example, even in a well-studied organism like *E. coli*, functions of 30% of its genes still remains unknown (Blattner *et al.*, 1997). The RpoS regulon has always been associated with stress related functions. In recent years the role of RpoS regulon members in other cellular processes such as energy metabolism, central intermediary metabolism are being revealed. A few years ago we started a project to characterize the genes of the RpoS regulon. We identified 48 genes under RpoS control and many of these newly identified genes were not assigned any function. Recently, it has been suggested that some of the RpoS regulated genes with unknown functions have homology to the genes involved in transport and catabolism of several carbon and nitrogen sources. All this leads to many unanswered questions, for example, what are the exact functions of these genes? Are these genes involved in nutrient scavenging and metabolism in stationary phase? The aim of this project is to identify additional members of the RpoS regulon and to determine whether any of these RpoS regulated genes are required for nutrient scavenging or metabolism.

Figure 3. A diagramatic representation of genetic screening method used to identify RpoS-dependent genes in *Escherichia coli*. An *rpoS*::*Tn*10 mutation is introduced into a Hfr strain by transduction and then conjugated into a F^- recepient carrying random lacZ fusions. The strains were then screened for β-galactosidase activity on X-Gal plates and for the loss of catalase activity. The fusions from the strains thus selected were sequenced and the identity of the gene was found by BLAST algorithm.

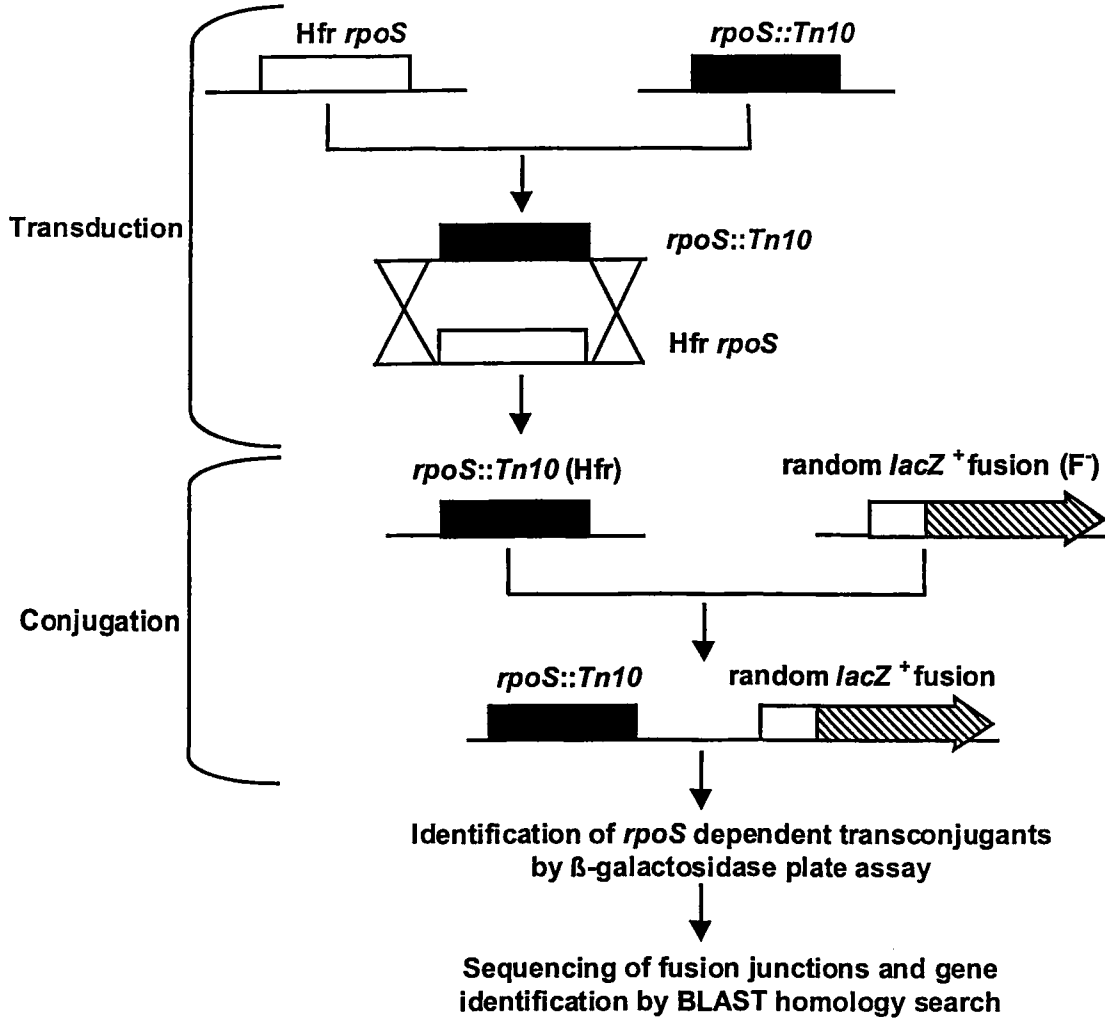

Hfr rpoS
rpoS::Tn10
Transduction
rpoS::Tn10
Hfr rpoS
rpoS::Tn10 (Hfr)
random lacZ + fusion (F-)
Conjugation
rpoS::Tn10
random lacZ + fusion
Identification of rpoS dependent transconjugants by ß-galactosidase plate assay
Sequencing of fusion junctions and gene identification by BLAST homology search

Table 1. *E. coli* strains and plasmid used in this study.

Strains and Plasmid	Genotype	Source or Reference
Strains		
GC4468	Δ*lacU*169 *rpsL*	(Schellhorn and Stones, 1992)
GC122	As GC4468, but Φ(*rpoS*::Tn*10*)	(Schellhorn and Hassan, 1988)
MC4100	Δ(*argF-lacZ*)205 *araD*139 *flbB*5301 *relA*1 *rpsL*150 *thi ptsF*25	(Schellhorn and Stones, 1992)
HS1002-HS1100	As GC4468, but carries RpoS-dependent operon-*lacZ* fusions	(Schellhorn *et al.*, 1998)
Plasmid		
pMM*katF3*	Carries *rpoS* (*katF*) gene	(Mulvey *et al.*, 1988)

2.0 MATERIALS AND METHODS

Chemicals and Media.

Chemicals used in this study were obtained from Fisher Scientific, Ltd (Toronto, Ontario, Canada), Sigma Chemicals Co. (St. Louis, Mo.), or Gibco BRL (Burlington, Ontario, Canada). Antibiotics, amino acids, sugars and other non-autoclavable solutions were filter sterilized using Gelman Sciences (Ann Arbor, Mi.) Acrodisc sterile filters (pore size, 0.45 μm). All liquid and solid media were prepared according to Miller, 1992 and where appropriate, the media was supplemented with kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; tetracycline, 15 μg/ml; and/or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 50 μg/ml. All amino acids used for this study were of L configuration while sugars were of D configuration except for arabinose.

Growth conditions.

Expression assays and amino acid and carbon utilization studies were performed using derivatives of *E. coli* (Table 1). Cultures were grown overnight in M9 minimal medium containing appropriate antibiotics. Cell growth was monitored spectrophotometrically (Novospec II, Pharmacia LKB Biochrom, Cambridge, UK) by measuring optical density at 600 nm (OD_{600}). For β-galactosidase expression studies, the cultures were maintained in early

exponential phase (OD_{600} <0.2) for at least 8 generations prior to the start of each experiment. Bacterial cultures were grown at 37°C and shaken at 200 rpm, sampled, and assayed for β-galactosidase activity at the times indicated. For amino acid and carbon utilization studies, the plates were incubated at 37°C for 3 days.

2.1 Isolation of RpoS-dependent genes.

To identify RpoS-dependent *lacZ* (*rsd-lacZ*) fusions, mutants containing individual random λp*lac*Mu insertions were conjugated with an Hfr strain containing an *rpoS*::Tn10 mutation that is transferred a few min after the point of origin during conjugation (Schellhorn *et al.*, 1998). $LacZ^+$ mutant strains that yielded transconjugants having reduced blue color on X-Gal-containing plates were considered to contain presumptive RpoS-dependent fusions. Of several hundred identified mutants, 105 could be efficiently complemented when transformed with an RpoS-expressing plasmid (determined by examining catalase activity in colonies grown on LB plates and flooded with 30% hydrogen peroxide). The fusion mutations were then $P1_{vir}$ transduced (Miller, 1992) into GC4468 for further study. A small proportion of the strains in the operon fusion mutant bank carried double fusions (~2%). During conjugation loss of a high expressing $lacZ^+$ allele by recombination, occasionally produced transconjugants that were Kan^R (due to retention of a second operon fusion) and possessed reduced *lacZ* activity. These strains were identified by the catalase

complementation test and eliminated from further study. A diagrammatic representation of this method is shown in Fig. 3.

2.2 Enzyme assays.

β-galactosidase activity was assayed using whole cells and ONPG as a substrate (o-nitrophenyl-β-D-galactopyranoside) (Miller, 1992). Units of activity were calculated as [1,000 x OD_{420}]/[time of incubation (min) x volume (ml) x OD_{600}] and are expressed in Miller units (Miller, 1992).

2.3.0 λ Lysogen induction.

2.3.1 Liquid lysate preparation.

Strains carrying *rsd-lacZ* fusion were inoculated into LB medium containing streptomycin and kanamycin and grown overnight at 37°C at 200 rpm. Cells were subcultured into 50 ml of LB medium and grown to an OD_{600} of 0.3, centrifuged and re-suspended in 10 ml of 10 mM $MgSO_4$. Lambdoid prophage induction was carried out by irradiating the cultures at 25 mW for 30 s (approximately $35J/m^2$). Irradiated cultures were transferred into 50 ml flasks containing 5 ml LL medium and shaken at 200 rpm until lysis (3 to 5 h) (Roy *et al.*, 1995). The lysate was then treated with chloroform (0.3% final concentration), shaken vigorously, and centrifuged at 10,000 x g for 10 min to remove the cell debris. Chloroform (0.3% final concentration) was added to the phage-containing supernatant and was stored at 4°C.

2.3.2 Plate lysate preparation.

To obtain a high-titer of phages, *E. coli* MC4100 was grown overnight in LB medium containing 0.2% maltose. The overnight culture was then subcultured into 10 ml of LB medium and grown to an OD_{600} of 0.3. This exponential phase culture was centrifuged and the pellet was resuspended in 5 ml cell suspension buffer (Maniatis *et al.*, 1982). One hundred µl of cells was mixed with 100 µl of lysate and incubated at 37°C for 30 min. After incubation, 8 ml of soft agarose (0.6%) was added and mixed thoroughly. The mixture was then poured on bottom agarose plates (1.5%), allowed to set and incubated at 37°C until confluent plaques were observed (6-8 h). Ten ml of suspension media (SM) buffer (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 1 M Tris Cl (pH 7.5) and 2% gelatin per liter) was added to the plates and incubated at room temperature with shaking for 2 h for passive diffusion of phages into the buffer. The buffer containing phages were transferred into clean 15 ml Corex tubes and chloroform (0.3% final concentration) was added, shaken vigorously and centrifuged at 10,000 x g for 10 min to remove the cell debris. DNase (10 µg/ml final concentration) and RNase (1 µg/ml final concentration) was added to the lysate and incubated at 37°C for 3 h. Chloroform (0.3% final concentration) was added to the lysate and stored at 4°C until use.

2.4 DNA template preparation and sequencing of λ fusion junctions.

DNA was extracted from the phage by phenol:chloroform extraction and ethanol precipitation (Maniatis *et al.*, 1982). Sequencing of bacterial DNA proximal to the λ fusion junction was performed by the MOBIX central facility (McMaster University, Hamilton, Ontario). The amount of DNA used in each sequencing reaction was approximately 1μg and was sequenced using Mu c end primer (5'-CCCGAATAATCCAATGTCCTCCCGG-3') (Roy *et al.*, 1995). The identities of the sequences obtained were determined by performing alignments with the BLASTN algorithm (Altschul *et al.*, 1990). The predicted functions of the genes thus identified are either based on SWISSPROT (Bairoch and Apweiler, 2000) or ECOCYC (Karp *et al.*, 2002) databases as indicated in the text.

2.5 Amino acid utilization studies.

All the strains were grown overnight at 37°C in microtiter plates containing M9 minimal media. All the 19 amino acids were provided individually either as a sole carbon or as a sole nitrogen source. Amino acids were used at 0.2% when provided as a sole carbon source and 0.1% when provided as a nitrogen source. For strains HS1072 and HS1072p (arginine auxotrophs), M9 minimal media was supplemented with 10 μg/ml arginine. Overnight cultures were diluted in M9 salts buffer and replica plated onto M9 minimal agar plates containing amino acids as sole carbon source or nitrogen source and X-Gal.

2.6 Carbon utilization studies.

All strains were grown overnight at 37°C in microtiter plates containing M9 minimal media. Sugars were supplemented at a concentration of 0.4%. Overnight cultures were then diluted in M9 salts buffer and replica plated onto M9 minimal agar plates containing different sugars as the sole source of carbon and X-Gal. For HS1072 and HS1072p (arginine auxotrophs), M9 minimal media was supplemented with 10 μg/ml arginine.

3.0 RESULTS

3.1 Identities of RpoS-dependent genes.

We identified several hundred *lacZ* mutants of which 105 contained RpoS-dependent fusions selected on the basis of complementation tests (Schellhorn *et al.*, 1998). All the strains containing *rsd-lacZ* fusions that were in duplicates were eliminated and strains with mutations in distinct genes were selected for further study. Many of the distinct *rsd-lacZ* fusions identified mapped to genes that are in operons. Though all the operon members were not identified as RpoS-dependent, the identification of single genes of an operon indicates that the whole operon may be under RpoS control. If this is taken into consideration the number of RpoS-dependent genes identified in this study stands close to 80. Of the 48 strains that contain fusions to distinct RpoS-dependent genes, 10 were sequenced and reported in a previous study (Schellhorn *et al.*, 1998). Here we report the identities of remaining fusions.

Strain HS1002 contains fusion in *yjbJ*. YjbJ is non-essential for the growth of *E. coli* in rich and minimal media (Link *et al.*, 1997). Recently, the crystal structure of YjbJ has been elucidated and it forms a four-helix bundle structure (Pineda-Lucena *et al.*, 2002). The *yjbJ* gene is similar to *csbD* (*ymwH*) of *B. subtilis*. The *csbD* gene forms a two-member operon with *csbC*, which has high identity to a Class I sugar transporter of the major facilitator (MFS) superfamily.

Both these genes are controlled by σ^B, a transcription factor that is expressed during stresses in *B. subtilis* (Akbar *et al.*, 1999). The function of YjbJ is unknown.

The *rsd*1008-*lacZ* (strain HS1008) mutation is located in *aldB*. The *aldB* gene codes for aldehyde dehydrogenase, which convert aldehydes to corresponding acids. The *aldB* gene has high similarity to *acoD* of *Alcaligenes eutrophus* and *aldA* of *Vibrio cholera* (Xu and Johnson, 1995). These genes code for broad-spectrum aldehyde dehydrogenases (Baldoma and Aguilar, 1987). Apart from RpoS, other identified regulators of *aldB* include Crp and Fis (Tani *et al.*, 2002; Xu and Johnson, 1995), which are positive and negative regulators respectively. The aldehyde dehydrogenase activity and physiological function of AldB in *E. coli* have yet to be confirmed (Xu and Johnson, 1995).

Strain HS1011 contains a fusion to *ygaU*, a gene with unknown function. YgaU is similar to the PBSX prophage proteins, XkdP and YqbP (Bairoch and Apweiler, 2000), found in *B. subtilis* (Krogh *et al.*, 1998).

The *rsd*1014 mutation (strain HS1014) maps to *katE* encoding catalase hydroperoxidase II, which converts highly reactive hydrogen peroxide into hydrogen and water (Von Ossowski *et al.*, 1991). In addition to RpoS, Lrp positively regulates transcription of *katE* (Tani *et al.*, 2002).

The fusion in HS1019 maps to the gene, *yqhE*. This gene codes for 2,5-di-keto-gluconate reductase A, which catalyzes the conversion of 2,5-di-keto-

guconate to 2-keto-L-gluconate (Yum *et al.*, 1999). Keto-gluconates can serve as sole carbon and energy sources in many bacteria (Yum *et al.*, 1999).

Sequencing of the HS1020 *rsd-lacZ* fusion and subsequent homology search revealed that the fusion is located in *mltB,* which codes for a membrane-bound lytic transglycosylase B (Ehlert *et al.*, 1995). This enzyme cleaves the glycosidic bonds between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan layer of the cell wall. Lytic transglycosylases play an important role in mucopeptide recycling in bacteria (Ehlert *et al.*, 1995).

The *lacZ* fusion in HS1022 is located in *narY. E. coli* has three functional nitrate reductases, one periplasmic nitrate reductase (NapA) and two membrane-bound nitrate reductases, nitrate reductase A (NRA) and nitrate reductase Z (NRZ) (Stewart, 1993). Nitrate reductase Z is encoded by the members of the *narZYWV* operon (Stewart, 1993). Both the nitrate reductases are tetramers composed of an alpha, a beta and two gamma subunits. The *narY* gene codes for the beta subunit of nitrate reductase Z that functions as an electron acceptor during anaerobic growth on nitrate in *E. coli* (Chang and Cronan, Jr., 1999).

The fusion in HS1026 is located in *ybaY,* a gene of unknown function. YbaY belongs to the class of proteins that are involved in glycoprotein/polysaccharide metabolism in *E. coli* (Karp *et al.*, 2002).

The fusion in HS1033 is located in *ydaM.* The YdaM sequence reveals two PAS and a PAC domain (Bairoch and Apweiler, 2000). These domains are found

in many signaling proteins where they act as signal sensors and receptors (Zinser and Kolter, 1999). The function of YdaM is unknown.

The *lacZ* mutation in HS1035 mapped to *ydcS*. The *ydcS* gene is part of a four member operon (*ydcSTUV*) and codes for a putative binding protein dependent ABC transporter (Karp *et al.*, 2002). YdcS is similar to polyamine transporters, PotD/PotF (Schellhorn *et al.*, 1998) and is under the positive control of RpoS (Schellhorn *et al.*, 1998). The function of this gene has not been demonstrated, however, microarray analysis has revealed that *ydcSTUV* operon is positively regulated by nitrogen assimilation control protein (Nac), which induces genes required for nitrogen scavenging under nitrogen limited conditions (Zimmer *et al.*, 2000).

The *rsd-lacZ* fusion in HS1036 is located in *ydcK*. YdcK is a hypothetical protein of unknown function and exhibits similarity to bacterial acetyltransferases (Bairoch and Apweiler, 2000).

Strain HS1024 contains a fusion in *ygaF*. This gene is found upstream of the *gab* operon, which is required for γ-amino-butyric acid (GABA) utilization in *E. coli* (Niegemann *et al.*, 1993). Recently, *ygaF* was shown to be upregulated in an *E. coli* septicemia strain in mice (Khan and Isaacson, 2002). The function of YgaF is unknown.

Sequencing of *rsd-lacZ* fusions in strains HS1010, HS1037 and HS1057 revealed that the fusions are located in *gabP*, *gabD-gabT* intergenic region and *gabD* respectively. The *gab* genes form an operon (*gabDTPC*) that encodes the

γ-amino-butyric acid (GABA) degradation pathway in *E. coli* (McFall and Newman, 1996). The *gabT* gene codes for glutamate:succinic semialdehyde transaminase that catalyzes the first step in the GABA catabolism, converting 4-aminobutanoate and 2-oxoglutarate to glutamate, and succinate semialdehyde. The latter is ultimately converted to succinate, by succinate semialdehyde dehydrogenase encoded by *gabD* (Niegemann *et al.*, 1993). The *gabP* gene codes for a GABA permease protein that functions as a GABA transporter in this pathway (Niegemann *et al.*, 1993). The *gabP* gene was previously reported as RpoS-dependent (Schellhorn *et al.*, 1998). There are three 35-bp repetitive extragenic palindromic (REP) sequences between *gabT* and *gabP*. These REP elements may play a role in repressing the transcription of this operon (Niegemann *et al.*, 1993). The YgaE encoded by *ygaE* gene is a regulatory protein belonging to the GntR family of transcriptional regulators (Bairoch and Apweiler, 2000). This gene is found upstream of the *gab* operon and functions as a repressor (Schneider *et al.*, 2002). The *ygaE* gene has been recently designated as *gabC* (Schneider *et al.*, 2002). Nitrogen assimilation control (Nac) protein acts as a positive regulator of this operon (Zimmer *et al.*, 2000).

The *lacZ* fusion in HS1042 is located in *yhjY*. YhjY is predicted to be a putative lipase involved in fatty acid and phospholipid metabolism in *E. coli* (Karp *et al.*, 2002).

Strain HS1049 carries a fusion in *yeaG* that encodes for a protein of unknown function. YeaG shows high homology to a serine protease kinase,

PrkA, which is a part of the phosphotransferase system (PTS)-associated proteins in *B. subtilis* (Reizer *et al.*, 1999). Recently, *yeaG* was shown to be activated in artificial seawater medium in *E. coli* (Rozen *et al.*, 2001). This gene is under the positive control of RpoS in *S. typhimurium* (Ibanez-Ruiz *et al.*, 2000) and in *E. coli* (Rozen *et al.*, 2001).

The *rsd-lacZ* fusion in HS1050 is located in *phnP*. The *phnP* gene is part of a fourteen-member operon, *phnCDEFGHIJKLMNOP,* which is required for phosphonate utilization in *E. coli* (Metcalf and Wanner, 1993). Genes from *phnG* to *phnM* code for the membrane-bound subunit of carbon-phosphorus lyase complex (Yakovleva *et al.*, 1998). Genes *phnP* and *phnN* are non-essential for phosphonate utilization but they may code for accessory proteins of the carbon-phosphorus lyase complex (Metcalf and Wanner, 1993). The genes *phnF* and *phnO* may have regulatory functions (Metcalf and Wanner, 1993).

The *rsd-lacZ* fusion in HS1059 mapped to *talA*, encoding the enzyme transaldolase A, that catalyzes the conversion of sedoheptulose-7-P and glyceraldehyde-3-P to fructose-6-P and erythrose-4-P (Sprenger, 1995). The *talA* gene forms an operon with *tktB*, which codes for transketolase B. These two enzymes act together to maintain the metabolite balance in the pentose-phosphate pathway (Sprenger, 1995). The CreBC two-component regulator positively regulate *talA* expression (Avison *et al.*, 2001).

The *rsd*1061-*lacZ* mutation mapped to *aroM*. The function of this gene is unknown, but it may be required for aromatic amino acid biosynthesis (DeFeyter

et al., 1986). AroM forms an operon with *aroL*, which together codes for the enzymes of shikimate kinase II pathway (DeFeyter *et al.*, 1986).

The *lacZ* fusion in strains HS1063 and HS1075 mapped to the genes *ugpE* and *ugpC*, respectively. These genes are part of the five-member operon with *ugpBAECQ* that codes for proteins required for the uptake of glycerol-3-phosphate (G-3-P), glycerol and glycerol phosphate phosphodiesters (Boos, 1998). The *ugpE* gene codes for the membrane-bound component of an ABC transport system responsible for G-3-P uptake and *ugpC* codes for the ATP binding protein of a G-3-P transporter (Boos, 1998). The *ugp* operon is a member of the Pho regulon (required for phosphate uptake and utilization) and is positively regulated by the PhoBR two component regulator (Boos, 1998).

Mutations in strains HS1077 and HS1081 mapped to the genes *yhiV* and *yhiU*, respectively. These two genes comprise an operon that codes for a multidrug efflux pump (Tsukagoshi and Aono, 2000). Over-expression of *yhiU* and *yhiV* confers resistance to a variety of antimicrobial compounds (Nishino and Yamaguchi, 2002) and increases resistance to solvents like nonane and octane (Tsukagoshi and Aono, 2000). These genes were previously identified as RpoS-dependent (Schellhorn *et al.*, 1998) and are positively regulated by the response regulator EvgA of EvgSA two-component regulatory system (Nishino and Yamaguchi, 2002).

Strain HS1067 has a fusion in *ybiO*, encoding member of the UPF0003 protein family (Bairoch and Apweiler, 2000). Other proteins in this family include

KefA (potassium efflux system) of *E. coli* and MJ0170 and MJ1143 proteins of *Methanococcus jannaschii*. The function of YbiO is unknown.

The *rsd*1072-*lacZ* mutation mapped to *argH*, a gene that forms a part of the *argCBH* operon. These genes code for enzymes required for the biosynthesis of arginine. The *argH* gene codes for argino-succinate lyase, which converts L-argino-succinate to L-arginine and fumarate, the final step in arginine biosynthesis (Glansdorff, 1996). The *arg* operon is negatively regulated by a hexameric repressor protein, ArgR (Tian *et al.*, 1992).

The *lacZ* fusion in HS1073 is located in *ylil*. Ylil is a putative dehydrogenase and shows similarity to glucose dehydrogenase B of *Acinetobacter calcoaceticus* (Shin *et al.*, 1997). The *ylil* gene is known to be RpoS-dependent (Schellhorn *et al.*, 1998), but the physiological function of Ylil is unknown.

The *lacZ* fusion in HS1078 is located in *uspB* which codes for a 14-kDa protein induced during stationary phase and is upregulated by RpoS (Farewell *et al.*, 1998). Mutation in *uspB* increases the susceptibility of *E. coli* to ethanol in stationary phase and over-expression of *uspB* causes cell death in stationary phase (Farewell *et al.*, 1998). It has been proposed that UspB may play a role in sensing or changing the membrane composition during stationary phase and thereby protecting the cells from the damaging effects of ethanol (Farewell *et al.*, 1998).

The mutation in HS1079 is identified in *ldcC* encoding lysine decarboxylase C (Lemonnier and Lane, 1998). There are two types of lysine decarboxylases in

E. coli: (i) CadA, an inducible lysine decarboxylase, and (ii) LdcC, originally thought to be constitutively expressed (Yamamoto *et al.*, 1997) but was subsequently found to be under RpoS control (Kikuchi *et al.*, 1998). LdcC is expressed in very low levels in *E. coli*, and catalyzes the decarboxylation of lysine to cadaverine and CO_2 (Lemonnier and Lane, 1998).

The *rsd*1080 mutation mapped to *appB* which shows strong similarity to the *cydB* gene encoding subunit II of cytochrome oxidase I of *E. coli* (Green *et al.*, 1988). The *appB* gene forms an operon with *appBCyccBappA* and codes for subunit II of cytochrome oxidase II (Dassa *et al.*, 1991). The *appB* gene is induced during stationary phase, phosphate starvation, aerobic to anaerobic shifts and is under the control of AppR (Dassa *et al.*, 1991). Though the exact function of AppB is unknown, *cyo* (cytochrome o oxidase), *cyd* (cytochrome d oxidase) and *appB* triple mutants are sensitive to oxygen suggesting that AppB may function as a oxygen detoxifying enzyme or an alternative cytochrome oxidase along with AppC (Dassa *et al.*, 1991).

The fusion in HS1082 mapped to *aidB*. Expression of the *aidB* gene is induced by alkylating agents, anaerobiosis and by acetate at acidic pH (Landini *et al.*, 1996). Normal transcription of *aidB* requires the formation of a complex consisting of meAda (methylated Ada) and RpoS (Landini and Volkert, 1995). Though the function of AidB is not completely understood, it may play a role in detoxifying nitroguanidines or toxic intermediates of nitroguanidines (Landini *et al.*, 1994).

The *rsd*1084-*lacZ* mutation is located in a gene of unknown function, *yehX*. This gene codes for a hypothetical ATP-binding protein of the ABC transporter family and is a member of the *yehZXYW* operon (Bairoch and Apweiler, 2000).

In strain HS1090, the *lacZ* fusion mapped to the gene *yphA*. The *yphA* gene product shows similarity to a hypothetical transmembrane protein YqjF of *E. coil* (Bairoch and Apweiler, 2000), but the function of these genes is not known.

The *lacZ* mutation in HS1091 is located in *osmY*. OsmY encodes a periplasmic protein induced by high osmolarity (Yim and Villarejo, 1992). Though the exact function of OsmY is unknown, mutations in *osmY* increase the susceptibility of *E. coli* to hyperosmotic conditions (Yim and Villarejo, 1992). RpoS positively regulates *osmY* transcription (Yim *et al.*, 1994), while Lrp (leucine responsive protein), Crp (cAMP receptor protein) and IHF (Integration host factor) are negative regulators of *osmY* (Colland *et al.*, 2000).

The *lacZ* fusion in HS1094 is located in *otsA* encoding trehalose-6-phosphate synthase, which forms a two-member operon with *otsB,* encoding trehalose-6-phosphate phosphatase (Strom and Kaasen, 1993). Trehalose biosynthesis is a two-step process where glucose-6-phosphate and UDP-glucose is converted to trehalose-6-P by trehalose-6-phosphate synthase, which is then converted to trehalose by trehalose-6-phosphate phosphatase (Strom and Kaasen, 1993). Biosynthesis of trehalose is induced in response to high osmolarity and upon entry into stationary phase (Strom and Kaasen, 1993). Trehalose can serve as an osmoprotectant in the absence of other compatible

solutes and if other compatible solutes are present, trehalose can be used as a carbon source (Strom and Kaasen, 1993). Transcription of *osmY* is highly RpoS dependent and is also induced by Lrp (Tani *et al.*, 2002).

The *rsd*1095-*lacZ* fusion mapped to *ecnB* that codes for a bacteriolytic lipoprotein known as entericidin B (Bishop *et al.*, 1998). Along with *ecnA*, *ecnB* forms an antidote/toxin gene pair termed as "addiction module" that induce programmed cell death of bacterial populations in stationary phase (Bishop *et al.*, 1998). The *ecnB* gene is induced by high osmolarity in stationary phase and is positively regulated by RpoS, while EnvZ/OmpR two-component regulator negatively regulates the *ecn* locus (Bishop *et al.*, 1998).

The *lacZ* mutation in HS1099 mapped to *yhjD*. YhjD shows similarity to tRNA processing ribonucleases (Tani *et al.*, 2002), however the exact function of YhjD is unknown.

The *rsd*1092-*lacZ* mutation is located in *yfcG*. The *yfcG* genes codes for a hypothetical glutathione-S-transferase like protein whose function is unknown (Bairoch and Apweiler, 2000). SdiA, a quorum sensing regulator protein, positively regulate *yfcG* transcription (Wei *et al.*, 2001).

The *lacZ* fusion in HS1045 is located in *yhiN*. YhiN shows similarity to a highly conserved transmembrane protein (HI0933) of unknown function in *H. influenzae* (Bairoch and Apweiler, 2000).

Table 2. Growth phase-dependent expression of RpoS-dependent operon-*lacZ* fusions in strains grown in rich medium*

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*rpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1002	*yjbJ*	6.2 ± 0.4	3.2 ± 0.1	2	74.4 ± 3.4	3.4 ± 0.1	22
HS1006	*yjbE*	4.2 ± 0.3	2.1 ± 0.0	2	45.2 ± 1.6	2.1 ± 0.1	22
HS1008	*aldB*	5.0 ± 0.1	1.8 ± 0.1	3	91.4 ± 3.3	4.3 ± 0.1	22
HS1010	*gabP*	5.1 ± 0.3	2.8 ± 0.0	2	111.3 ± 3.2	4.7 ± 0.1	24
HS1011	*ygaU*	15.4 ± 1.3	18.9 ± 0.9	1	144.3 ± 2.0	33.5 ± 1.8	4
HS1012	*ygdI*	13.8 ± 0.7	3.9 ± 0.9	4	54.5 ± 2.4	7.6 ± 1.5	7
HS1014	*katE*	12.7 ± 0.0	1.9 ± 0.0	7	80.3 ± 0.7	2.0 ± 0.1	40
HS1019	*yqhE*	7.4 ± 1.4	10.5 ± 0.4	2	91.4 ± 8.7	12.2 ± 0.4	8
HS1020	*mltB*	12.3 ± 0.5	38.1 ± 2.0	0.3	90.1 ± 2.2	52.5 ± 1.0	2
HS1022	*narY*	3.4 ± 0.1	2.5 ± 0.0	1	28.3 ± 1.3	3.0 ± 0.2	10
HS1024	*ygaF*	3.1 ± 0.1	10.2 ± 0.7	0.3	49.7 ± 4.4	18.8 ± 1.1	3
HS1026	*ybaY*	29.8 ± 1.6	10.1 ± 0.1	3	243.0 ± 14.5	16.4 ± 0.5	15
HS1028	*yjgR*	14.4 ± 1.2	7.4 ± 0.1	2	63.0 ± 2.9	11.5 ± 0.9	6
HS1033	*ydaM*	8.6 ± 0.4	0.9 ± 0.0	10	35.2 ± 0.8	2.2 ± 0.0	16
HS1035	*ydcS*	6.7 ± 0.1	2.4 ± 0.0	3	21.7 ± 0.7	3.9 ± 0.0	6

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*rpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1036	*ydcK*	0.7 ± 0.0	1.8 ± 0.1	0.4	46.2 ± 0.3	3 ± 0.1	15
HS1037	*gabD-gabT*	0.7 ± 0.0	0.6 ± 0.0	1	11 ± 0.3	1.3 ± 0.0	8
HS1042	*yhjY*	11.7 ± 0.3	3.6 ± 0.0	3	66.8 ± 2.8	4.5 ± 0.1	15
HS1045	*yhiN*	16.1 ± 1.5	11.1 ± 0.3	1	60.1 ± 1.0	13.0 ± 0.2	5
HS1049	*yeaG*	11.7 ± 0.6	2.2 ± 0.0	5	42.2 ± 4.6	3.3 ± 0.1	13
HS1050	*phnP*	17.4 ± 0.3	5.4 ± 0.1	3	51.1 ± 1.2	6.5 ± 0.3	8
HS1054	*yebF*	23.0 ± 0.5	2.7 ± 0.0	9	225.1 ± 2.7	5.5 ± 0.0	41
HS1056	*yodC*	7.1 ± 0.6	1.9 ± 0.3	4	166.1 ± 10.5	4.0 ± 0.2	41
HS1057	*gabD*	4.6 ± 0.4	3.7 ± 0.2	1	245.2 ± 4.0	15.7 ± 0.6	16
HS1059	*talA*	3.2 ± 0.2	3.3 ± 0.2	1	106.5 ± 1.6	5.3 ± 0.4	20
HS1061	*aroM*	2.1 ± 0.1	2.1 ± 0.0	1	74.8 ± 1.1	5.0 ± 0.3	15
HS1063	*ugpE*	2.7 ± 0.6	3.5 ± 0.0	1	47.0 ± 2.7	8.1 ± 0.0	6
HS1064	*nlpA-yicM*	5.9 ± 0.4	7.8 ± 0.2	1	30.7 ± 0.1	11.1 ± 0.0	3
HS1066	*argH-oxyR*	12.3 ± 0.5	14.8 ± 1.3	1	30.0 ± 1.1	18.9 ± 0.4	2
HS1067	*ybiO*	2.4 ± 0.6	1.0 ± 0.0	2	11.9 ± 0.0	1.0 ± 0.0	13
HS1071	*yhjG*	12.8 ± 3.4	6.0 ± 0.4	2	159.0 ± 4.5	9.4 ± 0.2	17
HS1072	*argH*	9.2 ± 0.3	12.4 ± 1.3	1	33.9 ± 0.4	18.3 ± 0.2	2

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*rpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1073	*ylil*	2.5 ± 0.0	1.0 ± 0.1	3	20.3 ± 1.1	1.2 ± 0.1	17
HS1075	*ugpC*	2.7 ± 0.1	1.3 ± 0.1	2	26 ± 0.5	1.3 ± 0.0	20
HS1077	*yhiV*	2.1 ± 0.1	1.9 ± 0.1	1	101.4 ± 3.5	3.3 ± 0.5	31
HS1078	*uspB*	4.5 ± 0.4	5.6 ± 0.5	1	30.0 ± 0.9	7.1 ± 0.2	4
HS1079	*ldcC*	4.9 ± 0.3	3.4 ± 0.6	1	29.8 ± 0.8	4.5 ± 0.9	7
HS1080	*appB*	2.7 ± 0.1	0.9 ± 0.0	3	112.8 ± 2.5	4.6 ± 0.0	25
HS1081	*yhiU*	6.0 ± 4.2	2.1 ± 0.1	3	112.3 ± 13.4	2.9 ± 0.1	39
HS1082	*aidB*	8.8 ± 5.4	3.0 ± 0.1	3	138.0 ± 39.9	3.8 ± 0.0	36
HS1084	*yehX*	3.6 ± 1.9	1.5 ± 0.1	2	58.3 ± 6.3	2.1 ± 0.1	28
HS1090	*yphA*	6.0 ± 2.0	2.7 ± 0.1	2	64.5 ± 5.6	2.7 ± 0.3	24
HS1091	*osmY*	15.9 ± 0.9	6.0 ± 0.2	3	264.9 ± 17.7	7.5 ± 0.3	35
HS1092	*yfcG*	3.3 ± 1.0	1.2 ± 0.2	3	62.3 ± 12.3	1.7 ± 0.2	37
HS1094	*otsA*	8.5 ± 0.4	3.1 ± 0.1	3	165.2±5.2	5.4 ± 0.2	31
HS1095	*ecnB*	15.0 ± 2.5	7.3 ± 0.5	2	144.3 ± 5.4	6.3 ± 0.4	23
HS1099	*yhjD*	29.2 ± 1.3	37.9 ± 0.6	1	172.3 ± 1.3	33.6 ± 1.2	5
HS1100	*yjiN*	8.6 ± 0.6	6.2 ± 0.7	1	66.8 ± 4.1	15.6 ± 1.6	4

*Strains were grown overnight in LB broth, subcultured, and maintained in exponential phase for 8 generations prior to sampling. Cultures were sampled it exponential phase (OD_{600} 0.3) and stationary phase (OD_{600} 1.5). All the values are shown as ± standard error.

Table 3. Growth phase-dependent expression of RpoS-dependent operon-*lacZ* fusions in strains grown in minimal medium*

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*RpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1002	*yjbJ*	20.2 ± 1.1	3.6 ± 2.6	6	53.7 ± 3.0	0.3 ± 0.0	179
HS1006	*yjbE*	23.9 ± 0.8	1.0 ± 0.1	24	63.3 ± 1.2	1.1 ± 0.0	58
HS1008	*aldB*	4.3 ± 0.1	0.3 ± 0.1	14	8.2 ± 0.2	0.4 ± 0.0	21
HS1010	*gabP*	6.9 ± 2.0	0.5 ± 0.0	14	26.8 ± 0.8	1.5 ± 0.1	18
HS1011	*ygaU*	58.9 ± 5.7	6.9 ± 0.2	9	198.4 ± 7.3	16 ± 0.9	12
HS1012	*ygdI*	8.4 ± 0.0	4.7 ± 0.4	2	21.3 ± 0.7	4.0 ± 0.1	5
HS1014	*katE*	23.4 ± 1.4	0.3 ± 0.0	78	79.4 ± 7.1	1.2 ± 0.1	66
HS1019	*yqhE*	20.2 ± 0.5	3.4 ± 0.0	6	48.3 ± 1.8	4.6 ± 0.4	11
HS1020	*mltB*	3.5 ± 0.3	5.3 ± 0.7	1	26.1 ± 0.9	16.3 ± 0.9	2
HS1022	*narY*	0.9 ± 0.2	1.2 ± 0.1	1	5.4 ± 1.0	2.4 ± 1.0	2
HS1024	*ygaF*	2.3 ± 0.3	1.0 ± 0.1	2	16.9 ± 3.1	2.5 ± 0.1	7
HS1026	*ybaY*	16.1 ± 3.0	1.5 ± 0.6	11	132.7 ± 5.1	11.0 ± 5.1	12
HS1028	*yjgR*	3.5 ± 0.9	1.5 ± 0.3	2	27.6 ± 3.9	8.1 ± 3.9	3
HS1033	*ydaM*	20.8 ± 2.1	2.1 ± 0.0	10	38.2 ± 1.6	1.5 ± 0.2	25

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*rpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1035	*ydcS*	2.0 ± 0.2	1.1 ± 0.1	2	18.9 ± 4.7	12.7 ± 4.7	2
HS1036	*ydcK*	5.2 ± 0.3	1.9 ± 0.2	3	26.7 ± 0.4	3.1 ± 0.0	9
HS1037	*gabD-gabT*	1.9 ± 0.1	0.3 ± 0.0	6	3.7 ± 0.1	0.6 ± 0.0	6
HS1042	*yhjY*	3.0 ± 1.2	1.2 ± 0.2	3	29.1 ± 9.7	3.2 ± 0.7	9
HS1045	*yhiN*	12.7 ± 0.5	1.6 ± 0.0	8	16.3 ± 1.2	2.5 ± 1.2	7
HS1049	*yeaG*	12.3 ± 0.6	0.5 ± 0.0	25	13.7 ± 1.7	1.4 ± 0.0	10
HS1050	*phnP*	2.7 ± 0.0	1.0 ± 0.0	3	2.2 ± 1.1	1.4 ± 1.1	2
HS1054	*yebF*	44.6 ± 2.5	3.6 ± 0.2	13	87.7 ± 1.1	3.6 ± 0.2	25
HS1056	*yodC*	9.78 ± 0.7	0.9 ± 0.0	11	23.6 ± 1.8	1.4 ± 0.4	17
HS1057	*gabD*	12.4 ± 0.9	1.3 ± 0.2	10	27.1 ± 3.0	2.1 ± 3.0	13
HS1059	*talA*	13.3 ± 2.0	0.9 ± 0.1	15	42.6 ± 1.6	1.6 ± 0.6	26
HS1061	*aroM*	1.81 ± 0.2	0.3 ± 0.0	6	31.9 ± 1.2	0.4 ± 1.2	78
HS1063	*ugpE*	7.3 ± 0.3	0.6 ± 0.1	12	10.8 ± 0.4	1.9 ± 0.7	6
HS1064	*nlpA-yicM*	8.8 ± 1.7	6.1 ± 0.4	1	23.1 ± 1.0	6 ± 0.1	4
HS1066	*argH-oxyR*	88.3 ± 4.9	81.4 ± 8.4	1	102.5 ± 9.0	102.4 ± 6.2	1
HS1067	*ybiO*	7.0 ± 1.0	0.4 ± 0.1	18	15.8 ± 0.2	0.6±0.0	26
HS1071	*yhjG*	27.7 ± 2.7	1.8 ± 0.2	15	95.7 ± 3.3	6.3 ± 0.2	15

		β-galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
Strain	Gene	WT	*rpoS*⁻	RpoS dep.	WT	*rpoS*⁻	RpoS dep.
HS1072	*argH*	42.8 ± 1.9	24.5 ± 1.0	2	90.8 ± 1.9	17.9 ± 1.8	5
HS1073	*ylil*	13.4 ± 0.6	0.4 ± 0.1	34	22.0 ± 0.5	0.6±0.0	37
HS1075	*ugpC*	3.2 ± 0.2	0.7 ± 0.0	5	9.9 ± 0.3	1.1 ± 0.0	9
HS1077	*yhiV*	13.2 ± 1.6	1.2 ± 0.1	11	86.6 ± 11.7	22.5 ± 4.0	4
HS1078	*uspB*	21.1 ± 5.2	2.0 ± 0.3	11	66.5 ± 11.7	22.5 ± 4.0	8
HS1079	*ldcC*	18.1 ± 2.4	1.3 ± 0.1	14	46.6 ± 1.9	4.3 ± 0.4	11
HS1080	*appB*	8.4 ± 1.5	1.6 ± 0.1	5	9.3 ± 1.5	1.3 ± 0.0	7
HS1081	*yhiU*	34.0 ± 8.3	2.5 ± 0.5	14	117.1 ± 3.2	14.0 ± 2.5	8
HS1082	*aidB*	22.2 ± 3.1	1.0 ± 0.1	20	55.5 ± 3.2	2.6 ± 0.1	33
HS1084	*yehX*	23.1 ± 2.6	1.0 ± 0.1	23	55.5 ± 3.2	2.6 ± 0.1	21
HS1090	*yphA*	25.2 ± 2.1	1.0 ± 0.1	25	67.5 ± 2.6	3.7 ± 0.0	18
HS1091	*osmY*	110.2 ± 2.2	2.3 ± 0.2	48	210.4 ± 3.4	3.3 ± 0.0	64
HS1092	*yfcG*	16.3 ± 0.8	0.6 ± 0.0	27	67.9 ± 4.2	1.3 ± 0.2	52
HS1094	*otsA*	80.1 ± 7.5	2.3 ± 0.1	35	159.8 ± 8.1	2.8 ± 0.2	57
HS1095	*ecnB*	48.9 ± 1.7	1.9 ± 0.1	26	134.0 ± 2.9	1.8 ± 74.4	74
HS1099	*yhjD*	47.3 ± 4.9	20.9 ± 0.5	2	87.4 ± 0.4	9.5 ± 0.8	34
HS1100	*yjiN*	30.2 ± 1.7	4.0 ± 0.2	8	52.2 ± 0.4	9.5 ± 0.8	6

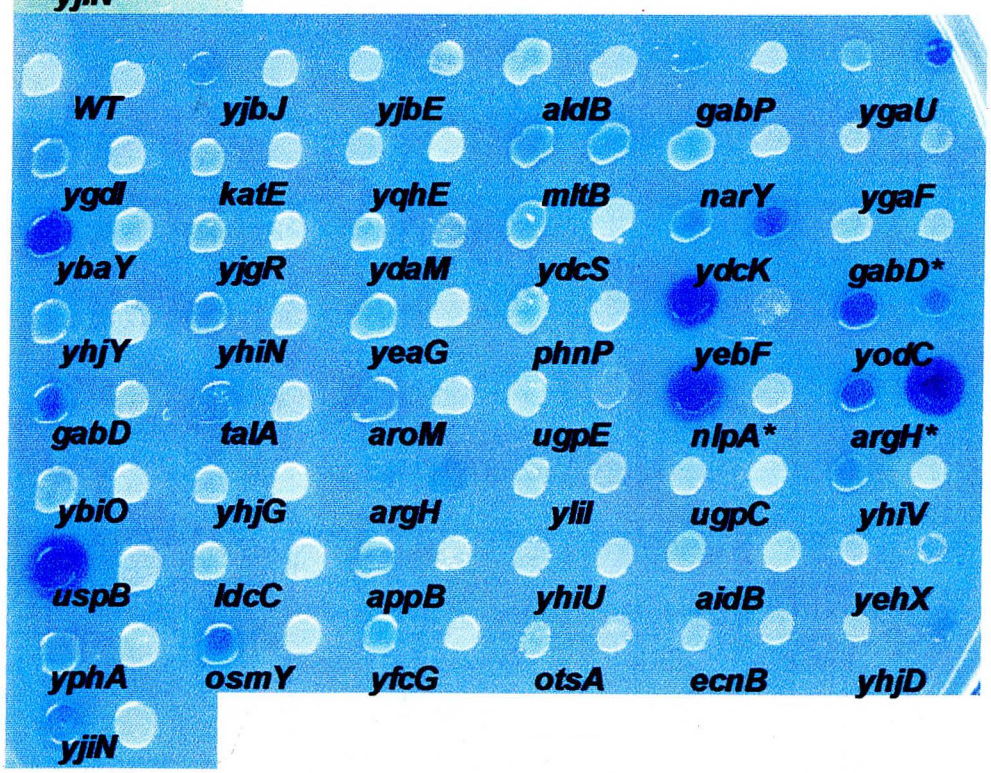
* Strains were grown overnight in M9 minimal broth, subcultured, and maintained in exponential phase for 8 generations prior to sampling. Cultures were sampled in exponential phase (OD_{600} 0.2) and stationary phase (OD_{600} 1.3). All the values are shown as ± standard error.

Figure 4. Qualitative expression of *rsd-lacZ* fusion in strains grown in A) Rich media and B) Minimal media. WT indicates the wild type strains GC4468 and GC122. The + and – indicates *rsd-lacZ* fusion strains in *rpoS*$^{+}$ and *rpoS*$^{-}$ backgrounds. *gab** - *gabD-gabT* intergenic region, *nlpA** - *nlpA-yicM* intergenic region and *argH** - *argH-oxyR* intergenic region.

A


WT yjbJ yjbE aldB gabP ygaU
ygdI katE yqhE mltB narY ygaF
ybaY yjgR ydaM ydcS ydcK gabD*
yhjY yhiN yeaG phnP yebF yodC
gabD talA aroM ugpE nlpA* argH*
ybiO yhjG argH ylil ugpC yhiV
uspB ldcC appB yhiU aidB yehX
yphA osmY yfcG otsA ecnB yhjD
yjiN

B


WT yjbJ yjbE aldB gabP ygaU
ygdI katE yqhE mltB narY ygaF
ybaY yjgR ydaM ydcS ydcK gabD*
yhjY yhiN yeaG phnP yebF yodC
gabD talA aroM ugpE nlpA* argH*
ybiO yhjG argH ylil ugpC yhiV
uspB ldcC appB yhiU aidB yehX
yphA osmY yfcG otsA ecnB yhjD
yjiN

Unlike the genes described above which have either a known or a predicted function, the *lacZ* fusions in strains HS1006, HS1012, HS1028, HS1056, HS1071 and HS1100 mapped to genes *yjbE*, *ygdI*, *yjgR*, *yodC*, *yhjY* and *yjiN* respectively, which have no known or predicted function.

3.2 Expression of *rsd-lacZ* fusion strains.

To quantify RpoS dependence of all the 48 *rsd-lacZ* fusion strains, β-galactosidase activity was measured in strains grown in minimal and rich media. The RpoS dependence of the strains in stationary phase varied from 2 to more than 100 in minimal media (Table 3) and from 2 to nearly 40 in rich media (Table 2). In exponential phase, *rsd-lacZ* fusion strains grown in minimal media exhibited higher RpoS dependence than strains grown on rich media. Many known RpoS-dependent genes such as *katE*, *otsA*, *osmY* exhibited high RpoS dependence in both rich and minimal media. The qualitative *lacZ* expression profile of all the *rsd-lacZ* fusion strains along with the wild-type strains is shown in Fig 3. There is a good concordence between quantitative and qualitative β-galactosidase assays. For example, in HS1091 (*osmY-lacZ*) there is a marked difference in the level of β-galactosidase expression in wild-type and mutant on both minimal and rich media plates which is seen as the degree of blueness of each colony (Fig 3). Quantitative measurement of β-galactosidase activity also shows a significant difference in the levels of expression in HS1091wild-type ad mutant strains. The wild type strain had a β-galactosidase activity of 210.4 miller units while the mutant showed only 3.3 units of activity in minimal media (Table

3). The β-galactosidase activity was also significantly different in rich media, wild-type had a activity of 264.9 units while the units of activity of the mutant strain was only 7.5 (Table 2).

3.3 Amino acid utilization studies.

To examine the ability of the *rsd-lacZ* fusion strains to utilize amino acids they were grown on minimal agar plates containing amino acids as sole carbon or nitrogen source. When cysteine, methionine, phenylalanine, valine, leucine or histidine were provided as either carbon or nitrogen source all the strains including the wild types exhibited very poor growth suggesting that these amino acids cannot be utilized. When proline, glutamic acid or lysine were given as nitrogen source the *rpoS* mutants exhibited better growth than *rpoS* wild-type strains. In minimal media containing γ-amino butyrate (GABA) as nitrogen source, strains HS1037p (*gabD-gabT* intergenic-*lacZ rpoS*::*Tn10*) and HS1057p (*gabD-lacZ rpoS*::*Tn10*) exhibited very poor growth. This is because fusions in these two strains are located in the genes that are essential for GABA utilization and fusions to these genes inactivates them thereby impairing the ability to utilize GABA. The better growth of other *rpoS* mutant strains may be due to enhanced nutrient scavenging (Zinser and Kolter, 1999).

3.3.0 Carbon utilization studies.

The *rsd-lacZ* fusion strains in *rpoS* wild-type and *rpoS* mutant backgrounds were tested for their ability to utilize a variety of carbon sources. This experiment was done to determine whether any of the newly identified RpoS-dependent

genes are involved in carbon metabolism. In M9-minimal media containing either glucose or glycerol as a sole carbon source, all the strains exhibited good growth except for HS1072 and HS1072p. These two strains have *lacZ* insertion in *argH* gene, which inactivates this gene, making these strains auxotrophic for arginine. Therefore these strains were unable to grow on any of the carbon sources tested.

3.3.1 Pentose utilization.

Three pentose sugars, D-ribose, D-xylose and L-arabinose were used in this study. As expected the arginine auxotrophs (HS1072 and HS1072p) were not able to grow on any of the pentose sugars. All other strains exhibited normal growth on L-arabinose and D-xylose as carbon sources. In D-ribose HS1026p (*ybaY-lacZ rpoS::Tn*10) failed to grow. To confirm this phenotype associated with HS1026p, the strains HS1026, HS1026p and HS1026ot (HS1026p complemented with plasmid pMMKatF3) were re-plated onto ribose minimal media plates. HS1026p again failed to grow, while HS1026 and HS1026ot grew normally confirming that RpoS may be required for this strain to utilize ribose as carbon source.

3.3.2 Carboxylates.

Growth of all the *rsd-lacZ* fusion strains was tested on acetate, succinate and fumarate as sole carbon source. On acetate minimal media plates with the exception of arginine auxotrophs all strains exhibited good growth. When succinate was used as carbon source, all the RpoS mutants grew better than

RpoS wild-type strains. This type of growth pattern was also observed in fumarate. HS1099 (*yhjD-lacZ*) and HS1099p (*yhjD-lacZ rpoS::Tn10*) did not grow on fumarate minimal media. This phenotype was confirmed by streaking the strains on fumarate minimal media.

A common observation in RpoS mutant strains grown in minimal media with glucose as a carbon source was the formation of mucoid colonies. These mucoid colonies formed in all the amino acids tested except in plates containing cysteine, methionine, phenylalanine, valine, leucine or histidine in which both RpoS wild-type and mutants failed to grow. In minimal media plates containing amino acids, pentoses or carboxylates as a carbon source RpoS mutants failed to form mucoid colonies indicating that glucose may be a main component in formation of these colonies.

4.0 DISCUSSION

The number of identified members of the RpoS regulon is almost 100 genes (Ishihama, 2000). The relatively large size of the RpoS regulon has made its complete characterization difficult. There are several approaches by which members of a regulon can be characterized. Techniques such as 2-D gel electrophoresis can identify differentially expressed proteins based on mass and charge (Oliver, 2002). Limitations to this approach include difficulty in identifying proteins that are expressed at very low levels, masking of one protein spot by another leading to an underestimation of the total number of proteins separated, and loss of detectable protein spots from treatment of the gels under denaturing conditions (Oliver, 2002). Micro- and macro-array analysis can be used to identify members of a large regulon like RpoS although to date there are only a few reports where it has been used to characterize regulon members by comparing RNA expression profiles of wild-type and isogenic regulator mutants. Most of the studies done using that have used micro-array analysis have instead examined gene expression under different growth conditions, which gives indirect information about a regulon. There are a few drawbacks to this method. The arrays sometimes fail to detect the expression of genes: For example, when used to examine osmotic stress-dependent genes of *E. coli*, three genes of the *kdp* operon (Weber and Jung, 2002) which are known to be upregulated during osmotic stress on the basis of Northern blot analysis were not detected

(Jung *et al.*, 2001). This finding indicates that there is a potential for arrays to give incomplete gene expression profiles. Gene expression levels measured using microarrays may not be consistent. For example expression of *osmY* increased 3-fold under osmotic stress using macro-array analysis while Northern blots showed that *osmY* expression increased 6-fold under similar conditions (Weber and Jung, 2002). In spite of these disadvantages, microarrays are very useful because (i) the total complement of all genes can be studied simultaneously under different conditions (Richmond *et al.*, 1999), and (ii) they allow one to determine if an entire metabolic pathway is coordinately regulated (Tao *et al.*, 1999) or to delineate a functional metabolic pathway (Oh *et al.*, 2002). Microarrays have been used successfully to characterize gene functions; for example, based on array data and mutation analysis *maeB*, was found to be required for acetate metabolism in *E. coli* (Oh *et al.*, 2002). The genetic screening technique used in this study provides a direct estimate of gene expression, unlike microarrays, since the expression of *lacZ* reporter gene relies solely on the promoter activity of the gene to which *lacZ* is inserted (Bremer *et al.*, 1984). Moreover, as a reporter, *lacZ* is a sensitive indicator of gene expression. Even genes with low expression can be detected easily using *β*-galactosidase assays and direct sequencing of the fusion junctions makes this technique more useful (Roy *et al.*, 1995). Moreover, the *lacZ* insertion in the genes can be exploited for studying the function of a gene, since it leads to insertional inactivation. This technique has some limitations such as formation of

double fusions, which can be circumvented easily and a rare chance of translocation from the initial site of insertion (Bremer *et al.*, 1984). We have used this technique successfully to characterize genes of the RpoS regulon and identified 48 genes under RpoS control. Gene expression studies were carried out on all 48 *rsd*-lacZ fusion strains in rich and minimal media to determine the RpoS dependence of these newly characterized genes. As expected most of the fusions were highly expressed during stationary phase in cells grown in rich and minimal media. Many fusions, exhibited high RpoS-dependence during exponential phase especially in minimal media. In minimal media, RpoS is expressed early in exponential phase. Minimal media is deficient in amino acids and this induces the RelA-mediated stringent response (Tao *et al.*, 1999). As a result, there is an accumulation of ppGpp, which in turn induces *rpoS* expression (Gentry *et al.*, 1993). Micro-array analysis of cultures grown in minimal media also revealed that RpoS and RpoS regulon members are upregulated earlier in minimal media (Tao *et al.*, 1999) which is consistent with our expression data. In rich media, the expression of most of the *rsd-lacZ* fusions was much lower in exponential phase. This is likely due to the fact that rich media contains most of the nutrients required for the growth therefore cells are under less stress and reach stationary phase much later than in minimal media (Tao *et al.*, 1999). Its also clear from the expression data that genes required for stress protection such as *otsA*, *osmY* and *katE* are expressed highly in both minimal and rich media

Table 4. Classification of proteins encoded by RpoS-dependent genes identified in this study based on Blattner's functional categories

Functional categories	# of proteins in each category	Proteins
Cell structure	1	YbaY
Transport and binding proteins	3	GabP, UgpE, UgpC
Putative transport proteins	2	YdcS, YehX
Energy metabolism	3	NarY, AppB, LdcC
DNA replication, recombination, modification and repair	1	AidB
Cell processes (including Adaptation, protection)	8	KatE, UspB, OsmY, YhiV, YhiU, MltB, OtsA, EcnB
Amino acid biosynthesis and metabolism	2	ArgH, AroM
Fatty acid and phospholipid metabolism	1	YhjY
Carbon compound catabolism	2	YqhE, AldB
Central intermediary metabolism	4	GabD, PhnP, TalA, YfcG
Putative enzymes	1	YliI
Hypothetical, unclassified, unknown	17	YjbJ, YjbE, YgaU, YgdI, YgaF, YjgR, YdaM, YdcK, YhiN, YebF, YjiN, YeaG, YodC, YbiO, YhjG, YphA, YhjD

especially in stationary phase, underlying the importance of RpoS in stress protection.

E. coli proteins have been grouped into 22 classes based on their known and/or predicted functions (Blattner *et al.*, 1997). The 48 RpoS-regulated genes identified in this study fall into 12 of these functional classes (Table 4), many of them (17 genes) into the class hypothetical, unclassified and unknown proteins followed by the class cell processes (8 genes).

4.1 Cell structure.

In stationary phase, changes occur in cellular morphology, membrane and cell-wall composition of *E. coli* (Huisman *et al.*, 1996). RpoS plays an important role in controlling the genes that bring about these changes, which may help *E. coli* to withstand stresses under sub-optimal growth conditions. An important feature of stationary phase cells is their round morphology, a consequence of RpoS-mediated induction of *bolA* (Santos *et al.*, 1999). RpoS also regulates other cell structure related genes such as *ftsQAZ* required for cell septum formation (Sitnikov *et al.*, 1996), *cfa* required for cyclopropane fatty acid synthesis which accumulates in the cell membrane during stationary phase and has been implicated in acid resistance (Chang and Cronan, Jr., 1999), and *csgAB* required for the formation curli fimbriae (Olsen *et al.*, 1993).

Glycolipids and polysaccharides are mostly found on the surface of gram-negative bacteria. Some of the important glycolipids include the lipopolysaccharide (LPS), the capsular polysaccharide, and the enterobacterial

common antigen (ECA) collectively termed as exopolysaccharides (Rick and Silver, 1996). These glycolipids are highly immunogenic and are responsible for the typical symptoms of gram-negative bacterial infection (Rick and Silver, 1996). They also play a significant role in virulence and in protecting the bacteria against the host immune response (Rick and Silver, 1996). Bacterial biofilms are also composed of these exopolysaccharides, and recently RpoS has been reported to negatively regulate biofilm formation in *E. coli* (Corona-Izquierdo and Membrillo-Hernandez, 2002). These evidences suggest that RpoS may have a role in regulating glycolipid/polysaccharide metabolism in *E. coli.* YbaY, a cell structure-related protein classified as required for glycolipid/polysaccharide metabolism (Karp *et al.*, 2002), was found to be RpoS-dependent in this study. The role of YbaY in glycolipid/polysaccharide metabolism remains to be established.

4.2 Transport and binding proteins.

RpoS upregulates several transporters including the GABA permease, GabP (Metzer and Halpern, 1990), and glycine, betaine and proline uptake proteins, ProP (Mellies *et al.*, 1995) and ProU. Two RpoS-dependent fusions mapped to the genes, *ugpE* and *ugpC* that form the part of *ugpBAECQ* operon. The genes of the *ugp* operon code for a binding-protein dependent adenosine triphosphate-binding cassette (ABC) transporter that was first identified in phenotypic revertants of *glpT* mutants (Boos, 1998). These revertants were able to utilize glycerol-3-phosphate (G3P) unlike the *glpT* mutants (Boos, 1998). The *ugp* operon is a member of the Pho regulon regulated by PhoBR a typical two-

component system containing a sensor kinase (PhoR) and a cognate response regulator (PhoB) (Wanner and Chang, 1987). Activated PhoB protein, phosphorylated in response to external phosphate limitation, induces the members of its regulon including *ugp* (Schweizer and Boos, 1985). The promoter region of *ugp* also contains a CRP binding site, which suggests that this operon may respond to carbon starvation conditions (Boos, 1998). Though Ugp enables G3P to be used as a sole carbon source, the main function of the Ugp transporters is to scavenge phosphates (Boos, 1998). When the Ugp transport system is operating, the cellular concentration of phosphates increases with a concomitant expulsion of G3P. It has been reported that Ugp transporters are expressed even in the presence of excess phosphates (Boos, 1998). If the main purpose of the Ugp transporter is for phosphate scavenging, why should it be expressed in the presence of excess phosphates? The presence of a CRP binding site and the finding that two genes of this operon are RpoS regulated suggests that this type of expression may be for the uptake of G3P as a carbon source (Boos, 1998).

4.3 Fatty acid and phospholipid metabolism.

During stasis, membrane lipids are catabolized and utilized as an energy source by starving bacteria (DiRusso and Nystrom, 1998). Though the exact mechanism by which the membrane lipids are catabolized is unknown, it may be brought about by the action of starvation-inducible phospholipases (DiRusso and Nystrom, 1998). Phospholipases catabolize the membrane lipids via the β-

oxidation pathway (DiRusso and Nystrom, 1998). One of the well-characterized phospholipases in *E. coli* is PldA, which catalyzes the hydrolysis of phosphotidylcholine (Brok *et al.*, 1994). To our knowledge, no other phospholipases have been shown to be induced during stasis. Our data shows that YhjY, a putative lipase, is under the control of RpoS suggesting that lipid turnover may be stationary phase inducible. If the lipase activity of this protein is verified experimentally then this may be the first report of a stationary phase-inducible lipase.

4.4 Cell processes (including adaptation, protection).

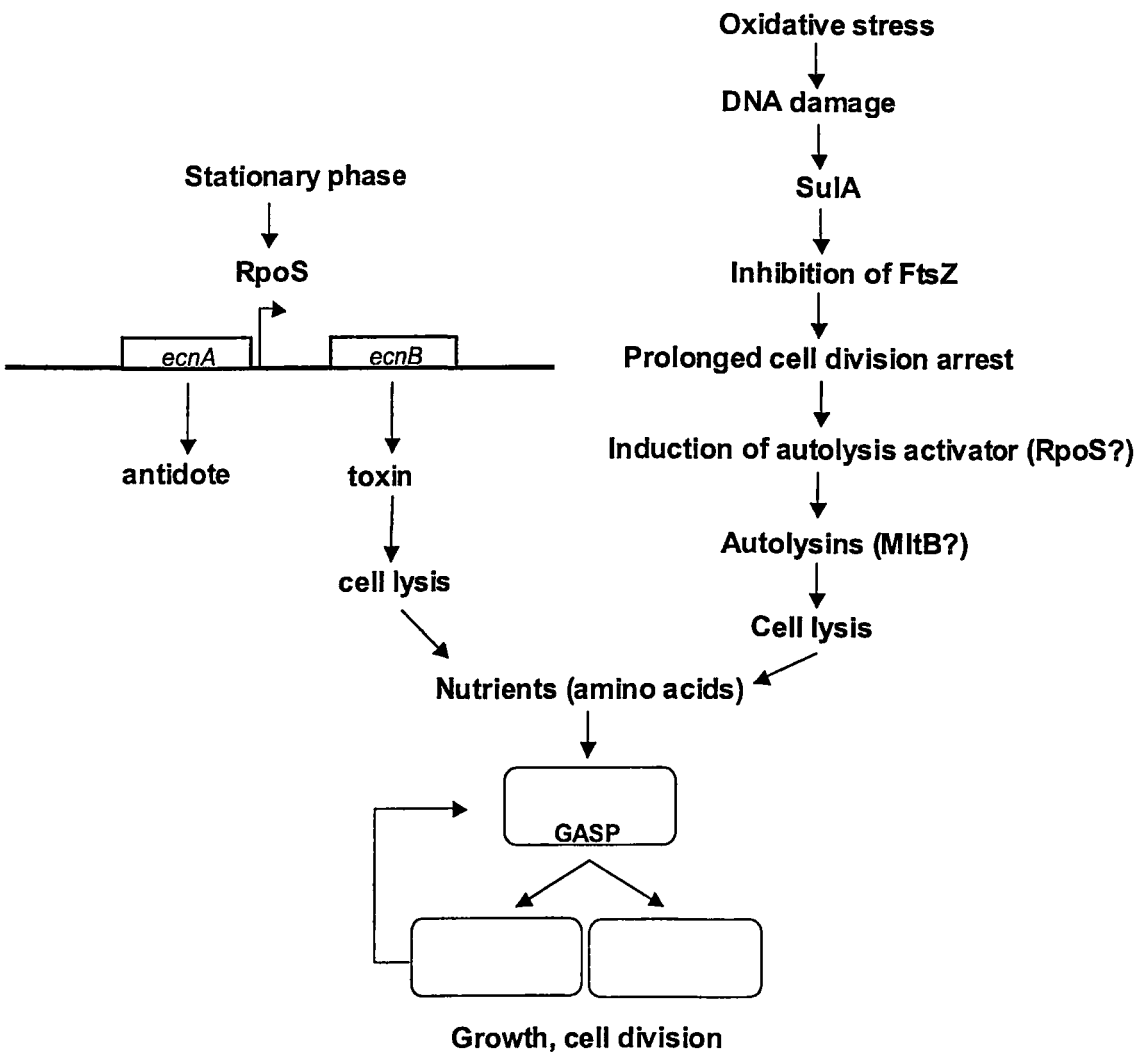
RpoS plays a major role in protecting cells from stresses during stationary phase (Hengge-Aronis, 1996). The function of RpoS as a master regulator of stress responses is well studied and has been extensively reviewed (Hengge-Aronis, 1993; Hengge-Aronis, 1996; Loewen and Hengge-Aronis, 1994). Of the 48 genes identified in this study, eight are in this class indicating the importance of RpoS-dependent genes in cellular processes. Of these 8 genes, 6 were previously reported to be RpoS-dependent, while the other two genes, *yhiV* and *mltB* were identified as RpoS-dependent in this study (Table 5). The *yhiV* gene along with *yhiU* codes for a multi drug-efflux pump, overexpression of which increases resistance to organic solvents and antibiotics (Hengge-Aronis, 1993; Hengge-Aronis, 1996; Tsukagoshi and Aono, 2000). The *mltB* gene codes for a lytic transglycosylase (autolysin) required for mucopeptide recycling during cell wall growth in *E. coli* (Ehlert *et al.*, 1995). MltB may also function in cell lysis

during DNA inducded damage (Lewis, 2000). But the activator that induces this autolysin remains unknown. Identification of *mltB* gene to be under RpoS control makes it very tempting to propose that RpoS may be the unknown activator (Fig 5). Addiction module encoded by *encAB* locus is also under the control of RpoS (Bishop *et al.*, 1998) and is implicated in stationary phase cell lysis (Bishop *et al.*, 1998). The control of autolysins and addiction modules by RpoS indicates that this lysis phenomenon can be integrated with the GASP phenotype model proposed by Zinser and Kolter, (1999). Cell lysis induced by these proteins are important in stationary phase since the nutrients released from the lysed cells provide valuable nutrients to surviving population of GASP mutants which have enhanced nutrient scavenging capability than the parental cells. These GASP mutants utilize the released nutrients and take over the parental cells thereby establishing a new generation of cells with better survival capability than the parental cells (Fig 5).

4.5 DNA replication, recombination, modification and repair.

During stationary phase, the chromosome of *E. coli* undergoes modifications brought about by nucleoid-associated proteins such as Dps (Wolf *et al.*, 1999) and H-NS (Atlung and Ingmer, 1997). Dps is produced in large amounts during stationary phase and is positively controlled by RpoS (Almiron *et al.*, 1992). Dps co-crystallizes with the DNA forming ferritin-like structure and effectively deprives the cell of iron required for the fenton reaction that leads to oxidative damage to the DNA (Wolf *et al.*, 1999). H-NS another nucleoid-

Figure 5. An integrated model for stationary phase cell lysis and GASP phenomenon. Cell lysis in stationary phase can be brought about by "addiction modules" such as EcnAB or by autolysins such as MltB. Stationary phase cell lysis has been implicated as a physiological phenomenon to overcome nutrient starvation. Nutrients released by cell lysis can then be utilized by surviving population of cells such as Growth Advantage Stationary Phase (GASP) mutants, which have better survival capability than the parental strains.



Adapted from Bishop et al, 1998, Lewis, 2000 and Zinser and Kolter, 1999

associated protein that is upregulated by RpoS (Barth *et al.*, 1995), compacts DNA (Dame *et al.*, 2000) and modulates gene expression (Atlung and Ingmer, 1997). RpoS also plays a role in protecting DNA from damage (Landini and Volkert, 2000). Under starvation conditions, endogenous DNA methylating agents are produced in *E. coli* by nitrosation reactions that leads to methylation of DNA (Taverna and Sedgwick, 1996). Methylated DNA mispairs with thymine during DNA replication leading to GC-to-AC transition mutations (Taverna and Sedgwick, 1996). There are various mechanisms to counteract DNA damage during stationary phase. One of these is by the induction of the adaptive response by the Ada protein that acts as a methyl transferase and in its methylated form, Ada acts as a transcriptional regulator of the Ada operon genes, *ada*, *aidB*, *alkA* and *alkB* (Landini and Busby, 1999). RpoS also induces adaptive response gene *aidB* during stationary phase (Landini and Busby, 1999). Even though RpoS by itself can direct the transcription of *aidB*, interaction of RpoS with methylated Ada (meAda) increases expression of *aidB* (Landini and Busby, 1999). AidB is proposed to be an inactivator of nitrosoguanidines or their intermediates (Landini *et al.*, 1994). Microarray analysis of mitomycin C (MMC) treated *E. coli* cells has revealed that numerous stationary phase genes including RpoS are induced by MMC treatment confirming the role of RpoS in alleviating DNA damage (Khil and Camerini-Otero, 2002).

4.6 Energy metabolism.

Energy metabolism in *E. coli* occurs through the activity of various dehydrogenases that transfer electrons from the donors to a common quinone pool from where they are directed to electron acceptors (Gennis and Stewart, 1996). The proton motive force thus generated is used for all energy requiring cellular functions such as solute transport, flagellar rotation, or ATP synthesis (Gennis and Stewart, 1996). *E. coli* is a facultative anaerobe and has evolved energy metabolizing systems for both aerobic respiration and fermentation (Atlung *et al.*, 1997). *E. coli* has two terminal oxidases, cytochrome bo, encoded by the *cyo* operon and cytochrome bd encoded by the *cyd* operon (Atlung *et al.*, 1997). The *cyo* operon operates under conditions of high oxygen concentration and has low-affinity to oxygen while *cyd* has high affinity to oxygen and operates under microaerobic conditions (Gennis and Stewart, 1996). A third cytochrome oxidase encoded by *appB* has been identified in *E. coli* (Sturr *et al.*, 1996). The *appB* gene is in operon with *appA* (pH 2.5 acid phosphatase) and encodes a second cytochrome bd-type oxidase (Sturr *et al.*, 1996). This gene is regulated by RpoS (Dassa *et al.*, 1991) and by a protein belonging to the AraC family of transcriptional regulators, AppY (Atlung *et al.*, 1997). AppY itself is subject to regulation by RpoS (Atlung *et al.*, 1997); this illustrates the interconnections and complexity of gene regulation during stationary phase. What might be the physiological significance of RpoS upregulation of cytochrome oxidases during stationary phase? In stationary phase, the concentration of cells in a culture

increases and as a consequence the amount of oxygen available to the cells may be reduced creating microaerobic conditions and upregulation of cytochrome bd II and AppB, which has high affinity for oxygen may be required for efficient electron transport under stationary phase microaerobic conditions.

4.7 Amino acid biosynthesis and metabolism.

Two amino acid biosynthetic genes, *argH* and *aroM* were newly identified to be under RpoS control in this study. The *argH* gene is a member of the *argCBH* operon and codes for arginino-succinate lyase, required for arginine biosynthesis in *E. coli* (Cunin *et al.*, 1986). Arginine biosynthesis is important during stationary phase because it serves as a precursor for polyamine synthesis (Gerard *et al.*, 1999), which protect DNA from oxidative damage by scavenging free radicals (Ha *et al.*, 1998). The *astCADBE* operon codes for the arginine catabolic pathway and is required for growth in arginine as a sole nitrogen source (Kiupakis and Reitzer, 2002). This operon is also under the control of RpoS (Fraley *et al.*, 1998) as well as RpoN (Kiupakis and Reitzer, 2002). Some of the well-known amino acid catabolic operons that have protective functions in *E. coli* are arginine decarboxylase (encoded by *adiA*) and glutamate decarboxylase (encoded by *gadABC*) (Castanie-Cornet *et al.*, 1999). These operons are part of the acid resistance (AR) system in *E. coli* and are regulated by RpoS (Castanie-Cornet *et al.*, 1999). The gene *aroM* forms an operon with *aroL*, which codes for the shikimate kinase II (DeFeyter *et al.*, 1986). This enzyme is required for the conversion of shikimate to shikimate-3-phosphate (S-3-P). S-3-P which is

ultimately converted to chorismate by *aroA* and *aroC* gene products (Pittard, 1996). Chorismate is the branching point for the synthesis of three aromatic amino acids, tyrosine, phenylalanine and tryptophan (Pittard, 1996). Though *aroM* is co-transcribed with *aroL* the exact function of AroM remains unknown (DeFeyter *et al.*, 1986).

4.8 Carbon compound catabolism.

The role of RpoS in stress response and protection is well-established, but recently the importance of RpoS in stationary phase metabolism is being revealed (Saier, 1998). Two RpoS-dependent genes indentified in this study, *yqhE* and *aldB* are involved in carbon catabolism. The *aldB* gene, is RpoS dependent (Xu and Johnson, 1995) and codes for a broad-spectrum aldehyde dehydrogenase that converts aldehydes to corresponding acids. The physiological role of AldB in stationary phase is unknown but is proposed to be involved in detoxification of alcohols and aldehydes produced during stationary phase (Xu and Johnson, 1995). The *yqhE* gene, newly identified to be RpoS dependent in this study, codes for 2,5-diketo-D-gluconic acid reductase A (Yum *et al.*, 1999). This enzyme is part of the keto-gluconate utilization pathway and converts 2,5-diketo-D-gluconate into 2-keto-L-gluconate (2-KLG). 2-KLG thus produced is ultimately converted to D-gluconate, which enters the Entner-Doudoroff and the pentose-phosphate pathways (Yum *et al.*, 1999). Other carbon catabolic genes regulated by RpoS include, *otsBA* (Kaasen *et al.*, 1992), *treA* (Hengge-Aronis *et al.*, 1991) and *glgS* (Hengge-Aronis, 1993). The *otsBA* operon

is induced under osmotic stress and by entry into stationary phase (Kaasen *et al.*, 1992). This operon codes for the enzymes required for trehalose synthesis. Under osmotic stress, trehalose accumulates in the periplasm and serves as an osmo-protectant but in the absence of osmotic stress, trehalose is converted to glucose by trehalase encoded by *treA* (Strom and Kaasen, 1993). Carbon-starved *E. coli* cells accumulate glycogen, which acts as a carbon reserve during starvation (Huisman *et al.*, 1996). Glycogen is synthesized from ADP-glucose by ADP-glucose pyrophosphorylase and glycogen synthetase encoded by *glgC* and *glgA* respectively; these genes are regulated by cAMP/CRP (Preiss, 1996). The *glgS* is another gene required for glycogen synthesis and is under the positive control of RpoS (Hengge-Aronis and Fischer, 1992). Regulation by RpoS has been proposed to provide an alternative mechanism of carbon catabolite control in *E. coli* (Saier, 1998), especially in stationary phase metabolism.

4.9 Central intermediary metabolism.

We identified 4 genes (*gabD*, *phnP*, *talA* and *yfcG*) that fall under this functional group; none of these has been previously reported to be RpoS-dependent. The *gab* operon (*gabDTP*) genes are required for GABA utilization in *E. coli* (Metzer and Halpern, 1990). These genes convert γ-amino butyric acid (GABA) into glutamate and succinate (Niegemann *et al.*, 1993). Succinate and glutamate thus produced can be utilized as a carbon source via the TCA cycle. Glutamate protects cells against acid stress and this pathway may be the only mechanism by which internal glutamate pools are generated (Hersh *et al.*, 1996).

The *phnP* gene along with 13 other genes codes for phosphonate transport and catabolism (Yakovleva *et al.*, 1998). Although *E. coli* cannot utilize phosphonates as carbon sources, they can be used as a sole phosphorus source (Yakovleva *et al.*, 1998). The *talA* gene codes for transaldolase A of the pentose phosphate pathway and is found in an operon with *tktB* (Sprenger, 1995). Together these two genes encode enzymes that catalyze the final step of the pentose phosphate pathway (Sprenger, 1995). Intermediates of the pentose phosphate pathway serve as precursors for the biosynthesis of aromatic amino acids and also for heptoses found in lipopolysacchrides (Sprenger, 1995). The exact reason why *talA* is under RpoS regulation is not clear but it may be required for replenishing glycolytic pathway during stationary phase by providing the necessary intermediates from the pentose phosphate pathway. The *yfcG* gene codes for a putative glutathione-S-transferase (GST) (Karp *et al.*, 2002). GSTs are found in both eukaryotes and in prokaryotes. In eukaryotes GSTs function as detoxifying enzymes (Vuilleumier and Pagni, 2002). The role of GST in prokaryotes is not well characterized but has been implicated in the degradation of xenobiotics (Vuilleumier and Pagni, 2002). The SspA protein of *E. coli*, closely related to GST (Vuilleumier and Pagni, 2002), is induced in response to starvation and stress (Williams *et al.*, 1994). SspA interacts with the RNA polymerase holoenzyme (RNAP) and has been proposed to modulate RNAP activity during transition from exponential to stationary phase (Williams *et al.*, 1994). Other genes of the central intermediary pathway controlled by RpoS include *poxB*, encoding a pyruvate

oxidase which converts pyruvate directly to acetate (Chang *et al.*, 1994), *acnA*, that codes for aconitase A of the TCA cycle (Cunningham and Guest, 1998) and *sucCD*, encoding succinyl-coA synthetase which converts succinyl-CoA to succinate (Cunningham and Guest, 1998).

4.10 Hypothetical, Unclassified, Unknown.

Of the 48 RpoS-dependent genes identified in this study, 17 have unknown or hypothetical function. It is not surprising to find many of the newly identified RpoS-dependent genes in this class since 30% of *E. coli* genes have no assigned function (Blattner *et al.*, 1997). The *yjbJ* gene has a very high RpoS dependence in stationary phase, especially when grown in minimal media, and is one of the most abundant proteins during stationary phase (Link *et al.*, 1997). Moreover, this gene has homology to a sigma factor B regulated gene (*csbD*) of *B. subtilis* (Akbar *et al.*, 1999) suggesting the commonality of stress-related induction of this gene. Some of the RpoS-dependent genes of this group have recently been shown to be upregulated under different growth conditions: the *yeaG* gene is induced in artificial sea-water medium (Rozen *et al.*, 2001), while *ybaF* is induced under *in vivo* conditions in mice (Khan and Isaacson, 2002). Though the physiological functions of these genes were not revealed by these studies, the growth conditions mentioned above are stressful conditions emphasizing that some of these RpoS regulated genes may have a role in adaptation or in counteracting stress.

4.11 Carbon utilization.

The functions of many of the RpoS-dependent genes identified in this study are either unknown or predicted based on the homology to other known bacterial genes. In an attempt to elucidate the functions of these genes we assayed the growth of all the strains on different carbon sources to determine whether any of the strains have differentiable phenotypes. One of the *rsd-lacZ* fusions mapped to *talA*, *en*coding transaldolase A which converts sedoheptulose-7-P and glyceraldehyde-3-P to fructose-6-P and erythrose-4-P, the final step in the pentose phosphate pathway (Sprenger, 1995), therefore, we tested this and other strains for their ability to grown on pentose sugars. Mutants of this gene have not been reported so far (Sprenger, 1995) and moreover, *talA* is in an operon with *tktB*, we suspected that mutations in *talA* might have a polar effect on *tktB*. Both transaldolase and transketolase enzymes are crucial in the final steps of pentose phosphate pathway, which feed into the glycolytic cycle (Sprenger, 1995). Pentose sugars such as ribose, xylose and arabinose can only be utilized via the pentose phosphate pathway (Sprenger, 1995). These three pentoses are converted to ribulose-5-P involving two different pathways, one that converts D-xylose and L-arabinose into ribulose-5-phosphate and the other which converts D-ribose to ribulose-5-phosphate (Sprenger, 1995). We expected to that *talA* mutants would be impaired in ribose utilization since catabolism of these pentoses forms ribulose-5-phosphate, which enters the pentose phosphate pathway (Sprenger, 1995). However, this strain grew normally on all pentoses

tested. This may be due to the presence of another transaldolase enzyme encoded by *talB*, which has same function as TalA (Sprenger, 1995). Mutations in *talA* may be masked by the function of TalB. A phenotype that was observed in this study was the inability of HS1026p (*ybaY-lacZ rpoS*::*Tn*10) to utilize D-ribose as carbon source while HS1026 (*ybaY-lacZ*) grew normally. The *ybaY* gene codes for a hypothetical protein of unknown function, that may be involved in glycoprotein/polysaccharide metabolism (Karp *et al.*, 2002). The failure of the *ybaY-lacZ rpoS*::*Tn10* mutant to grow on D-ribose is intriguing since ribose, xylose and arabinose are converted to ribulose-5-P, which enters the pentose phosphate pathway (Sprenger, 1995).

We also tested the growth of all 48 strains on carboxylates to determine whether they are impaired in carboxylate utilization. On succinate and fumarate minimal media, all the *rpoS*$^-$ strains showed better growth than *rpoS*$^+$ strains except *yhjD* mutants which failed to grow on fumarate. There are two main pathways by which succinate and fumarate are metabolized in *E. coli*: (i) conversion of succinate to fumarate (aerobic pathway) which is catalyzed by succinate dehydrogenase (*sdhABCD*) (Cunningham and Guest, 1998) and (ii) Conversion of succinate to fumarate (anaerobic pathway) by fumarate reductase encoded by *frdABCD* (Cunningham and Guest, 1998). This latter pathway is positively regulated by Fnr and NarL (Cunningham and Guest, 1998). The *sdhABCD and frdABCD* operon genes have homology and catalyze the same reaction (Cronan, Jr. and LaPorte, 1996), however the two operons are

differentially regulated. The *sdhABCD* operon is active during aerobiosis while *frdABCD* is active during anaerobiosis (Cronan, Jr. and LaPorte, 1996). The *sdhABCD* operon is repressed by RpoS (Xu and Johnson, 1995) and this might be the reason for better growth of *rpoS* mutants on succinate and fumarate. The exact mechanism by which RpoS exerts negative regulation is unknown (Tsui *et al.*, 1997).

4.12 Amino acid utilization.

E. coli employs several strategies to overcome starvation in stationary phase. Starving *E. coli* cells utilize internal macromolecules like lipids and proteins as nutrient sources (Nystrom *et al.*, 1996). This has to be tightly regulated since uncontrolled utilization of these macromolecules can be detrimental to the cells (Nystrom *et al.*, 1996). *E. coli* can also survive starvation by scavenging nutrients from the environment. To test whether any of the newly identified RpoS regulated genes were involved in amino acid scavenging and utilization; we tested the growth of all the 48 *rsd-lacZ* fusion strains on individual amino acids.

On some amino acids, when given as a sole of carbon or nitrogen source, *rpoS* mutants exhibited better growth than RpoS wild-type strains. This may be due to the better nutrient scavenging ability of the RpoS mutants. Recently, it has been demonstrated that growth advantage stationary phase (GASP) mutants, that carry a mutation in *rpoS*, have better nutrient scavenging ability than wild-type strains (Zinser and Kolter, 1999). The nutrients for these GASP mutants

may be provided by the lysing cells (Zinser and Kolter, 1999). *E. coli* undergoes programmed cell death in stationary phase releasing nutrients including amino acids into the medium (Blasco *et al.*, 1990). These nutrients can then be scavenged and utilized for growth by the surviving population of cells (Zinser and Kolter, 1999). Programmed cell death in prokaryotes is brought about by toxin/antitoxin protein pairs termed "addiction modules" (Engelberg-Kulka and Glaser, 1999). Most of these addiction modules are plasmid encoded but some are chromosome-borne (Engelberg-Kulka and Glaser, 1999). MazEF is a chromosomally encoded addiction module induced by ppGpp under nutrient limited conditions (Aizenman *et al.*, 1996). Another chromosomally encoded addiction module found in *E. coli* is EcnAB (Bishop *et al.*, 1998). RpoS positively regulates the expression of *ecnB* the toxin-encoding gene of the *ecnAB* addiction module (Bishop *et al.*, 1998). The positive regulation of genes encoding addiction modules by RpoS and ppGpp suggests that they may be required for inducing cell lysis in dying cells during stationary phase, thereby providing crucial nutrients for surviving cells (Bishop *et al.*, 1998). The reason why GASP mutants have better nutrient scavenging ability has not been established (Zinser and Kolter, 1999).

Another phenotype observed in RpoS mutants is the formation of mucoid colonies when grown on glucose minimal media. This phenotype may be due to the production and excretion of extracellular polysaccharides, composed of lipopolysaccharides (LPS) and colanic acid (Stevenson *et al.*, 1996). Extracellular

polysaccharides are implicated in several functions such as swarming motility (Harshey and Matsuyama, 1994), resistance to desiccation (Ophir and Gutnick, 1994) and biofilm formation (Danese *et al.*, 2000). Recently, it has been found that *rpoS* mutants have better biofilm forming ability than *rpoS* wild-type strains (Corona-Izquierdo and Membrillo-Hernandez, 2002). Mutations in *fliC* or *flhD* (encoding flagella related proteins) and *motAB* (encoding motility related functions) impair the initial steps of biofilm formation (Pratt and Kolter, 1998).

On methionine, phenylalanine, histidine, leucine, cysteine and valine as sole carbon or nitrogen source all the *rsd-lacZ* fusion and the wild-type strains exhibited very poor growth. The inability of *E. coli* to grow on these amino acids as carbon sources may be due to the toxic effect of some amino acids or may be due to the inability in utilizing them. Cysteine in high concentrations exerts a bacteriostatic effect by inhibiting threonine deaminase, the first enzyme in the isoleucine biosynthetic pathway (Harris, 1981). Furthermore, excess cysteine induces transient amino acid starvation (Sorensen and Pedersen, 1991). Valine inhibits growth by inactivating acetohydroxy acid synthase, a key enzyme in the biosynthesis of isoleucine and valine (Sutton *et al.*, 1981), and leucine exerts its toxic effect by inactivating threonine deaminase and acetohydroxy acid synthase (Quay *et al.*, 1977). *E. coli* cannot utilize histidine as a carbon source as it lack genes required for histidine utilization (McFall and Newman, 1996).

5.0 APPENDICES.

5.1 Whole cell β-galactosidase assay.

- Grow strains (in triplicates) in 96-well microtiter plates containing either M9 minimal media (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl. Adjust pH to 7.4 sterilize by autoclaving and add: 2 ml 1 M $MgSO_4$, and 0.1 ml 1M $CaCl_2$) containing 0.4% glucose or LB medium containing appropriate anitbiotics depending on the type of medium the cells has to be grown.
- Incubate the microtiter plate at 37°C overnight.
- After overnight incubation, inoculate 10 μl of the overnight culture into 25 ml flasks (in triplicates) containing 10 ml M9 minimal medium (0.4% glucose) or LB medium.
- Incubate the flasks in an orbital shaker (Innova 4000 incubator shaker, New Brunswick Scientific) at 37°C at 200 rpm.
- To perform β-galactosidase assay on exponential phase cells ($O.D._{600}$ 0.3 for minimal media cultures and $O.D._{600}$ 0.2 for LB cultures), aliquot 5 ml of culture into 15 ml falcon tubes containing 10 μl of 15 mg/ml chromphenicol. Mix thoroughly and centrifuge (Sorvall RT6000B refrigerated centrifuge, Du Pont instruments) at 3600 rpm for 15 min. Discard the supernatant completely and make up the volume to 0.5 ml by adding M9 buffer (same composition as M9 minimal media but without NH_4Cl and glucose) containing chloramphenicol (150 μg/ml). Resuspend the pellet by vortexing. Store at 4°C.
- To perform β-galactosidase assay on stationary phase cells ($O.D._{600}$ 1.4 for minimal media cultures and $O.D._{600}$ 1.2 for LB media), aliquot the remaining culture into 15 ml falcon tubes containing appropriate amount of 15 mg/ml chromphenicol depending on the amount of culture. Mix thoroughly and store at 4°C until use.
- Aliquot 1 ml of Z-buffer [0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M Mg_2SO_4, 0.03 M, β –mercaptoethanol (pH 7)] in 5 ml borosilicate tubes.
- To this, add 50 μl of sample followed by 25 μl of SDS (0.1%) and 50 μl of chloroform.
- Vortex vigorously for approximately 10 s.
- Add 200 μl of 4 mg/ml ONPG to the sample, mix thoroughly and allow the reaction to occur. Note the time
- After the color changes approximately 1X LB yellow, stop the reaction with 500 μl of 1M Na_2CO_3 solution. Note the time.
- Mix the tubes and allow them to sit for a few minutes. Aliquot 1 ml into cuvettes and measure the $O.D._{420}$.
- Calculate the β-galactosidase activity using [1000 x $O.D._{420}$]/ [time in min x volume (ml) x $O.D._{600}$].

5.2 Replica-plating technique.

- Inoculate the strains to be replica plated in 96-well microtiter plates containing M9 minimal media containing 0.4% glucose and appropriate antibiotics.
- Incubate the microtiter plate overnight at 37°C.
- Dilute the overnight culture into another microtitre plate containing M9 buffer solution.
- After dilution, immerse sterile (sterilize the replica-plating block by dipping it into ethanol and flaming it) replica-plating block into the wells and carefully plate it on to appropriate media plates.
- Following precautions should be taken while replica plating:
  (i) Always keep the prongs of the block immersed in a plate containing ethanol.
  (ii) Evaporate the ethanol completely by flaming the prongs and cool the prongs before immersing in the culture.
  (iii) If the prongs are too hot or if it contains any residual ethanol it will kill the cells.

5.3 λ lysate preparation (Liquid lysate method).

- Inoculate the strains containing λ *plac*Mu fusion in 1 ml of LB medium containing appropriate antibiotics.
- Incubate overnight at 37°C.
- Inoculate 0.5 ml of overnight culture into 50 ml fresh LB and grow it to an $O.D._{600}$ of 0.3 (exponential phase).
- Transfer the exponential phase culture into large tubes and centrifuge (Sorvall RT6000B refrigerated centrifuge, Du Pont insturments) at 3600 rpm for 15 min.
- Discard the supernatant and resuspend the pellet in 10 ml of 10 mM $MgSO_4$.
- Transfer the resuspended solution into large Petri plates (15 cm diameter).
- Irradiate the plates containing resuspended cultures (lids off) under UV-light at 25 mW for 30 s (approximately $35J/m^2$).
- Swril the plates gently while irradiating to ensure even irradiation of the cells.
- Then transfer the irradiated cultures into 25 ml flasks and add 5 ml of 3X LL medium (90 g tryptone, 45 g yeast extract, 45 g NaCl, 60 mg each adenine, cytosine, guanine and thymine per liter, pH 7.3). Mix thoroughly and incubate at 37°C with shaking (200 rpm) until lysis occurs (usually takes 3 to 5 h).

- Transfer the lysate into 30 ml COREX tubes. Add chloroform (0.3 % final concentration) and mix thoroughly to kill the surviving cells.
- Centrifuge (Sorvall RC-%B refrigerated superspeed centrifuge, Du Pont instruments) the tubes at 10,000 x g to remove the cell debris.
- Trasfer the supernatant into clean 15 ml tubes and add DNase (10 μg/ml final concentration) and RNase (1 μg/ml final concentration) to remove chromosomal DNA and RNA. Incubate the supernatant containing λ phage particles at 37°C for 3 h.
- Add chloroform (0.3% final concentration) to the supernatant and store it at 4°C until use.

5.4 Plate lysate method.

- Inoculate *E. coli* MC4100 strain in 5 ml LB containing 0.2% maltose and incubate overnight at 37°C with shaking at 200 rpm.
- Inoculate 100 μl of overnight culture into 10 ml LB and incubate it at 37°C to an $O.D._{600}$ of 0.3 (exponential phase).
- Centrifuge (Sorvall RT 6000B refrigerated centrifuge, Du Pont instruments) this exponential phase culture at 3600 rpm for 15 min.
- Discard the supernatant and resuspend the pellet in 5 ml of cell suspension buffer (5 mM $CaCl_2$, 10 mM $MgSO_4$).
- Transfer 100 μl of the resuspended cells into 15 ml red-capped tubes and mix with 100 μl of λ lysate (obtained from liquid lysate preparation).
- Mix thoroughly and incubate at 30°C for 30 min.
- After incubation, add 8 ml of soft agarose (0.6%) to the tube and mix thoroughly by inverting the tube several times. (Make sure that agarose is not too hot and also ensure thorough mixing).
- Pour the mixture onto a prewarmed (warmed at 37°C in an incubator) bottom agarose (1.5%) plates (15 cm diameter) and spread evenly by gently tilting the plates.
- Allow the soft agarose to set and then incubate the plates at 37°C until confluent plaques appear (usually takes 6 to 8 h). (Note: Agarose is preferred for making plates since agar contains impurities that will interfere with sequencing).
- After confluent plaques appear on the plates, flood it with 10 ml of suspension media (SM) buffer (5.8 g NaCl, 2 g $MgSO_4$, 1 M Tris Cl (pH 7.5) and 2% gelatin per liter) and incubate at room temperature with gentle shaking for 2 h for passive diffusion of phages into the buffer.
- Transfer the buffer containing phages into a 15 ml COREX tube, wash the plate again with 2 ml of SM buffer and keep it in a slanting position for a few minutes and then collect the accumulated SM buffer using a 1 ml pipette and transfer into the same 15 ml COREX tube.

- Add chloroform (0.3% final concentration), mix thoroughly and centrifuge at 10,000 x g for 10 min.
- Transfer the supernatant into a 15 ml red-capped tube and add DNase (10 μg/ml final concentration) and RNase (1 μg/ml final concentration) to remove chromosomal DNA and RNA. Incubate the supernatant containing λ phage particles at 37°C for 3 h.
- Store the supernatant at 4°C with chloroform (0.3%).

5.5.0 λ DNA isolation.

5.5.1 Phenol:Chloroform method.

- Take 1 ml of phage containing supernatant into a 2 ml microfuge tube.
- Add equal volume of phenol:chloroform (25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol), mix thoroughly by inverting.
- Centrifuge at 10,000 x g (Biofuge A, Baxter scientific products) for 10 min.
- Remove the aqueous phase with a wide-bore pipette tip and transfer it into a new microfuge tube and add equal volumes of phenol:chloroform. (Note: Avoid the bottom organic layer which contain impurities while pipetting)
- Repeat steps 2, 3 and 4 twice.
- After phenol:chloroform extraction, transfer the aqueous phase into a new microfuge tube and add 1 ml of chloroform.
- Mix thoroughly and centrifuge (Biofuge A, Baxter scientific products) at 10,000 x g for 10 min (Do the chloroform wash twice).
- Transfer the aqueous phase into a new tube and add two volumes of ice-cold 100% ethanol and 50 μl of 3 M sodium acetate (pH 5.2).
- Mix thoroughly and keep the tube in -20°C for 30 min or at 4°C overnight for DNA precipitation.
- Centrifuge (Biofuge A, Baxter scientific products) the tube at 10,000 x g for 30 min at 4°C. Carefully remove the ethanol completely without disturbing the pellet.
- Add 1 ml of 70% ethanol to wash the pellet and centrifuge (Biofuge A, Baxter scientific products) at 10,000 x g for 10 min. Repeat this step twice.
- Remove the supernatant completely. Air-dry the pellets for about 15 min (Note: Don't dry the pellet with residual ethanol since it may interefere with sequencing. After discarding ethanol, remove the residual ethanol by centrifuging the tube at 10,000 x g for 10 s. Remove the ethanol that accumulates at the bottom of the tube using a pipette).
- Resuspend the DNA pellet in sterile ddH_2O and tap the bottom of the tube until the pellet is completely dissolved.
- Store the DNA at -20°C until use.

6.0 REFERENCES

Addinall, S.G., Bi, E., and Lutkenhaus, J. (1996) FtsZ ring formation in *fts* mutants. *J Bacteriol* 178: 3877-3884.

Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996) An *Escherichia coli* chromosomal "addiction module" regulated by guanosine 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proc Natl Acad Sci U S A* 93: 6059-6063.

Akbar, S., Lee, S.Y., Boylan, S.A., and Price, C.W. (1999) Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor sigmaB. *Microbiology* 145 (Pt 5): 1069-1078.

Aldea, M., Hernandez-Chico, C., de la Campa, A.G., Kushner, S.R., and Vicente, M. (1988) Identification, cloning, and expression of *bolA*, an *ftsZ*-dependent morphogene of *Escherichia coli. J Bacteriol* 170: 5169-5176.

Ali, A.T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J Bacteriol* 181: 6361-6370.

Almiron, M., Link, A.J., Furlong, D., and Kolter, R. (1992) A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli. Genes Dev* 6: 2646-2654.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410.

Altuvia, S., Almiron, M., Huisman, G., Kolter, R., and Storz, G. (1994) The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol Microbiol* 13: 265-272.

Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L., and Storz, G. (1997) A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* 90: 43-53.

Arfin, S.M., Long, A.D., Ito, E.T., Tolleri, L., Riehle, M.M., Paegle, E.S., and Hatfield, G.W. (2000) Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factor. *J Biol Chem* 275: 29672-29684.

Atlung, T., and Ingmer, H. (1997) H-NS: a modulator of environmentally regulated gene expression. *Mol Microbiol* 24: 7-17.

Atlung, T., Knudsen, K., Heerfordt, L., and Brondsted, L. (1997) Effects of sigmaS and the transcriptional activator AppY on induction of the *Escherichia coli hya* and *cbdAB-appA* operons in response to carbon and phosphate starvation. *J Bacteriol* 179: 2141-2146.

Avison, M.B., Horton, R.E., Walsh, T.R., and Bennett, P.M. (2001) *Escherichia coli* CreBC is a global regulator of gene expression that responds to growth in minimal media. *J Biol Chem* 276: 26955-26961.

Bairoch, A., and Apweiler, R. (2000) The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Res* 28: 45-48.

Balandina, A., Claret, L., Hengge-Aronis, R., and Rouviere-Yaniv, J. (2001) The *Escherichia coli* histone-like protein HU regulates *rpoS* translation. *Mol Microbiol* 39: 1069-1079.

Baldoma, L., and Aguilar, J. (1987) Involvement of lactaldehyde dehydrogenase in several metabolic pathways of *Escherichia coli* K12. *J Biol Chem* 262: 13991-13996.

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001) Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. *J Mol Biol* 305: 673-688.

Barth, M., Marschall, C., Muffler, A., Fischer, D., and Hengge-Aronis, R. (1995) Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in *Escherichia coli. J Bacteriol* 177: 3455-3464.

Beltrametti, F., Kresse, A.U., and Guzman, C.A. (1999) Transcriptional regulation of the *esp* genes of enterohemorrhagic *Escherichia coli. J Bacteriol* 181: 3409-3418.

Bishop, R.E., Leskiw, B.K., Hodges, R.S., Kay, C.M., and Weiner, J.H. (1998) The entericidin locus of *Escherichia coli* and its implications for programmed bacterial cell death. *J Mol Biol* 280: 583-596.

Blasco, F., Iobbi, C., Ratouchniak, J., Bonnefoy, V., and Chippaux, M. (1990) Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by the *narGHJI* operon. *Mol Gen Genet* 222: 104-111.

Blattner, F.R., Plunkett, G., III, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* 277: 1453-1474.

Bohringer, J., Fischer, D., Mosler, G., and Hengge-Aronis, R. (1995) UDP-glucose is a potential intracellular signal molecule in the control of expression of sigma S and sigma S-dependent genes in *Escherichia coli*. *J Bacteriol* 177: 413-422.

Boos, W. (1998) Binding protein-dependent ABC transport system for glycerol 3-phosphate of *Escherichia coli*. *Methods Enzymol* 292: 40-51.

Bremer, E., Silhavy, T.J., Weisemann, J.M., and Weinstock, G.M. (1984) Lambda p*lac*Mu: a transposable derivative of bacteriophage lambda for creating *lacZ* protein fusions in a single step. *J Bacteriol* 158: 1084-1093.

Brok, R.G., Brinkman, E., van Boxtel, R., Bekkers, A.C., Verheij, H.M., and Tommassen, J. (1994) Molecular characterization of enterobacterial *pldA* genes encoding outer membrane phospholipase A. *J Bacteriol* 176: 861-870.

Brown, L., and Elliott, T. (1997) Mutations that increase expression of the *rpoS* gene and decrease its dependence on *hfq* function in *Salmonella typhimurium*. *J Bacteriol* 179: 656-662.

Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. (1996) The stringent response. In *Escherichia and Salmonella:cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtis III, John L. Ingraham, Edmund C. C. Lin, K.

Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D.C.: ASM press, pp. 1458-1496.

Castanie-Cornet, M.P., Penfound, T.A., Smith, D., Elliott, J.F., and Foster, J.W. (1999) Control of acid resistance in *Escherichia coli. J Bacteriol* 181: 3525-3535.

Chang, D.E., Smalley, D.J., and Conway, T. (2002) Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. *Mol Microbiol* 45: 289-306.

Chang, Y.Y., and Cronan, J.E., Jr. (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli. Mol Microbiol* 33: 249-259.

Chang, Y.Y., Wang, A.Y., and Cronan, J.E., Jr. (1994) Expression of *Escherichia coli* pyruvate oxidase (PoxB) depends on the sigma factor encoded by the *rpoS*(*katF*) gene. *Mol Microbiol* 11: 1019-1028.

Chapman, M.R., Robinson, L.S., Pinkner, J.S., Roth, R., Heuser, J., Hammar, M., Normark, S., and Hultgren, S.J. (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295: 851-855.

Chen, J.C., and Beckwith, J. (2001) FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. *Mol Microbiol* 42: 395-413.

Claret, L., and Rouviere-Yaniv, J. (1997) Variation in HU composition during growth of *Escherichia coli*: the heterodimer is required for long term survival. *J Mol Biol* 273: 93-104.

Colland, F., Barth, M., Hengge-Aronis, R., and Kolb, A. (2000) Sigma factor selectivity of *Escherichia coli* RNA polymerase: role for CRP, IHF and Lrp transcription factors. *EMBO J* 19: 3028-3037.

Corona-Izquierdo, F.P., and Membrillo-Hernandez, J. (2002) A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. *FEMS Microbiol Lett* 211: 105-110.

Coynault, C., Robbe-Saule, V., and Norel, F. (1996) Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. *Mol Microbiol* 22: 149-160.

Craig, N.L., and Nash, H.A. (1984) *E. coli* integration host factor binds to specific sites in DNA. *Cell* 39: 707-716.

Cronan, J.E., Jr., LaPorte, D. (1996) Tricarboxylic acid cycle and Glyoxylate bypass. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtis III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D.C.: ASM press, pp. 206-216.

Cunin, R., Glansdorff, N., Pierard, A., and Stalon, V. (1986) Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* 50: 314-352.

Cunningham, L., and Guest, J.R. (1998) Transcription and transcript processing in the *sdhCDAB-sucABCD* operon of *Escherichia coli*. *Microbiology* 144 (Pt 8): 2113-2123.

D'Ari, R., Jaffe, A., Bouloc, P., and Robin, A. (1988) Cyclic AMP and cell division in *Escherichia coli. J Bacteriol* 170: 65-70.

Dame, R.T., Wyman, C., and Goosen, N. (2000) H-NS mediated compaction of DNA visualised by atomic force microscopy. *Nucleic Acids Res* 28: 3504-3510.

Danese, P.N., Pratt, L.A., and Kolter, R. (2000) Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. *J Bacteriol* 182: 3593-3596.

Dassa, J., Fsihi, H., Marck, C., Dion, M., Kieffer-Bontemps, M., and Boquet, P.L. (1991) A new oxygen-regulated operon in *Escherichia coli* comprises the genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*). *Mol Gen Genet* 229: 341-352

.

Death, A., and Ferenci, T. (1994) Between feast and famine: endogenous inducer synthesis in the adaptation of *Escherichia coli* to growth with limiting carbohydrates. *J Bacteriol* 176: 5101-5107.

DeFeyter, R.C., Davidson, B.E., and Pittard, J. (1986) Nucleotide sequence of the transcription unit containing the *aroL* and *aroM* genes from *Escherichia coli* K-12. *J Bacteriol* 165: 233-239.

DiRusso, C.C., and Nystrom, T. (1998) The fats of *Escherichia coli* during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. *Mol Microbiol* 27: 1-8.

Drlica, K., and Rouviere-Yaniv, J. (1987) Histone-like proteins of bacteria. *Microbiol Rev* 51: 301-319.

Ehlert, K., Holtje, J.V., and Templin, M.F. (1995) Cloning and expression of a murein hydrolase lipoprotein from *Escherichia coli*. *Mol Microbiol* 16: 761-768.

Engelberg-Kulka, H., and Glaser, G. (1999) Addiction modules and programmed cell death and antideath in bacterial cultures. *Annu Rev Microbiol* 53: 43-70.

Fang, F.C., Libby, S.J., Buchmeier, N.A., Loewen, P.C., Switala, J., Harwood, J., and Guiney, D.G. (1992) The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc Natl Acad Sci U S A* 89: 11978-11982.

Farewell, A., Kvint, K., and Nystrom, T. (1998) *uspB*, a new sigmaS-regulated gene in *Escherichia coli* which is required for stationary-phase resistance to ethanol. *J Bacteriol* 180: 6140-6147.

Ferro-Luzzi Ames, G. (1986) Bacterial periplasmic transport systems: Structure, Mechanism, and Evolution. *Ann Rev Biochem* 55: 397-425.

Finkel, S.E., and Johnson, R.C. (1992) The Fis protein: it's not just for DNA inversion anymore. *Mol Microbiol* 6: 3257-3265.

Foley, I., Marsh, P., Wellington, E.M., Smith, A.W., and Brown, M.R. (1999) General stress response master regulator *rpoS* is expressed in human infection: a possible role in chronicity. *J Antimicrob Chemother* 43: 164-165.

Fraley, C.D., Kim, J.H., McCann, M.P., and Matin, A. (1998) The *Escherichia coli* starvation gene *cstC* is involved in amino acid catabolism. *J Bacteriol* 180: 4287-4290.

Franze de Fernandez, M.T., Hayward, W.S., and August, J.T. (1972) Bacterial proteins required for replication of phage Q ribonucleic acid. Pruification and properties of host factor I, a ribonucleic acid-binding protein. *J Biol Chem* 247: 824-831.

Gennis, R.B., and Stewart, V. (1996) Respiration. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 217-261.

Gentry, D.R., Hernandez, V.J., Nguyen, L.H., Jensen, D.B., and Cashel, M. (1993) Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. *J Bacteriol* 175: 7982-7989.

Gerard, F., Dri, A.M., and Moreau, P.L. (1999) Role of *Escherichia coli* RpoS, LexA and H-NS global regulators in metabolism and survival under aerobic, phosphate-starvation conditions. *Microbiology* 145 (Pt 7): 1547-1562.

Glansdorff, N. (1996) Biosynthesis of Arginine and Polyamines. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C .C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 408-433.

Green, G.N., Fang, H., Lin, R.J., Newton, G., Mather, M., Georgiou, C.D., and Gennis, R.B. (1988) The nucleotide sequence of the *cyd* locus encoding the two subunits of the cytochrome d terminal oxidase complex of *Escherichia coli. J Biol Chem* 263: 13138-13143.

Grogan, D.W., and Cronan, J.E., Jr. (1997) Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol Mol Biol Rev* 61: 429-441.

Ha, H.C., Sirisoma, N.S., Kuppusamy, P., Zweier, J.L., Woster, P.M., and Casero, R.A., Jr. (1998) The natural polyamine spermine functions directly as a free radical scavenger. *Proc Natl Acad Sci U S A* 95: 11140-11145.

Hammar, M., Arnqvist, A., Bian, Z., Olsen, A., and Normark, S. (1995) Expression of two *csg* operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. *Mol Microbiol* 18: 661-670.

Harris, C.L. (1981) Cysteine and growth inhibition of *Escherichia coli*: threonine deaminase as the target enzyme. *J Bacteriol* 145: 1031-1035

.

Harshey, R.M., and Matsuyama, T. (1994) Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci U S A* 91: 8631-8635.

Hengge-Aronis, R. (1993) Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli. Cell* 72: 165-168.

Hengge-Aronis, R. (1996) Regulation of gene expression during entry into stationary phase. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 1497-1512.

Hengge-Aronis, R. (2000) The general stress response in *Escherichia coli*. In *Bacterial stress response*. Storz, G., Hengge-Aronis, R. (eds). Washinton, D.C.: ASM press, pp. 161-178.

Hengge-Aronis, R. (2002) Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* 66: 373-95.

Hengge-Aronis, R., and Fischer, D. (1992) Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. *Mol Microbiol* 6: 1877-1886.

Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, M., and Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. *J Bacteriol* 173: 7918-7924.

Hersh, B.M., Farooq, F.T., Barstad, D.N., Blankenhorn, D.L., and Slonczewski, J.L. (1996) A glutamate-dependent acid resistance gene in *Escherichia coli*. *J Bacteriol* 178: 3978-3981.

Hirsch, M., and Elliott, T. (2002) Role of ppGpp in *rpoS* stationary-phase regulation in *Escherichia coli*. *J Bacteriol* 184: 5077-5087.

Huisman, G.W., Siegele, D.A., Zambrano, M.A., and Kolter R. (1996) Morphological and physiological changes during stationary phase. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris

Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 1672-1682.

Huisman, O., Faelen, M., Girard, D., Jaffe, A., Toussaint, A., and Rouviere-Yaniv, J. (1989) Multiple defects in *Escherichia coli* mutants lacking HU protein. *J Bacteriol* 171: 3704-3712.

Ibanez-Ruiz, M., Robbe-Saule, V., Hermant, D., Labrude, S., and Norel, F. (2000) Identification of RpoS regulated genes in *Salmonella enterica* serovar *typhimurium. J Bacteriol* 182: 5749-5756.

Ishihama, A. (2000) Functional modulation of *Escherichia coli* RNA polymerase. *Annu Rev Microbiol* 54: 499-518.

Iuchi, S. (1993) Phosphorylation/dephosphorylation of the receiver module at the conserved aspartate residue controls transphosphorylation activity of histidine kinase in sensor protein ArcB of *Escherichia coli. J Biol Chem* 268: 23972-23980.

Iuchi, S., and Lin, E.C. (1988) *arcA* (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. *Proc Natl Acad Sci U S A* 85: 1888-1892.

Jaffe, A., Vinella, D., and D'Ari, R. (1997) The *Escherichia coli* histone-like protein HU affects DNA initiation, chromosome partitioning via MukB, and cell division via MinCDE. *J Bacteriol* 179: 3494-3499.

Jung, K., Krabusch, M., and Altendorf, K. (2001) Cs(+) induces the *kdp* operon of *Escherichia coli* by lowering the intracellular K(+) concentration. *J Bacteriol* 183: 3800-3803.

Kaasen, I., Falkenberg, P., Styrvold, O.B., and Strom, A.R. (1992) Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli*: evidence that transcription is activated by *katF* (AppR). *J Bacteriol* 174: 889-898.

Kano, Y., Wada, M., Nagase, T., and Imamoto, F. (1986) Genetic characterization of the gene *hupB* encoding the HU-1 protein of *Escherichia coli*. *Gene* 45: 37-44.

Kaplan, R., and Apirion, D. (1975) Decay of ribosomal ribonucleic acid in *Escherichia coli* cells starved for various nutrients. *J Biol Chem* 250: 3174-3178.

Karp, P.D., Riley, M., Saier, M., Paulsen, I.T., Collado-Vides, J., Paley, S.M., Pellegrini-Toole, A., Bonavides, C., and Gama-Castro, S. (2002) The EcoCyc Database. *Nucleic Acids Res* 30: 56-58.

Khan, M.A., and Isaacson, R.E. (2002) Identification of *Escherichia coli* genes that are specifically expressed in a murine model of septicemic infection. *Infect Immun* 70: 3404-3412.

Khil, P.P., and Camerini-Otero, R.D. (2002) Over 1000 genes are involved in the DNA damage response of *Escherichia coli*. *Mol Microbiol* 44: 89-105.

Kikuchi, Y., Kurahashi, O., Nagano, T., and Kamio, Y. (1998) RpoS-dependent expression of the second lysine decarboxylase gene in *Escherichia coli*. *Biosci Biotechnol Biochem* 62: 1267-1270.

Kiupakis, A.K., and Reitzer, L. (2002) ArgR-independent induction and ArgR-dependent superinduction of the *astCADBE* operon in *Escherichia coli. J Bacteriol* 184: 2940-2950.

Kjelleberg, S., Hermansson, M., Marden, P., and Jones, G.W. (1987) The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annu Rev Microbiol* 41: 25-49.

Klauck, E., Lingnau, M., and Hengge-Aronis, R. (2001) Role of the response regulator RssB in sigma recognition and initiation of sigma proteolysis in *Escherichia coli. Mol Microbiol* 40: 1381-1390.

Krogh, S., Jorgensen, S.T., and Devine, K.M. (1998) Lysis genes of the *Bacillus subtilis* defective prophage PBSX. *J Bacteriol* 180: 2110-2117.

Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and Kornberg, A. (2001) Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli. Science* 293: 705-708.

Kuroda, A., Tanaka, S., Ikeda, T., Kato, J., Takiguchi, N., and Ohtake, H. (1999) Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in *Escherichia coli. Proc Natl Acad Sci U S A* 96: 14264-14269.

Landini, P., and Busby, S.J. (1999) Expression of the *Escherichia coli ada* regulon in stationary phase: evidence for *rpoS*-dependent negative regulation of *alkA* transcription. *J Bacteriol* 181: 6836-6839.

Landini, P., Hajec, L.I., Nguyen, L.H., Burgess, R.R., and Volkert, M.R. (1996) The leucine-responsive regulatory protein (Lrp) acts as a specific repressor for sigma s-dependent transcription of the *Escherichia coli aidB* gene. *Mol Microbiol* 20: 947-955.

Landini, P., Hajec, L.I., and Volkert, M.R. (1994) Structure and transcriptional regulation of the *Escherichia coli* adaptive response gene *aidB. J Bacteriol* 176: 6583-6589.

Landini, P., and Volkert, M.R. (1995) RNA polymerase alpha subunit binding site in positively controlled promoters: a new model for RNA polymerase-promoter interaction and transcriptional activation in the *Escherichia coli ada* and *aidB* genes. *EMBO J* 14: 4329-4335.

Landini, P., and Volkert, M.R. (2000) Regulatory responses of the adaptive response to alkylation damage: a simple regulon with complex regulatory features. *J Bacteriol* 182: 6543-6549.

Lange, R., Fischer, D., and Hengge-Aronis, R. (1995) Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli. J Bacteriol* 177: 4676-4680.

Lange, R., and Hengge-Aronis, R. (1991) Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S. *J Bacteriol* 173: 4474-4481.

Lange, R., and Hengge-Aronis, R. (1994) The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev* 8: 1600-1612.

Lange, R., and Hengge-Aronis, R. (1994) The *nlpD* gene is located in an operon with *rpoS* on the *Escherichia coli* chromosome and encodes a novel lipoprotein with a potential function in cell wall formation. *Mol Microbiol* 13: 733-743.

Lease, R.A., and Belfort, M. (2000) Riboregulation by DsrA RNA: trans-actions for global economy. *Mol Microbiol* 38: 667-672.

Lemonnier, M., and Lane, D. (1998) Expression of the second lysine decarboxylase gene of *Escherichia coli. Microbiology* 144 (Pt 3): 751-760.

Lewis, K. (2000) Programmed death in bacteria. *Microbiol Mol Biol Rev* 64: 503-514.

Link, A.J., Phillips, D., and Church, G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 179: 6228-6237.

Loewen, P.C., and Hengge-Aronis, R. (1994) The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* 48: 53-80.

Majdalani, N., Chen, S., Murrow, J., St John, K., and Gottesman, S. (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol Microbiol* 39: 1382-1394.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A* 95: 12462-12467.

Majdalani, N., Hernandez, D., and Gottesman, S. (2002) Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol Microbiol* 46: 813-826.

Maniatis, T., Fritsch E.F., and Sambrook, J. (1982) *Molecular cloning: A laboratory manual.* New York: Cold Spring Harbour Laboratory, New York.

Matin, A., Auger, E.A., Blum, P.H., and Schultz, J.E. (1989) Genetic basis of starvation survival in nondifferentiating bacteria. *Annu Rev Microbiol* 43: 293-316.

Maurizi, M.R., Clark, W.P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B., and Gottesman, S. (1990) Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli. J Biol Chem* 265: 12536-12545.

McCann, M.P., Kidwell, J.P., and Matin, A. (1991) The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli. J Bacteriol* 173: 4188-4194.

McFall, E., Newman, E.B. (1996) Amino acids as carbon sources. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 358-379.

Mellies, J., Wise, A., and Villarejo, M. (1995) Two different *Escherichia coli proP* promoters respond to osmotic and growth phase signals. *J Bacteriol* 177: 144-151.

Mengin-Lecreulx, D., and van Heijenoort, J. (1985) Effect of growth conditions on peptidoglycan content and cytoplasmic steps of its biosynthesis in *Escherichia coli. J Bacteriol* 163: 208-212.

Metcalf, W.W., and Wanner, B.L. (1993) Mutational analysis of an *Escherichia coli* fourteen-gene operon for phosphonate degradation, using TnphoA' elements. *J Bacteriol* 175: 3430-3442.

Metzer, E., and Halpern, Y.S. (1990) In vivo cloning and characterization of the *gabCTDP* gene cluster of *Escherichia coli* K-12. *J Bacteriol* 172: 3250-3256.

Miller, C.G. (1996) Protein degradation and proteolytic modification. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtis III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D.C.: ASM press, pp. 938-954.

Miller, J.H. (1992) A short course in bacterial genetics: A laboratory manual and handbookfor *Escherichia coli* and related bacteria. Cold Spring Harbor Press (ed). Cold Spring Harbor, N.Y.

Muffler, A., Fischer, D., and Hengge-Aronis, R. (1996) The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for *rpoS* translation in *Escherichia coli. Genes Dev* 10: 1143-1151.

Nelson, D.E., Ghosh, A.S., Paulson, A.L., and Young, K.D. (2002) Contribution of membrane-binding and enzymatic domains of penicillin binding protein 5 to maintenance of uniform cellular morphology of *Escherichia coli. J Bacteriol* 184: 3630-3639.

Niegemann, E., Schulz, A., and Bartsch, K. (1993) Molecular organization of the *Escherichia coli gab* cluster: nucleotide sequence of the structural genes *gabD* and *gabP* and expression of the GABA permease gene. *Arch Microbiol* 160: 454-460.

Nishino, K., and Yamaguchi, A. (2002) EvgA of the two-component signal transduction system modulates production of the *yhiUV* multidrug transporter in *Escherichia coli. J Bacteriol* 184: 2319-2323.

Notley, L., and Ferenci, T. (1996) Induction of RpoS-dependent functions in glucose-limited continuous culture: what level of nutrient limitation induces the stationary phase of *Escherichia coli*? *J Bacteriol* 178: 1465-1468.

Nystrom, T., Larsson, C., and Gustafsson, L. (1996) Bacterial defense against aging: role of the *Escherichia coli* ArcA regulator in gene expression, readjusted energy flux and survival during stasis. *EMBO J* 15: 3219-3228.

O'Connor, C.D., Adams, P., Alefounder, P., Farris, M., Kinsella, N., Li, Y., Payot, S., and Skipp, P. (2000) The analysis of microbial proteomes: strategies and data exploitation. *Electrophoresis* 21: 1178-1186.

Oh, M.K., Rohlin, L., Kao, K.C., and Liao, J.C. (2002) Global expression profiling of acetate-grown *Escherichia coli. J Biol Chem* 277: 13175-13183.

Oliver, S.G. (2002) Functional genomics: lessons from yeast. *Philos Trans R Soc Lond B Biol Sci* 357: 17-23.

Olsen, A., Arnqvist, A., Hammar, M., Sukupolvi, S., and Normark, S. (1993) The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli. Mol Microbiol* 7: 523-536.

Ophir, T., and Gutnick, D.L. (1994) A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl Environ Microbiol* 60: 740-745.

Parekh, B.S., and Hatfield, G.W. (1996) Transcriptional activation by protein-induced DNA bending: evidence for a DNA structural transmission model. *Proc Natl Acad Sci U S A* 93: 1173-1177.

Pineda-Lucena, A., Liao, J., Wu, B., Yee, A., Cort, J.R., Kennedy, M.A., Edwards, A.M., and Arrowsmith, C.H. (2002) NMR structure of the hypothetical protein encoded by the YjbJ gene from *Escherichia coli. Proteins* 47: 572-574.

Pittard, A.J. (1996) Biosynthesis of aromatic amino acids. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 458-484.

Pratt, L.A., and Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30: 285-293.

Pratt, L.A., and Silhavy, T.J. (1996) The response regulator SprE controls the stability of RpoS. *Proc Natl Acad Sci U S A* 93: 2488-2492.

Preiss, J. (1996) Regulation of glycogen synthesis. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtis III, John L. Ingraham, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D.C.: ASM press, pp. 1015-1024.

Quay, S.C., Dick, T.E., and Oxender, D.L. (1977) Role of transport systems in amino acid metabolism: leucine toxicity and the branched-chain amino acid transport systems. *J Bacteriol* 129: 1257-1265.

Rao, N.N., and Kornberg, A. (1996) Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli*. *J Bacteriol* 178: 1394-1400.

Reizer, J., Bachem, S., Reizer, A., Arnaud, M., Saier, M.H., Jr., and Stulke, J. (1999) Novel phosphotransferase system genes revealed by genome analysis - the complete complement of PTS proteins encoded within the genome of *Bacillus subtilis*. *Microbiology* 145 (Pt 12): 3419-3429.

Richmond, C.S., Glasner, J.D., Mau, R., Jin, H., and Blattner, F.R. (1999) Genome-wide expression profiling in *Escherichia coli* K-12. *Nucleic Acids Res* 27: 3821-3835.

Rick, P.D., Silver, R.P. (1996) Enterobacterial Common Antigen and Capsular Polysaccharides. In *Escherichia coli and Salmonella: cellular and molecular biology*. Frederick C. Neidhardt, Roy Curtiss III, Edmund C. C. Lin, K. Brooks Low, Boris Magasanik, William S. Reznikoff, Monica Riley, Moselio Schaechter, and H. Edwin Umbarger (eds). Washington, D. C.: ASM press, pp. 104-122.

Romeo, T., and Preiss, J. (1989) Genetic regulation of glycogen biosynthesis in *Escherichia coli*: in vitro effects of cyclic AMP and guanosine 5'-diphosphate 3'-diphosphate and analysis of in vivo transcripts. *J Bacteriol* 171: 2773-2782.

Rothfield, L.I., Shih, Y.L., and King, G. (2001) Polar explorers: membrane proteins that determine division site placement. *Cell* 106: 13-16.

Roy, R.N., Mukhopadhyay, S., Wei, L.I., and Schellhorn, H.E. (1995) Isolation and sequencing of gene fusions carried by lambda p*lac*Mu specialized transducing phage. *Nucleic Acids Res* 23: 3076-3078.

Rozen, Y., Dyk, T.K., LaRossa, R.A., and Belkin, S. (2001) Seawater Activation of *Escherichia coli* Gene Promoter Elements: Dominance of *rpoS* Control. *Microb Ecol* 42: 635-643.

Saier, M.H. (1998) Multiple mechanisms controlling carbon metabolism in bacteria. *Biotechnol Bioeng* 58: 170-174.

Santos, J.M., Freire, P., Vicente, M., and Arraiano, C.M. (1999) The stationary-phase morphogene *bolA* from *Escherichia coli* is induced by stress during early stages of growth. *Mol Microbiol* 32: 789-798.

Santos, J.M., Lobo, M., Matos, A.P., De Pedro, M.A., and Arraiano, C.M. (2002) The gene *bolA* regulates *dacA* (PBP5), *dacC* (PBP6) and *ampC* (AmpC), promoting normal morphology in *Escherichia coli. Mol Microbiol* 45: 1729-1740.

Schellhorn, H.E., Audia, J.P., Wei, L.I., and Chang, L. (1998) Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli. J Bacteriol* 180: 6283-6291.

Schellhorn, H.E., and Stones, V.L. (1992) Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. *J Bacteriol* 174: 4769-4776.

Schellhorn, H.E., and Hassan, H.M. (1988) Transcriptional regulation of *katE* in *Escherichia coli* K-12. *J Bacteriol* 170: 4286-4292.

Schmid, M.B. (1990) More than just "histone-like" proteins. *Cell* 63: 451-453.

Schneider, B.L., Ruback, S., Kiupakis, A.K., Kasbarian, H., Pybus, C., and Reitzer, L. (2002) The *Escherichia coli gabDTPC* Operon: Specific gamma-Aminobutyrate Catabolism and Nonspecific Induction. *J Bacteriol* 184: 6976-6986.

Schweder, T., Lee, K.H., Lomovskaya, O., and Matin, A. (1996) Regulation of *Escherichia coli* starvation sigma factor (sigma s) by ClpXP protease. *J Bacteriol* 178: 470-476.

Schweizer, H., and Boos, W. (1985) Regulation of *ugp*, the sn-glycerol-3-phosphate transport system of *Escherichia coli* K-12 that is part of the *pho* regulon. *J Bacteriol* 163: 392-394.

Sevcik, M., Sebkova, A., Volf, J., and Rychlik, I. (2001) Transcription of *arcA* and *rpoS* during growth of *Salmonella typhimurium* under aerobic and microaerobic conditions. *Microbiology* 147: 701-708.

Shiba, T., Tsutsumi, K., Yano, H., Ihara, Y., Kameda, A., Tanaka, K., Takahashi, H., Munekata, M., Rao, N.N., and Kornberg, A. (1997) Inorganic polyphosphate and the induction of *rpoS* expression. *Proc Natl Acad Sci U S A* 94: 11210-11215.

Shin, S., Song, S.G., Lee, D.S., Pan, J.G., and Park, C. (1997) Involvement of *iclR* and *rpoS* in the induction of *acs*, the gene for acetyl coenzyme A synthetase of *Escherichia coli* K-12. *FEMS Microbiol Lett* 146: 103-108.

Sitnikov, D.M., Schineller, J.B., and Baldwin, T.O. (1996) Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. *Proc Natl Acad Sci U S A* 93: 336-341.

Sledjeski, D.D., Gupta, A., and Gottesman, S. (1996) The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli. EMBO J* 15: 3993-4000.

Sledjeski, D.D., Whitman, C., and Zhang, A. (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. *J Bacteriol* 183: 1997-2005.

Sorensen, M.A., and Pedersen, S. (1991) Cysteine, even in low concentrations, induces transient amino acid starvation in *Escherichia coli. J Bacteriol* 173: 5244-5246.

Spira, B., and Yagil, E. (1999) The integration host factor (IHF) affects the expression of the phosphate-binding protein and of alkaline phosphatase in *Escherichia coli. Curr Microbiol* 38: 80-85.

Sprenger, G.A. (1995) Genetics of pentose-phosphate pathway enzymes of *Escherichia coli* K-12. *Arch Microbiol* 164: 324-330.

Stevenson, G., Andrianopoulos, K., Hobbs, M., and Reeves, P.R. (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J Bacteriol* 178: 4885-4893.

Stewart, V. (1993) Nitrate regulation of anaerobic respiratory gene expression in *Escherichia coli. Mol Microbiol* 9: 425-434.

Strom, A.R., and Kaasen, I. (1993) Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. *Mol Microbiol* 8: 205-210.

Sturr, M.G., Krulwich, T.A., and Hicks, D.B. (1996) Purification of a cytochrome bd terminal oxidase encoded by the *Escherichia coli app* locus from a delta *cyo* delta *cyd* strain complemented by genes from *Bacillus firmus* OF4. *J Bacteriol* 178: 1742-1749.

Sutton, A., Newman, T., Francis, M., and Freundlich, M. (1981) Valine-resistant *Escherichia coli* K-12 strains with mutations in the *ilvB* operon. *J Bacteriol* 148: 998-1001.

Tani, T.H., Khodursky, A., Blumenthal, R.M., Brown, P.O., and Matthews, R.G. (2002) Adaptation to famine: a family of stationary-phase genes revealed by microarray analysis. *Proc Natl Acad Sci U S A* 99: 13471-13476.

Tao, H., Bausch, C., Richmond, C., Blattner, F.R., and Conway, T. (1999) Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. *J Bacteriol* 181: 6425-6440.

Taverna, P., and Sedgwick, B. (1996) Generation of an endogenous DNA-methylating agent by nitrosation in *Escherichia coli. J Bacteriol* 178: 5105-5111.

Tian, G., Lim, D., Carey, J., and Maas, W.K. (1992) Binding of the arginine repressor of *Escherichia coli* K12 to its operator sites. *J Mol Biol* 226: 387-397.

Tsui, H.C., Feng, G., and Winkler, M.E. (1997) Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. *J Bacteriol* 179: 7476-7487.

Tsukagoshi, N., and Aono, R. (2000) Entry into and release of solvents by *Escherichia coli* in an organic-aqueous two-liquid-phase system and substrate specificity of the AcrAB-TolC solvent-extruding pump. *J Bacteriol* 182: 4803-4810.

Von Ossowski, I., Mulvey, M.R., Leco, P.A., Borys, A., and Loewen, P.C. (1991) Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. *J Bacteriol* 173: 514-520.

Vuilleumier, S., and Pagni, M. (2002) The elusive roles of bacterial glutathione S-transferases: new lessons from genomes. *Appl Microbiol Biotechnol* 58: 138-146.

Vulic, M., and Kolter, R. (2001) Evolutionary cheating in *Escherichia coli* stationary phase cultures. *Genetics* 158: 519-526.

Wanner, B.L., and Chang, B.D. (1987) The phoBR operon in *Escherichia coli* K-12. *J Bacteriol* 169: 5569-5574.

Weber, A., and Jung, K. (2002) Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. *J Bacteriol* 184: 5502-5507.

Wei, Y., Lee, J.M., Smulski, D.R., and LaRossa, R.A. (2001) Global impact of *sdiA* amplification revealed by comprehensive gene expression profiling of *Escherichia coli. J Bacteriol* 183: 2265-2272.

Williams, M.D., Ouyang, T.X., and Flickinger, M.C. (1994) Starvation-induced expression of SspA and SspB: the effects of a null mutation in *sspA* on *Escherichia coli* protein synthesis and survival during growth and prolonged starvation. *Mol Microbiol* 11: 1029-1043.

Wolf, S.G., Frenkiel, D., Arad, T., Finkel, S.E., Kolter, R., and Minsky, A. (1999) DNA protection by stress-induced biocrystallization. *Nature* 400: 83-85.

Xu, J., and Johnson, R.C. (1995) *aldB*, an RpoS-dependent gene in *Escherichia coli* encoding an aldehyde dehydrogenase that is repressed by Fis and activated by Crp. *J Bacteriol* 177: 3166-3175.

Yakovleva, G.M., Kim, S.K., and Wanner, B.L. (1998) Phosphate-independent expression of the carbon-phosphorus lyase activity of *Escherichia coli. Appl Microbiol Biotechnol* 49: 573-578.

Yamamoto, Y., Miwa, Y., Miyoshi, K., Furuyama, J., and Ohmori, H. (1997) The *Escherichia coli ldcC* gene encodes another lysine decarboxylase, probably a constitutive enzyme. *Genes Genet Syst* 72: 167-172.

Yamashino, T., Ueguchi, C., and Mizuno, T. (1995) Quantitative control of the stationary phase-specific sigma factor, sigma S, in *Escherichia coli*: involvement of the nucleoid protein H-NS. *EMBO J* 14: 594-602.

Yim, H.H., Brems, R.L., and Villarejo, M. (1994) Molecular characterization of the promoter of *osmY*, an *rpoS*-dependent gene. *J Bacteriol* 176: 100-107.

Yim, H.H., and Villarejo, M. (1992) *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli. J Bacteriol* 174: 3637-3644.

Yum, D.Y., Lee, B.Y., and Pan, J.G. (1999) Identification of the *yqhE* and *yafB* genes encoding two 2, 5-diketo-D-gluconate reductases in *Escherichia coli. Appl Environ Microbiol* 65: 3341-3346.

Zambrano, M.M., and Kolter, R. (1996) GASPing for life in stationary phase. *Cell* 86: 181-184.

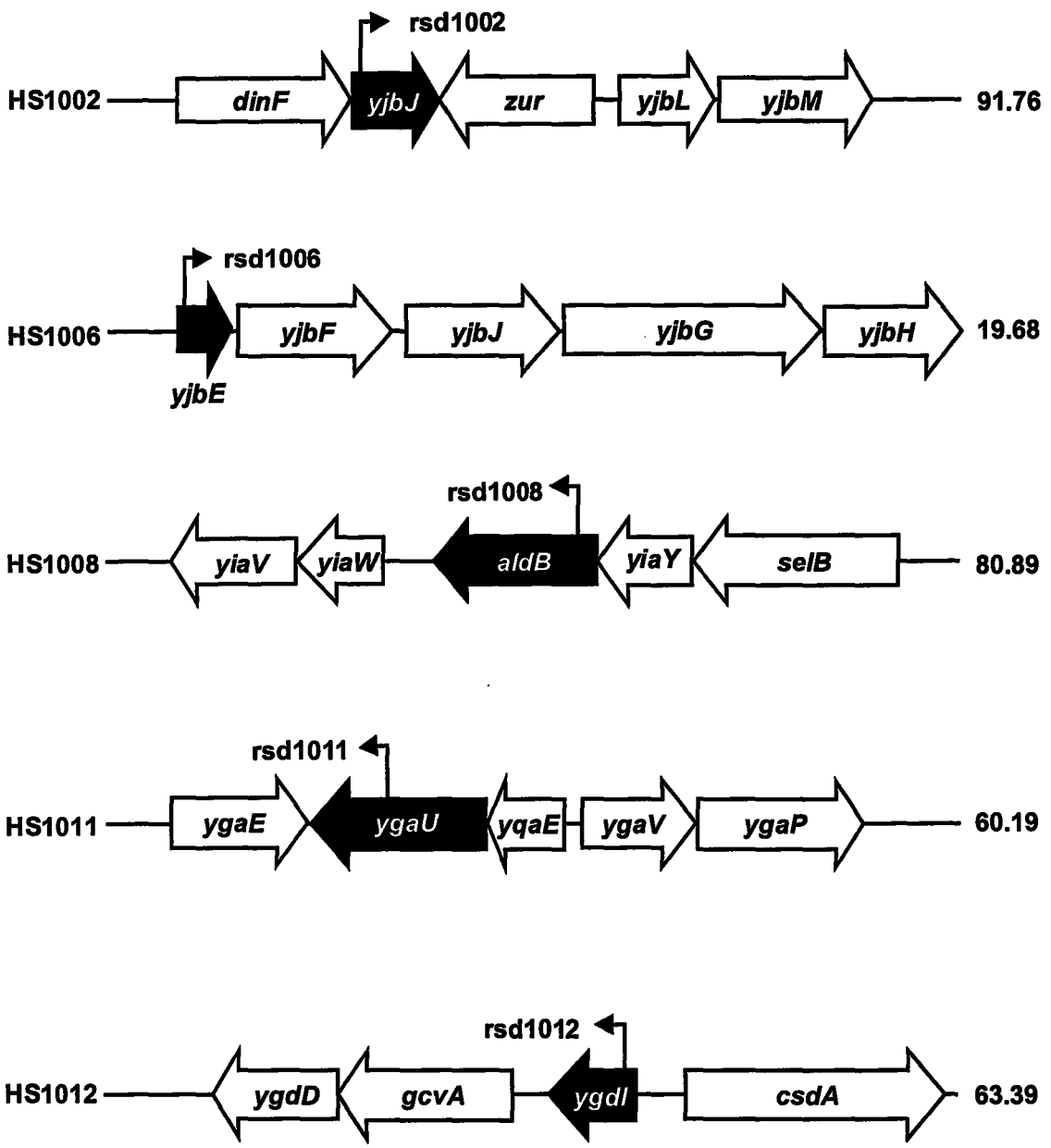
Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R., and Storz, G. (1998) The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein. *EMBO J* 17: 6061-6068.

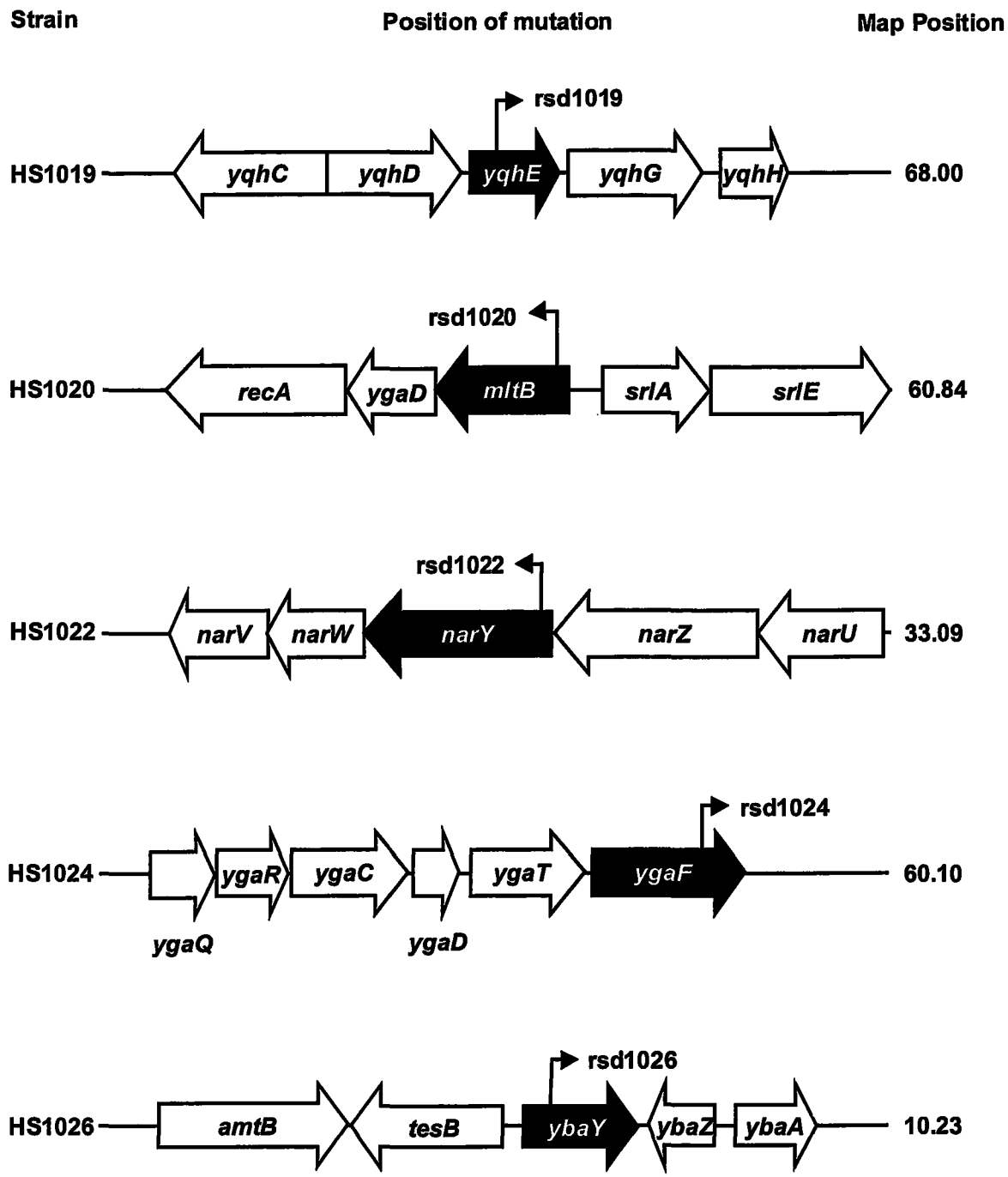
Zhou, Y., and Gottesman, S. (1998) Regulation of proteolysis of the stationary-phase sigma factor RpoS. *J Bacteriol* 180: 1154-1158.

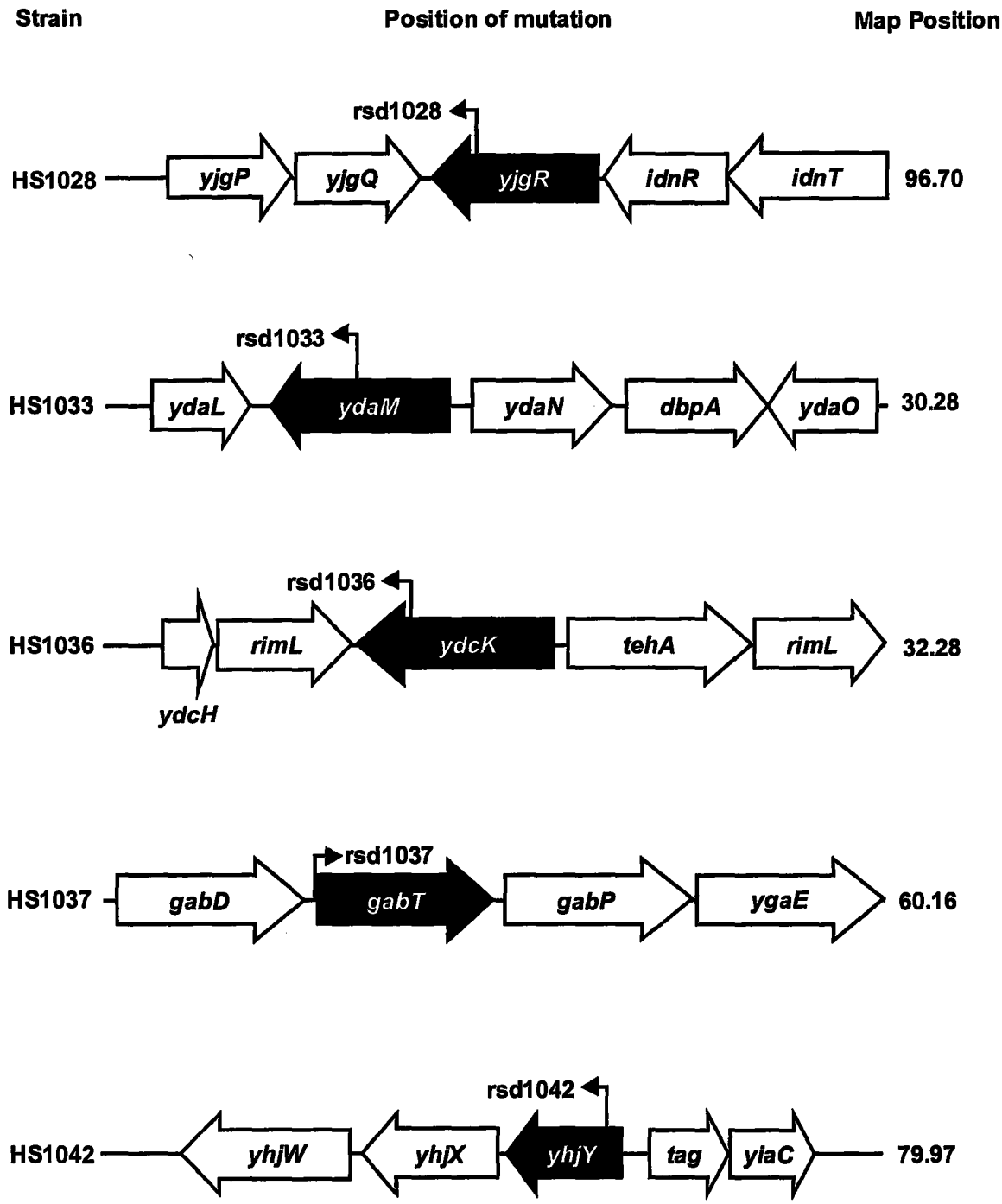
Zimmer, D.P., Soupene, E., Lee, H.L., Wendisch, V.F., Khodursky, A.B., Peter, B.J., Bender, R.A., and Kustu, S. (2000) Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. *Proc Natl Acad Sci U S A* 97: 14674-14679.

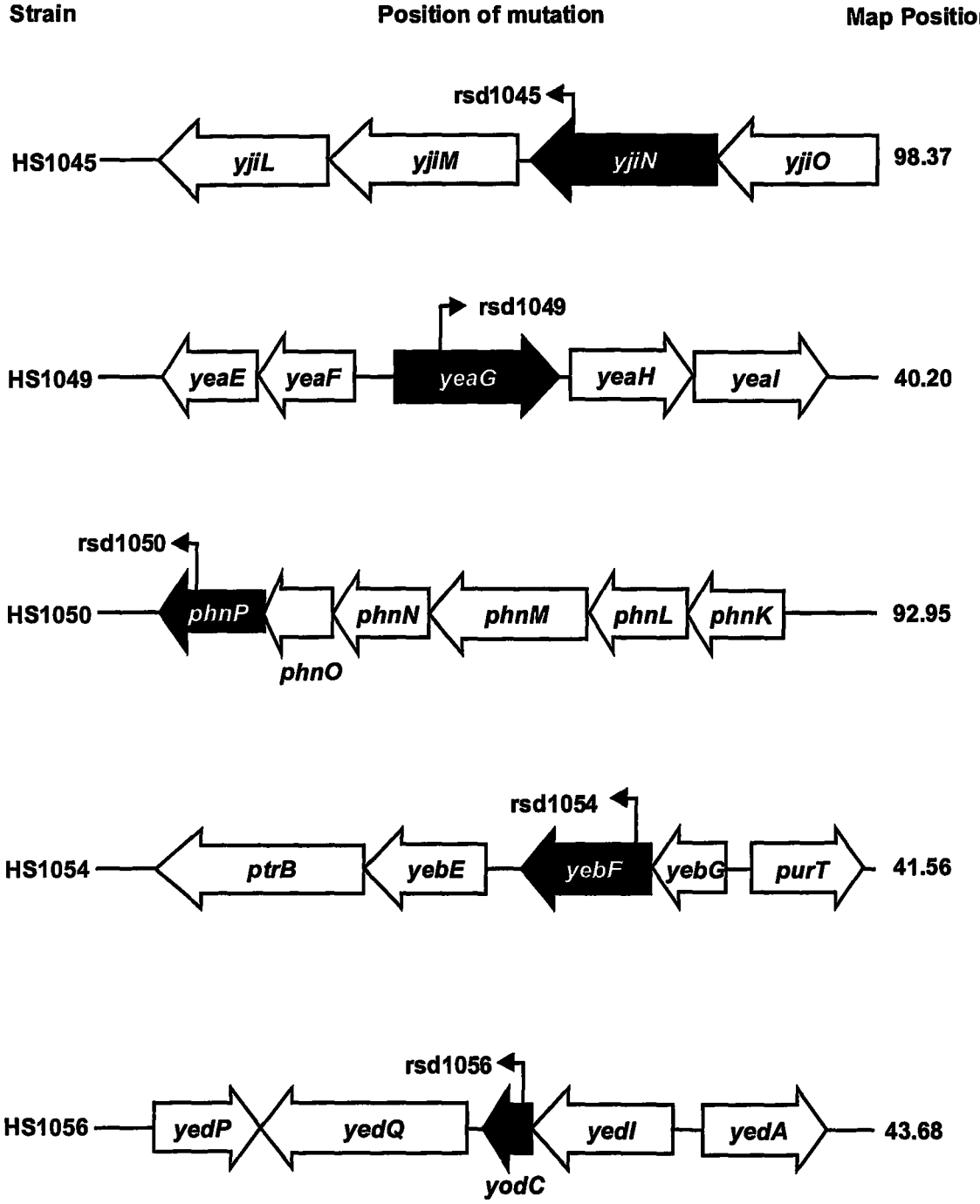
Zinser, E.R., and Kolter, R. (1999) Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. *J Bacteriol* 181: 5800-5807.

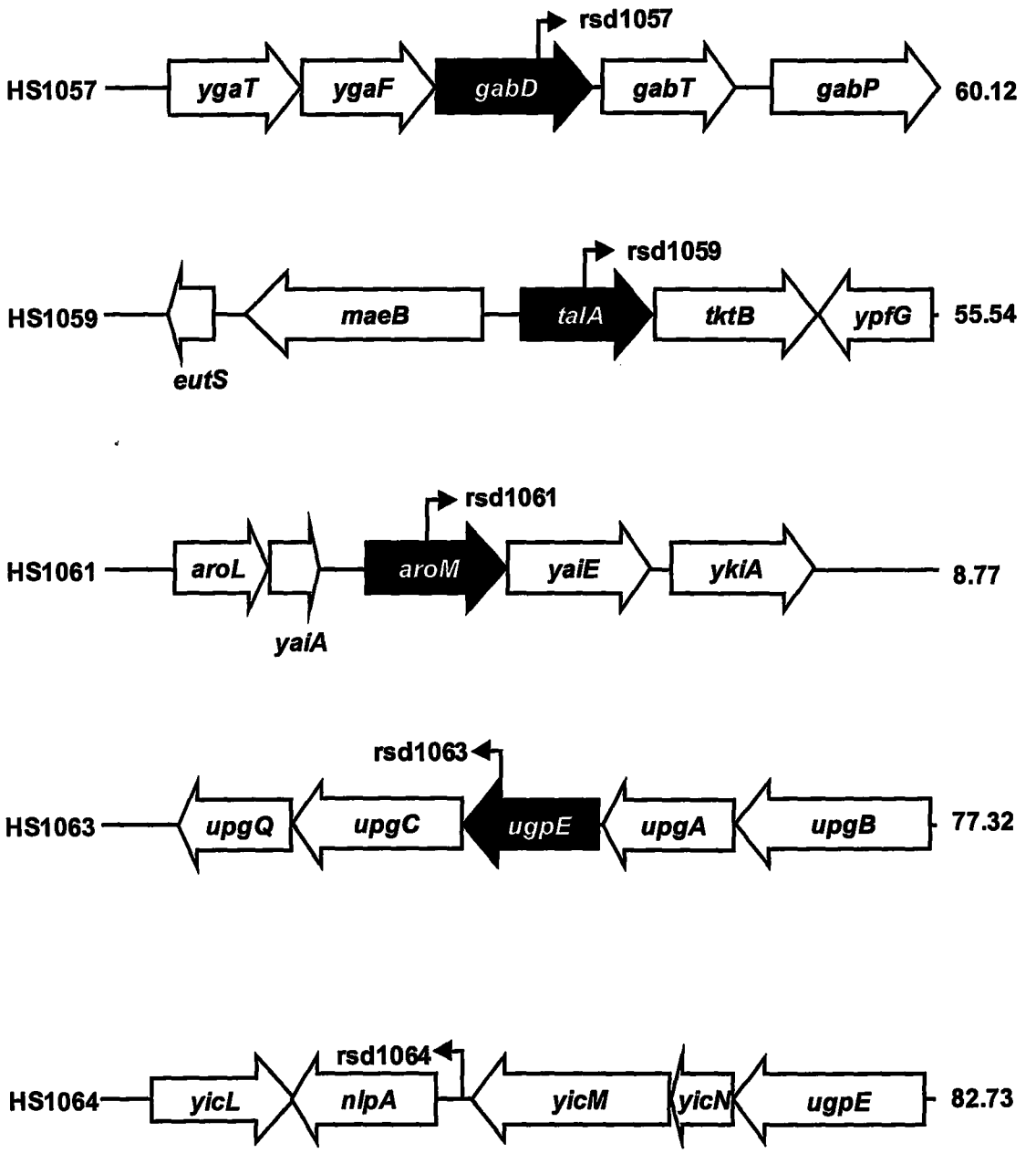
Figure 6. Location of *lacZ* fusion in RpoS-dependent genes. The RpoS-dependent genes are shown as thick black arrows and the location of fusion and the direction of transcription are shown as thin black arrows above each RpoS-dependent gene. The map position for RpoS-dependent genes were obtained from COLIBRI database.

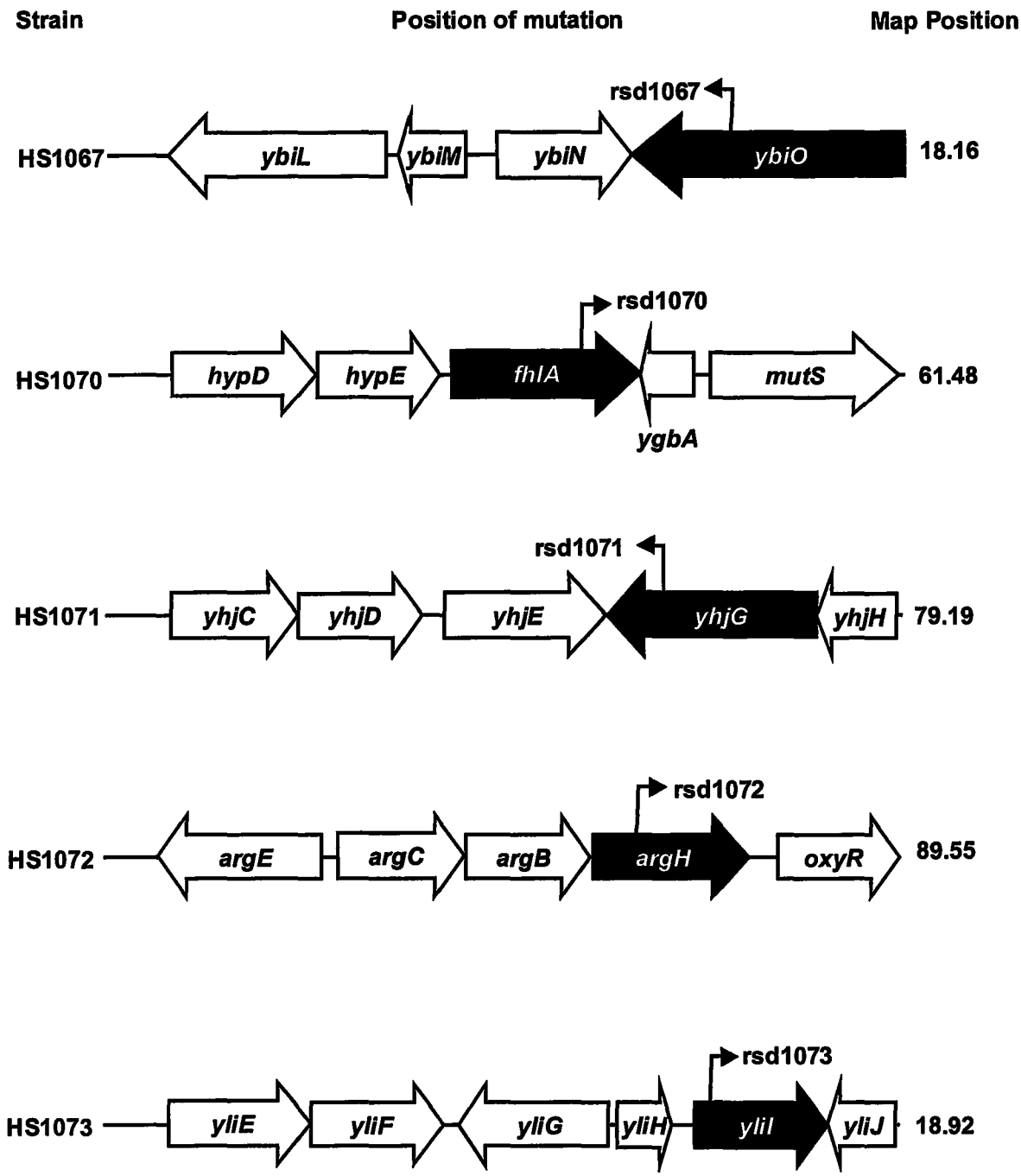

Strain
Position of mutation
Map Position
rsd1002
HS1002
dinF
yjbJ
zur
yjbL
yjbM
91.76
rsd1006
HS1006
yjbF
yjbJ
yjbG
yjbH
19.68
yjbE
rsd1008
HS1008
yiaV
yiaW
aldB
yiaY
selB
80.89
rsd1011
HS1011
ygaE
ygaU
yqaE
ygaV
ygaP
60.19
rsd1012
HS1012
ygdD
gcvA
ygdI
csdA
63.39

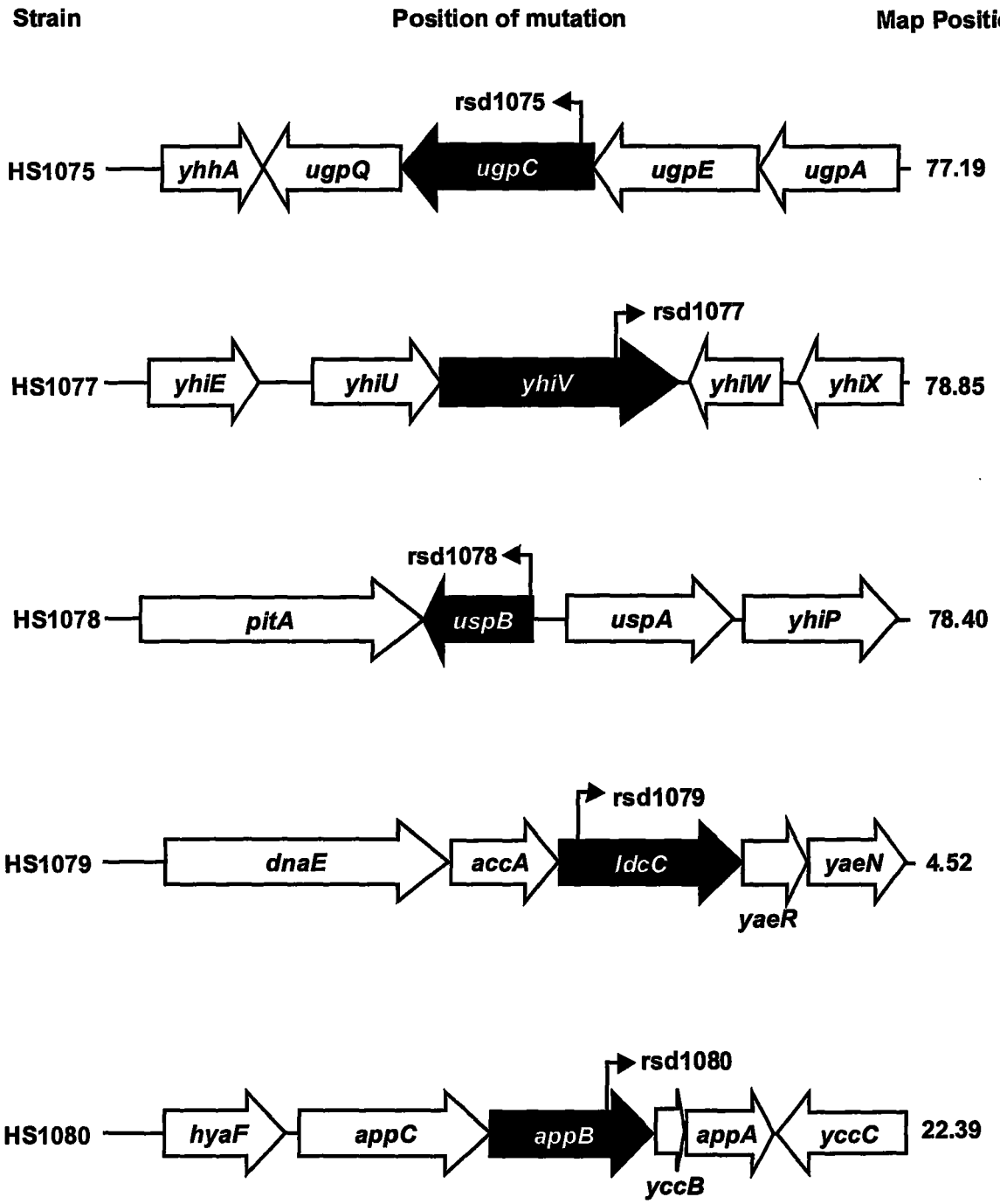

Strain
Position of mutation
Map Position
rsd1019
HS1019
yqhC
yqhD
yqhE
yqhG
yqhH
68.00
rsd1020
HS1020
recA
ygaD
mltB
srlA
srlE
60.84
rsd1022
HS1022
narV
narW
narY
narZ
narU
33.09
rsd1024
HS1024
ygaR
ygaC
ygaT
ygaF
60.10
ygaQ
ygaD
rsd1026
HS1026
amtB
tesB
ybaY
ybaZ
ybaA
10.23

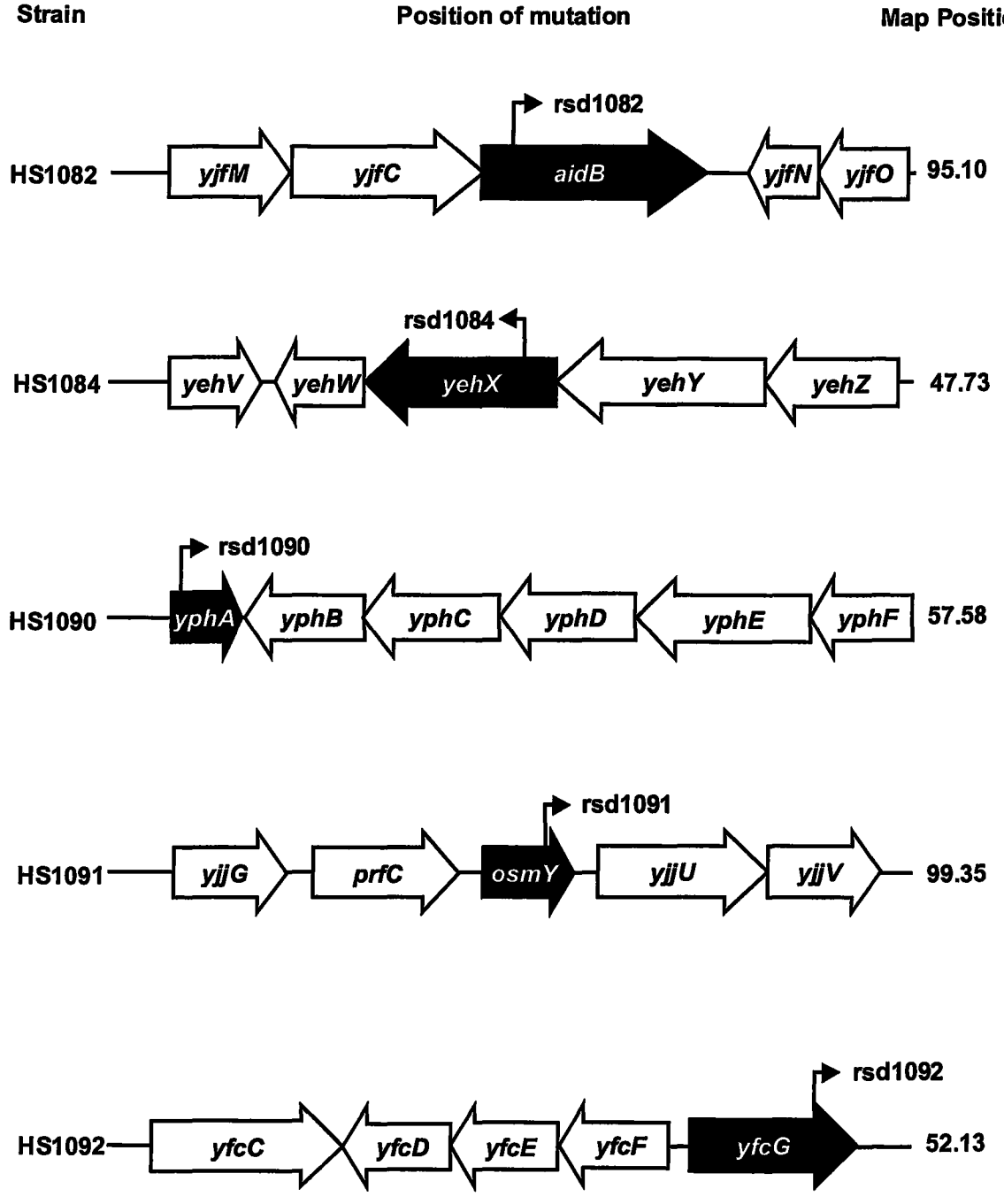

Strain
Position of mutation
Map Position
rsd1028
HS1028
yjgP
yjgQ
yjgR
idnR
idnT
96.70
rsd1033
HS1033
ydaL
ydaM
ydaN
dbpA
ydaO
30.28
rsd1036
HS1036
rimL
ydcK
tehA
rimL
32.28
ydcH
rsd1037
HS1037
gabD
gabT
gabP
ygaE
60.16
rsd1042
HS1042
yhjW
yhjX
yhjY
tag
yiaC
79.97


Strain
Position of mutation
Map Position
rsd1045
HS1045
yjiL
yjiM
yjiN
yjiO
98.37
rsd1049
HS1049
yeaE
yeaF
yeaG
yeaH
yeaI
40.20
rsd1050
HS1050
phnP
phnO
phnN
phnM
phnL
phnK
92.95
rsd1054
HS1054
ptrB
yebE
yebF
yebG
purT
41.56
rsd1056
HS1056
yedP
yedQ
yodC
yedI
yedA
43.68


Strain
Position of mutation
Map Position
rsd1057
HS1057
ygaT
ygaF
gabD
gabT
gabP
60.12
rsd1059
HS1059
maeB
talA
tktB
ypfG
55.54
eutS
rsd1061
HS1061
aroL
aroM
yaiE
ykiA
8.77
yaiA
rsd1063
HS1063
upgQ
upgC
ugpE
upgA
upgB
77.32
rsd1064
HS1064
yicL
nlpA
yicM
yicN
ugpE
82.73


Strain
Position of mutation
Map Position
rsd1067
HS1067
ybiL
ybiM
ybiN
ybiO
18.16
rsd1070
HS1070
hypD
hypE
fhlA
mutS
61.48
ygbA
rsd1071
HS1071
yhjC
yhjD
yhjE
yhjG
yhjH
79.19
rsd1072
HS1072
argE
argC
argB
argH
oxyR
89.55
rsd1073
HS1073
yliE
yliF
yliG
yliH
yliI
yliJ
18.92


Strain
Position of mutation
Map Position
rsd1075
HS1075
yhhA
ugpQ
ugpC
ugpE
ugpA
77.19
rsd1077
HS1077
yhiE
yhiU
yhiV
yhiW
yhiX
78.85
rsd1078
HS1078
pitA
uspB
uspA
yhiP
78.40
rsd1079
HS1079
dnaE
accA
ldcC
yaeN
yaeR
4.52
rsd1080
HS1080
hyaF
appC
appB
appA
yccC
yccB
22.39


Strain
Position of mutation
Map Position
rsd1082
HS1082
yjfM
yjfC
aidB
yjfN
yjfO
95.10
rsd1084
HS1084
yehV
yehW
yehX
yehY
yehZ
47.73
rsd1090
HS1090
yphA
yphB
yphC
yphD
yphE
yphF
57.58
rsd1091
HS1091
yjjG
prfC
osmY
yjjU
yjjV
99.35
rsd1092
HS1092
yfcC
yfcD
yfcE
yfcF
yfcG
52.13

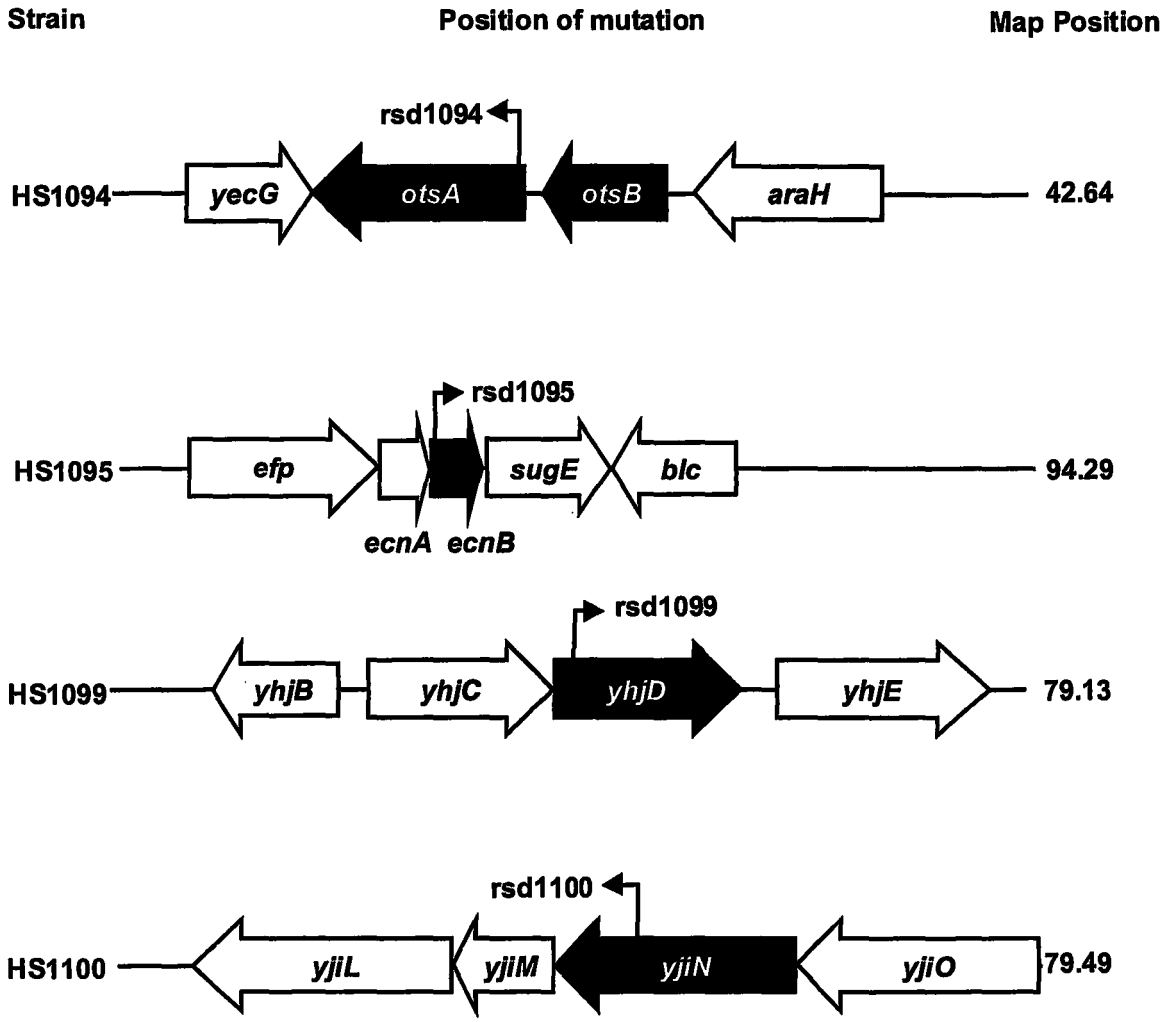

Strain
Position of mutation
Map Position
rsd1094
HS1094
yecG
otsA
otsB
araH
42.64
rsd1095
HS1095
efp
sugE
blc
94.29
ecnA
ecnB
rsd1099
HS1099
yhjB
yhjC
yhjD
yhjE
79.13
rsd1100
HS1100
yjiL
yjiM
yjiN
yjiO
79.49

Table 5. List of RpoS-dependent genes and their known and predicted functions

Gene	Blattner No.	Function*	Other Regulator(s)	Previously reported as RpoS dependent	RpoS Dependence		Reference(s)
					Rich Media	Minimal Media	
ybaY	b0453	Function unknown		No	15	12	
gabP	b2663	GABA transporter protein	GabC(-), Nac(+)	Yes (Schellhorn *et al.*, 1998)	24	18	(Niegemann *et al.*, 1993; Zimmer *et al.*, 2000)
ugpE	b3451	G-3-P transporter membrane bound component	PhoBR(+)	No	6	6	(Boos, 1998)
ugpC	b3450	ATP binding protein of G-3-P transporter	PhoBR(+)	No	20	9	(Boos, 1998)
ydcS	b1440	Putative binding protein of ABC transporter	Nac(+)	Yes (Schellhorn *et al.*, 1998)	6	2	(Zimmer *et al.*, 2000)
yehX	b2129	Hypothetical ATP-binding protein of ABC transporter		No	28	21	
narY	b1467	Beta subunit of nitrate reductase-II		Yes (Chang and Cronan, Jr., 1999)	10	2	(Blasco *et al.*, 1990)
appB	b0979	Codes for a subunit of cytochrome BD-II oxidase	AppR(+)	Yes (Dassa *et al.*, 1991)	25	7	(Dassa *et al.*, 1991)
ldcC	b0186	Lysine decarboxylase. Converts lysine to cadaverine and CO_2		Yes (Van Dyk *et al.*, 1998)	7	11	(Lemonnier and Lane, 1998)
aidB	b4187	Induced by alkylating agents. Protects DNA from alkylation damage		Yes (Landini *et al.*, 1996; Schellhorn *et al.*, 1998)	36	33	(Landini *et al.*, 1994; Landini *et al.*, 1996)
katE	b1732	Hydroperoxidase II	Lrp(+)	Yes (Mulvey *et al.*, 1988)	40	66	(Tani *et al.*, 2002)
uspB	b3494	Universal stress protein B. Required for ethanol resistance.		Yes (Farewell *et al.*, 1998)	4	8	(Farewell *et al.*, 1998)
osmY	b4376	Periplasmic protein induced by high osmolarity. Function unknown	Lrp(-), Crp(-), IHF(-)	Yes (Yim *et al.*, 1994)	35	64	(Colland *et al.*, 2000; Yim and Villarejo, 1992; Yim *et al.*, 1994)
yhiV	b3514	Codes for multidrug efflux pump	EvgA(+), Lrp(+)	No	31	4	(Nishino and Yamaguchi, 2002; Tani *et al.*, 2002; Tsukagoshi and Aono, 2000)
yhiU	b3513	Codes for multidrug efflux pump	EvgA(+)	Yes (Schellhorn *et al.*, 1998)	39	8	(Nishino and Yamaguchi, 2002; Tsukagoshi and Aono, 2000)

Table 5. List of RpoS-dependent genes and their known and predicted functions

Gene	Blattner No.	Function[a]	Other Regulator(s)	Previously reported as RpoS dependent	RpoS Dependence		Reference(s)
					Rich Media	Minimal Media	
mltB	b2701	Membrane bound lytic transglycosylase B		No	2	2	(Ehlert *et al.*, 1995)
otsA	b1896	Trehalose phosphate synthase	Lrp(+)	Yes (Strom and Kaasen, 1993)	31	57	(Strom and Kaasen, 1993; Tani *et al.*, 2002)
ecnB		Bacteriolytic lipoprotein entericidin B	EnvZ/OmpR (-)	Yes (Bishop *et al.*, 1998)	23	74	(Bishop *et al.*, 1998)
argH	b3960	Argino-succinate lyase.	ArgR(-)	No	2	5	(Tian *et al.*, 1992)
aroM	b0390	Function unknown.	TyrR(+)	No	15	78	(DeFeyter *et al.*, 1986)
yhjY	b3548	Putative lipase		No	15	9	
yqhE	b3012	2,5-diketo-D-gluconic acid reductase. Converts 2,5-KGD to 2-KLG.		No	8	11	(Yum *et al.*, 1999)
aldB	b3588	Aldehyde dehydrogenase B. Converts lactaldehyde and NAD^+ to lactate and NADH.		Yes (Xu and Johnson, 1995)	22	21	(Tani *et al.*, 2002; Xu and Johnson, 1995)
gabD	b2661	Succinate-semialdehyde dehydrogenase	GabC(-), Nac(+)	No	16	13	(Niegemann *et al.*, 1993; Zimmer *et al.*, 2000)
phnP	b4092	Membrane bound subunit of carbon-phosphorus lyase complex	PhnF and PhnO(+)	No	8	2	(Metcalf and Wanner, 1993)
talA	b2464	Transaldolase A. Converts Sed-7-P and Gly-3-P to Fru-6-P and Ery-4-P	CreBC(+)	No	20	26	(Avison *et al.*, 2001; Sprenger, 1995)
yfcG	b2302	Putative glutathione-S-transferase	SdiA(+)	No	37	52	(Wei *et al.*, 2001)
yiiI	b0837	Putative dehydrogenase		Yes (Schellhorn *et al.*, 1998)	17	37	(Schellhorn *et al.*, 1998)
yjbJ	B4045	Function unknown		No	22	179	
yjbE	b4026	Function unknown		No	22	58	
ygaU	b2665	Function unknown	Lrp(+)	No	4	12	(Tani *et al.*, 2002)
ygdI	b2809	Function unknown		No	7	5	
ygaF	b2660	Function unknown		No	3	7	
yjgR	b4263	Function unknown		No	6	3	
ydaM	b1341	Function unknown		No	16	23	
ydcK	b1428	Function unknown		No	15	9	
yhiN	b3492	Function unknown		No	5	7	
yebF	b1847	Function unknown		No	41	25	

Table 5. List of RpoS-dependent genes and their known and predicted functions

Gene	Blattner No.	Function[a]	Other Regulator(s)	Previously reported as RpoS dependent	RpoS Dependence		Reference(s)
					Rich Media	Minimal Media	
yjiN	b4336	Function unknown		No	4	6	
yeaG	b1783	Function unknown	Lrp(+)	Yes (Rozen *et al.*, 2001)	13	10	(Rozen *et al.*, 2001; Tani *et al.*, 2002)
yodC	b1957	Function unknown		No	41	17	
ybiO	b0808	Function unknown		No	13	26	
yhjG	b3524	Function unknown		No	17	15	
yphA	b2543	Function unknown		No	24	18	
yhjD	b3522	Function unknown	Lrp(+)	Yes (Rozen *et al.*, 2001)	5	34	(Rozen *et al.*, 2001)

[a]The known or predicted functions were assigned to each based on SWISSPROT or ECOCYC databases.