RPOS-DEPENDENT STATIONARY PHASE INDUCTION OF NITRATE REDUCTASE Z IN *E. COLI*

RPOS-DEPENDENT STATIONARY PHASE INDUCTION OF NITRATE REDUCTASE Z AND THE IDENTIFICATION OF RPOS-DIRECTED GENES

By

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Abstract

During entry into stationary phase, *Escherichia coli* expresses many genes which impart cellular resistance to numerous environmental stresses such as oxidative or acid stress. Many of these genes are regulated by the alternative sigma factor, RpoS. To identify additional genes regulated by RpoS, a phenotype independent genetic screen was previously employed (L. Wei Masters thesis). The identities of the ten most highly RpoS-dependent fusions were determined by DNA sequencing and subsequent sequence analysis using the BLAST algorithm. Three fusions map to genes previously known to be RpoS-dependent while the remaining seven represent new members of the regulon. The expression of many of the RpoS-dependent fusions remained growth phase dependent even in the *rpoS* background. This suggests that other growth phase regulatory factors in addition to RpoS may coordinate stationary phase gene expression. Upon sequencing the remaining *rsd* fusions, three mutants mapped to *narY* which is part of the *narZYWV* operon encoding the secondary nitrate reductase Z (NRZ). This operon was selected for further investigation since NRZ has been previously reported to be constitutively expressed. Expression studies using promoter::*lacZ* fusions and nitrate reductase assays reveal that NRZ is induced ten-fold at the onset of stationary phase and twenty-fold in the presence of nitrate. Like other *rsd* fusions, growth phase dependent expression was observed in an *rpoS* background indicating that other regulatory factors may be involved

in the regulation of NRZ. Northern analyses using probes specific to NRZ confirmed that transcription of NRZ is indeed dependent on RpoS. These results suggest that RpoS mediated regulation of NRZ may be an important physiological adaptation to reduced oxygen levels during transition to stationary phase.

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List of Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
CAI	Codon Adaptation Index
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
GABA	γ-aminobutyrate
λ	Bacteriophage Lambda
LB	Luria-Bertani
μg	Microgram
μl	Microlitre
mg	Milligram
ml	Milliliter
mM	Millimolar
μm	Micromolar
nm	Nanometer
NED	Napthyletheylenediamine dihydrochloride
NRA	Nitrate reductase A
NRZ	Nitrate reductase Z
OD	Optical density
PCR	Polymerase Chain Reaction
% w/v	Percent weight volume
REP	Repetitive extragenic palindromic elements
rpm	Revolutions per minute
RNA	Ribonucleic acid
UV	Ultraviolet

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CHAPTER 1. INTRODUCTION

1.1 Overview

In their natural environment, bacteria frequently experience nutrient limitation and as a result, have adapted complex mechanisms to cope with the environmental stresses associated with starvation. While some gram positive bacteria such as *Bacillus subtilis* respond to this stress by differentiating into highly resistant spores, non-differentiating bacteria such as *Escherichia coli* and *Salmonella typhimurium* enter a physiological state known as stationary phase (Hengge-Aronis, 1996b).

Entry into stationary phase involves a transition from a state of maximal growth and cell division to a basal level of metabolism and cell growth. During this transition, changes in cellular physiology and morphology occur which aids in increased viability and longevity of the culture. For example, *E. coli* cells assume a smaller and more spherical shape compared to the characteristic rod shape observed in exponential phase. The cytoplasm of the cells condenses, whereas the volume of the periplasm increases (Lange and Hengge-Aronis, 1991a; Reeve et al., 1984). Furthermore, the cell wall is more highly cross linked while the DNA becomes supercoiled (Balke and Gralla, 1987). Two-dimensional gel electrophoresis studies show that as many as thirty proteins are induced as cells enter stationary phase (Groat et al., 1986). Many of these proteins are involved in

general stress protection since stationary phase cells are characteristically resistant to multiple stresses associated with dormancy. These include heat shock, near-UV exposure and oxidative stress.

Expression of these stationary phase genes are under the control of the *rpoS* encoded σ^s subunit (also designated σ^{38} or RpoS) (Lange and Hengge-Aronis, 1991b; Mulvey and Loewen, 1989). As a component of RNA polymerase, σ^s recognizes a set of promoters that are poorly recognized *in vivo* by the "housekeeping sigma factor" σ^{70}. Over 40 genes/operons have been identified to be regulated by σ^s. These include *katE* which encodes for the catalase HPII (Sak et al., 1989; Schellhorn and Stones, 1992), exonuclease III (Sak et al., 1989) and the virulence genes (*vir*) in *Salmonella typhimurium* (Kowarz et al., 1994). Although the regulon is fairly large, expression of many genes are also dependent on additional co-regulators which interact with RpoS in a complex manner.

While σ^s may be the predominant sigma factor in stationary phase, RpoS and many RpoS-dependent genes are also induced under various stress conditions including osmotic stress (Lange and Hengge-Aronis, 1994b), acid stress (Lee et al., 1995) and heat shock (Jishage et al., 1996) during exponential growth. These observations, and the fact that RpoS regulated genes encompass a variety of stress related mechanisms have led to the view that RpoS should be regarded as a global stress response regulator that includes the response to starvation (Hengge-Aronis, 1996a).

It is evident that a great deal has yet to be learned about σ^s itself and the complex mechanisms of the genes it regulates. Future work will not only provide greater knowledge in the field of bacterial physiology, but may also lead to the development of

drugs for the treatment of bacterial infections and industrial products.

1.2 RpoS Homologues in other organisms.

The importance of RpoS for survival under stress related conditions in *E. coli* has led to the examination of similar RpoS systems in other bacteria. Homologues of *rpoS* have been identified in *Salmonella typhimurium* (Kowarz et al., 1994), *Shigella flexineri* (Small et al., 1994), *Pseudomonas aeruginosa* (Fujita et al., 1994), *Yersinia entercolitica* (Iriarte et al., 1995), *Erwinia carotovora* (Becker-Hapak et al., 1997) and *Vibrio choloerae* (Yildiz et al., 1998). Proteins of similar RpoS activity have also been reported in *Acetobacter methanolicus, Xanthomonas camprestris, Pseudomonas putida*, and *Rhizobium meliloti* (Miksch and Dobrowolski, 1995). To date, no genes that are homologous to *rpoS* have been reported in gram-positive organisms.

Studies of *rpoS* mutants in the various organisms have revealed that like *E. coli,* they are impaired in the ability to survive diverse environmental stresses such as exposure to hydrogen peroxide, hyperosmolarity and low pH. Thus, the role of RpoS in multiple stress protection is fairly conserved amongst bacteria. In some cases, RpoS contributes to the virulence and pathogenesis of the bacteria. For example, RpoS is important for acid tolerance in *Salmonella typhimurium* (Lee et al., 1995) and in *Shigella flexineri* (Small et al., 1994). Such conditions are comparable to the stomach where enteric bacteria must survive before entry into the intestines. In addition to acid tolerance, RpoS also regulates the expression of *spvR* in *S. typhimurium* (Kowartz et al., 1994). SpvR activates the expression of the plasmid borne virulence genes (*spvABCD*) (Kowarz et al., 1994) which are required for intracellular growth. In *Yersinia enterocolitica,* an RpoS homologue

regulates the enterotoxin gene *yst* which causes diarrhea (Iriarte et al., 1995). Furthermore, the antibiotic pyrrolnitrin in rhizosphere-inhabiting bacterium *Pseudomonas fluorescens Pf-5*, was also found to be regulated by RpoS (Sarniguet et al., 1995) which suppresses the growth of several soil borne pathogens. Expression of this gene was also found to be regulated by RpoS. When viewed together these studies demonstrate the significant role of RpoS in bacterial stress adaptation and virulence.

1.3 Variation of the *rpoS* sequence in *E.coli*

Numerous studies have reported variability in the *rpoS* gene which implies that there is a selection for attenuation of RpoS (Barth et al., 1995; Ivanova, et al., 1992; Jishage and Ishihama, 1997). One of the earliest reports which demonstrated plasticity in the *rpoS* sequence compared the *rpoS* sequence of different wild-type strains of *E. coli* (Ivanova et al., 1992). In this study, variations of the *rpoS* sequence included single nucleotide differences that did not affect the amino acid sequence, and other substitutions that caused single amino acid alterations. An *hns* mutant can acquire a mutation that resides in or close to the *rpoS* gene (Barth et al., 1995). This results in reduced RpoS expression by approximately 10-75% of wild type. Another study reported that levels of σ^s vary between different laboratory stocks of the same commonly-used wild type strain (W3110) (Jishage and Ishihama, 1997). Of the five types identified, the C-type strains lacked σ^s protein, while the B-type lineages carried a truncated form of the σ^s subunit. Sequence of the *rpoS* gene in the B-type strains revealed that there were three single base changes, of which one generated an amber codon. This amber mutation eliminates region 4 of the amino acid sequence that is necessary for promoter recognition (Jishage and

Ishihama, 1997). The survival of these strains under the artificial stresses associated with laboratory conditions is debatable. It is proposed that these stocks may have acquired additional mutations that can suppress the *rpoS* mutation (Jishage and Ishihama, 1997). Alternatively, these mutations in the *rpoS* sequence may confer some uncharacterized advantage for survival that may have been selected for over several years in these stock cultures (Jishage and Ishihama, 1997).

Perhaps the most dramatic variation in the *rpoS* sequence is a mutation that arises spontaneously in late stationary phase cells (Zambrano et al., 1993). Sequence analysis reveals that these mutants carry a 46 base pair duplication at the 3' end of the coding region, resulting in an elongated *rpoS* gene. Strains carrying this mutation exhibit a competitive growth advantage for survival under prolonged periods of starvation. This competitive advantage arises only in late stationary phase cells and was demonstrated by incubating mixed cultures of ten day old cells with one day old cells. The aged cells were found to take over the culture resulting in the subsequent death of the younger cells (Zambrano et al., 1993). The **g**rowth **a**dvantage of **s**tationary **p**hase cells was designated the **GASP** phenotype. Taken *in toto*, these studies suggest that mutations in the *rpoS* sequence under adverse conditions may be an adaptive response of the cell to a given stress condition.

1.4 Regulation of RpoS

RpoS expression increases during entry into stationary phase and it is the predominant sigma factor during this period of growth. Regulation of RpoS expression is controlled at the levels of transcription, translation and protein stability (See Figure 1 for an overview). Regulatory factors and signal molecules involved in RpoS regulation are discussed below.

1.4.1 Transcriptional Control

Transcriptional control of *rpoS* is fairly complex. The *rpoS* gene is the second gene of an operon along with *nlpD*, which encodes for a lipoprotein involved in morphological changes (Lange and Hengge-Aronis, 1994a). There are at least three promoters upstream of *rpoS* responsible for transcription initiation. Two of the promoters direct expression of both *nlpD* and *rpoS*, and likely contribute to the basal levels of σ^s expression observed during exponential phase. The third promoter (*rpoSp1*) is located within the *nlpD* structural gene. Deletion of the *rpoSp1* promoter region results in the reduction of *rpoS* transcription by twenty-fold during stationary phase. Thus, it is highly likely that *rpoSp1* is the main promoter from which transcription initiation occurs during stationary phase induction (Lange et al., 1995).

Changes in the environment are sensed by molecular signals which in turn induce *rpoS* expression. Although the molecular details remain unclear, cAMP-CRP, ppGpp and UDP-glucose have been implicated as potential signal molecules (Hengge-Aronis, 1996b).

1.4.1.1 cAMP-CRP

The cAMP-CRP complex is normally involved in catabolite repression. This

process involves the inhibition of alternative enzymes that are utilized in other metabolic processes when a preferred carbon source (e.g. glucose) is present. When cultures are grown in glucose, cAMP levels (synthesized by the *cya* encoded adenylate cyclase) are very low. Upon glucose depletion, as observed during starvation, the levels of cAMP increase. When cAMP forms a complex with the cAMP regulatory protein (CRP, coded by the *crp* gene), it can act as a transcriptional activator or repressor of a variety of genes/operons (eg. lactose operon) to replenish intracellular glucose levels.

Strains carrying a *cya* mutation exhibit increased *rpoS::lacZ* expression in exponential phase. The addition of cAMP decreases *rpoS::lacZ* activity, thereby demonstrating that cAMP represses *rpoS* expression (Lange and Hengge-Aronis, 1991b; Lange and Hengge-Aronis, 1994b). Cyclic-AMP also represses several RpoS dependent genes including *osmY* (Weichart et al., 1993) and *glgS* (Hengge-Aronis and Fischer, 1992). It is postulated that cAMP may interfere with transcription in order to avoid strong activation of *rpoS* and therefore, the entire stationary phase response in a carbon source downshift which can be managed by relieving catabolite repression (Lange and Hengge-Aronis, 1994b).

1.4.1.2 Guanosine tetraphosphate (ppGpp)

Guanosine tetraphosphate (ppGpp) induces a number of genes involved in the stringent response (reviewed in Cashel and Rudd, 1987). The accumulation of ppGpp depends on the products of two genes, *relA* and *spoT*, which respond to amino acid and carbon source limitation (Gentry et al., 1993). It was originally observed that ppGpp^0 (*ΔrelAspoT*) mutants exhibit a pleiotrophic phenotype similar to that of an *rpoS* mutant

which suggests a possible link between ppGpp and σ^s (Gentry et al., 1993; Lange et al., 1995). For example, the RpoS-dependent production of acid phosphatase (encoded by *appA*) is impaired in a ppGpp° mutant stain compared to its wild type parental strain during the transition into stationary phase (Gentry et al., 1993). RpoS levels were found to decrease during entry into stationary phase as determined by Western analyses (Gentry et al., 1993). Protein levels could be restored by artificial elevation of ppGpp obtained by a mutation in *spoT*, or by an overexpression of *relA* (Gentry et al., 1993). It is likely that ppGpp affects *rpoS* at the transcriptional level since expression of an transcriptional *rpoS::lacZ* fusion decreases in a ppGpp° background (Lange et al., 1995).

1.4.1.3 Homoserine lactone (HSL)

Homoserine lactone (HSL) was first implicated as a positive regulator of *rpoS* during the screening of a chromosomal library for genes involved in the repression of the RpoS-dependent *bolA::lacZ* fusion (Huisman and Kolter, 1994). One clone which repressed *bolA::lacZ* expression was found to encode the *rspA gene* (Huisman and Kolter, 1994). The RspA amino acid sequence exhibits homology to a lactonizing enzyme, suggesting that the RspA may reduce the levels of a starvation signal molecule (eg. lactone) responsible for *rpoS* induction. This therefore indicates that HSL may be an inducer of RpoS activity (Huisman and Kolter, 1994). Mutants unable to synthesize homoserine exhibit reduced σ^s levels as assayed by catalase activity. The addition of HSL into the growth media restores this activity (Huisman and Kolter, 1994).

HSL derivatives are produced and excreted by *Vibrio* and other marine species in response to population density. When HSL reaches a certain threshold concentration, a

signaling pathway is induced. In light of this, HSL in *E. coli* may allow the cell to sense population levels and its accumulation to a critical concentration trigger synthesis of *rpoS* (Huisman and Kolter, 1994).

1.4.1.4 UDP-glucose

In a screen to identify regulatory factors in addition to RpoS that control expression of an *osmY::lacZ* transcriptional fusion (an candidate RpoS dependent gene), a mutation in the *pgi* gene was isolated (Bohringer et al., 1995). The genes *pgi* (PGI, phosphoglucose isomerase), *pgm* (phosphoglucomutase) and *galU* (UDP-glucose pyrophophorylase), encode glycolytic enzymes catalyzing the reactions of precursor substrates to UDP-glucose (Bohringer et al., 1995). In a *pgi* mutant background the RpoS dependent genes *osmY* and *otsBA*, exhibit increased basal levels of expression in exponentially growing cells. To determine whether UDP-glucose directly influences RpoS expression, protein levels were monitored in strains deficient in *pgi*, *pgm*, and *galU*. These mutants exhibited a four-fold increase in levels of σ^s demonstrating that UDP-glucose is a negatively acting signal molecule that down modulates σ^s levels during exponential growth (Bohringer et al., 1995). Since UDP-glucose is a precursor for the osmoprotectant trehalose, it is proposed that UDP-glucose represses *rpoS* during exponential growth (Bohringer et al., 1995). Upon osmotic challenge, UDP-glucose levels drop, thereby alleviating *rpoS* repression and inducing trehalose synthesis.

1.4.1.5 Weak Acids

Experiments with gene fusions have demonstrated that *rpoS* transcription in early exponential phase cells can be induced by a dialyzable, heat stable factor present in spent

medium of stationary phase cultures (Mulvey et al., 1990). Several compounds that may accumulate in stationary phase cells have been tested for the ability to induce *rpoS* expression. These include weak acids such as acetic, propionic and benzoic acids leading to the suggestion that *rpoS* expression may be modulated by the internal pH of a cell (Schellhorn and Stones, 1992). Further support for this comes from a study by (Van Dyk et al., 1998) which found that many RpoS dependent genes were induced by internal acidification caused by the accumulation of α-ketoacids due to acetolactate synthase inhibition.

1.4.2 Translational Control

Studies using transcriptional and translational *rpoS::lacZ* fusions (with identical fusion joints and upstream regions) have shown that while expression of transcriptional fusions remains nearly constant, translational activity and protein levels increases upon entry into stationary phase (Lange and Hengge-Aronis, 1994b). This implicated that post-transcriptional mechanisms were involved in *rpoS* induction.

A stable secondary structure (comprising the Shine Dalgarno sequence, the initiation codon and a downstream box with complementarity to a 3' region in 16s rRNA) in the *rpoS* mRNA is predicted to affect translational regulation (Lange and Hengge-Aronis, 1994b). It has been hypothesized that the translational initiation region may be base-paired making it unaccessible to ribosomes (Lange and Hengge-Aronis, 1994b). However under inducing conditions, the proposed secondary structure may undergo a conformational change which allows translation of mRNA to proceed. Specific proteins that respond to stationary phase or other related stresses may either be involved in the

maintenance or resolution of these mRNA secondary structures (Lange and Hengge-Aronis, 1994b).

Two proteins that have been implicated in translational regulation of *rpoS* are H-NS (histone-like protein) and HF-1. H-NS is a DNA binding protein involved in the compact organization of the chromosome and the direct or indirect negative regulation of the expression of many genes (Hulton et al., 1990). A mutation in *hns* results in a seven fold increase in translation of *rpoS* mRNA during exponential phase (Barth et al., 1995; Yamashino et al., 1995). Furthermore, stationary phase or osmotic induction is no longer observed in an *hns* mutant. This suggests that H-NS represses RpoS translation during exponential growth which is relieved following stationary or osmotic induction. The mechanism by which H-NS represses σ^s is not clear although it may possibly interact with HF-1.

The *hfq* encoded HF-1 protein was originally identified as a host factor for phage Qβ RNA replication, however its physiological function in *E. coli* was not known (Fernandez et al., 1972; Muffler et al., 1996). Using Western blot analyses, (Muffler et al., 1996) demonstrated that RpoS levels were barely detectable throughout growth in an *hfq* mutant strain while protein levels increased five fold in the otherwise isogenic *hfq*$^+$ strain. Reduced activity of an *rpoS::lacZ* translational fusion in an *hfq* mutant background further demonstrated that HF-1 positively regulates *rpoS* translation upon entry into stationary phase (Muffler et al., 1997). During phage RNA synthesis, HF-1 induces a conformational change in the secondary structure of the 3' region of the mRNA for replication to occur (Barrera et al., 1993). In light of this, it is proposed that HF-1 may bind to *rpoS* mRNA to

induce conformational change of the secondary structure that would allow translation to proceed (Muffler et al., 1997).

Experiments to determine the nature of the HF-1 interaction with other proteins revealed that an *hns* mutation does not affect *rpoS* translation when present in an *hfq* mutant background (Muffler et al., 1997). This suggested that H-NS may affect RpoS translation by influencing the expression of HF-1, which in turn would alleviate the secondary structure in the *rpoS* mRNA for translation to occur. It has been reported that H-NS binds tightly to HF-1 *in vitro* (Kajitani et al., 1994), which raises the possibility that under *in vivo* conditions, H-NS may interfere with HF-1 activity by direct protein-protein interaction (Muffler et al., 1996). This would suggest a novel function of H-NS which up to now has been generally considered a DNA binding protein.

The small RNA OxyS has also been implicated in translational regulation of *rpoS*. OxyS was originally isolated as a small, untranslated RNA whose expression is dependent on OxyR (Altuvia et al., 1997) and is able to repress a number of genes including *rpoS* (Altuvia et al., 1997). In strains carrying a constitutively expressed plasmid-borne copy of *oxyS*, stationary phase expression of an *rpoS::lacZ* translational fusion is greatly reduced (Zhang et al., 1998). To determine whether OxyS interacts with other regulatory proteins, mutations in various genes used in conjunction with over-expression of *oxyS* were constructed, and *rpoS* expression was monitored. It was observed that OxyS was able to repress expression of *rpoS* in all backgrounds except in an *hfq* mutant background, indicating that OxyS expression requires a functional HF-1 (Zhang et al., 1998). Co-immunoprecipitation and gel mobility shift experiments revealed that HF-1 is able to bind

OxyS, probably at A-rich regions found in a 26 base pair linker region between stem loops b and c of oxyS. It is proposed that under conditions of oxidative stress, OxyS is induced and prevents HF-1 from activating RpoS and hence the entire regulon (Zhang et al., 1988). This likely serves to prevent redundant expression of some RpoS dependent genes which may already be induced by OxyR (e.g. *katG*) (Zhang et al., 1998).

In addition to stationary phase induction, RpoS is also induced under various stress conditions including exposure to sub-optimal temperatures (Sledjeski et al., 1996). This "cold shock" induction of RpoS is dependent on a small untranslated RNA called DsrA (Sledjeski et al., 1996). In a *dsrA* mutant background, an *rpoS::lacZ* translational fusion exhibits only very low levels of induction when cultures are grown at 20°C. (Sledjeski et al., 1996). Over-expression of *dsrA* can counteract H-NS mediated gene silencing (Sledjeski and Gottesman, 1995). Recently, it has been demonstrated that DsrA regulates translation of *rpoS* by an anti-antisense mechanism (Majdalani et al., 1998). Results of mutation studies reveal that the first stem loop of DsrA is complementary to the upstream leader portion of *rpoS* messenger RNA (Majdalani et al., 1998). This suggests that DsrA binding to the *rpoS* mRNA may increase translation efficiency by freeing the initiation region from the cis-acting antisense RNA (Majdalani et al., 1998).

1.4.3 σ^s Stability

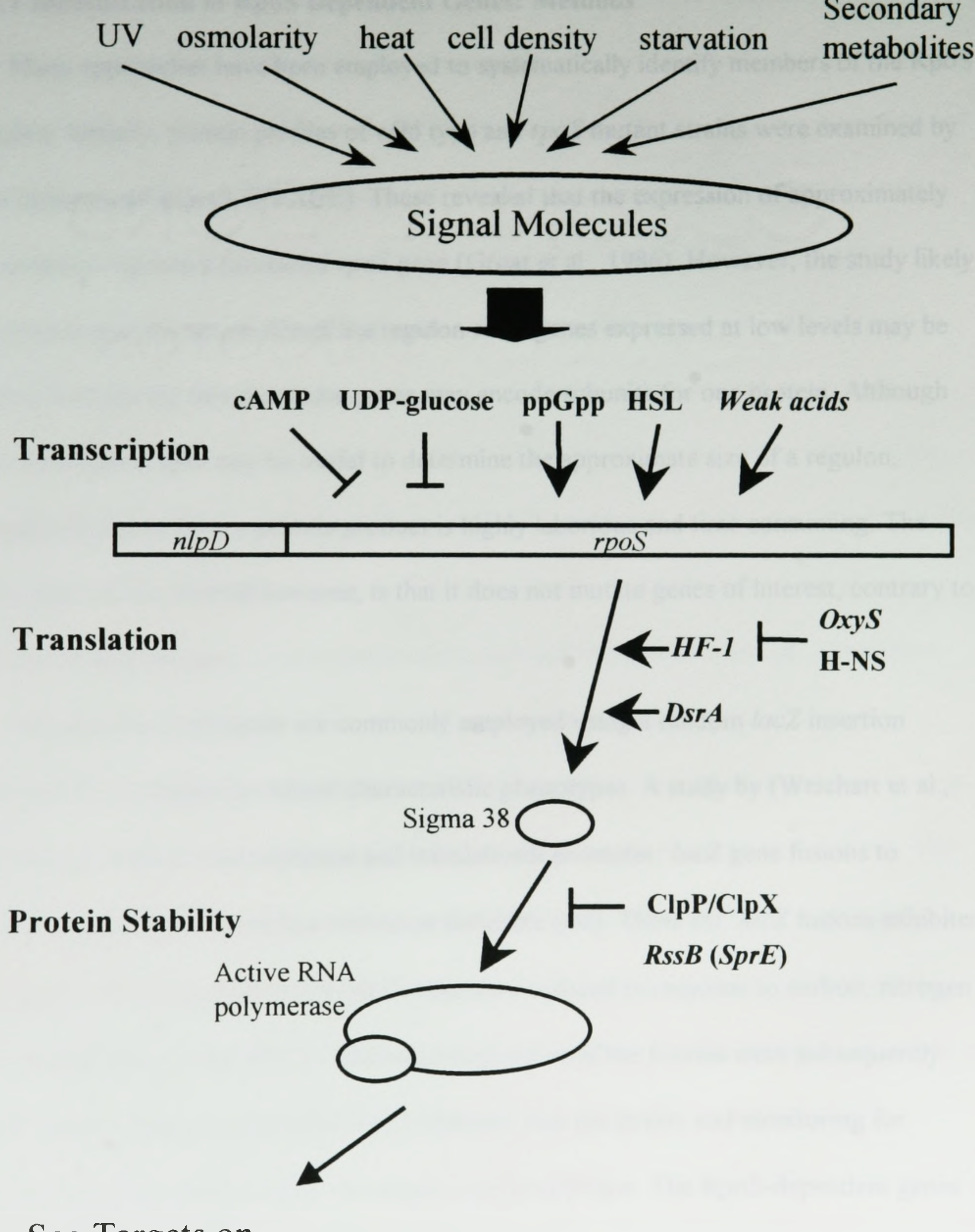
In exponential phase, σ^s is highly unstable with a half life of 1.4 - 2.5 min. Upon entry into stationary phase, σ^s half life increases to 16.5 min (Lange and Hengge-Aronis, 1994b). In response to osmotic upshift, σ^s stability also increases to a half-life of more than 45 min. (Lange and Hengge-Aronis, 1994b). RpoS turnover in exponential phase is

due to ClpXP protease (Schweder et al., 1996). In cells deficient of ClpX or its subunit ClpP, σ^s protein levels in exponential phase are comparable to levels found in stationary phase (Schweder et al., 1996). Using translational fusions containing different lengths of RpoS coding regions, it was found that amino acid residues 173 to 188 may directly or indirectly serve as a target for ClpXP protease (Schweder et al., 1996).

RpoS mediated turnover by ClpXP has been linked to the two component response regulator RssB. RssB is defined by the presence of an N-terminal receiver domain which is phosphorylated or dephosphorylated in response to signals transmitted by histidine sensor kinases which controls the activity of a C-terminal output domain (Muffler et al., 1996). RssB null mutants are phenotypically similar to *clpXP* mutants, exhibiting high levels of the σ^s protein in both exponential phase and stationary phase (Muffler et al., 1996). To determine whether RssB acts on the specific substrate RpoS or the ClpXP protease, a recent study compared the effects of an *rssB* mutation and *rssB* overexpression on RpoS and the λO protein (another protein degraded by ClpXP) (Zhou and Gottesman, 1998). It was found that RpoS turnover is influenced by RssB levels, while λO protein turnover is unaffected. Thus, it was concluded that RssB acts on the specific substrate RpoS rather than on the protease (Zhou and Gottesman, 1998). This suggests a possible model where RssB may act as a sensor to starvation and/or osmotic signals and bind to σ^s in a manner that allows for degradation by the ClpXP protease (Zhou and Gottesman, 1998). Although no sensor kinase has been identified that activates RssB, acetyl phosphate has been proposed as a potential phosphate donor to RssB (Bouche et al., 1998). Cells deficient in the synthesis of acetyl-phosphate exhibits increased RpoS stability during exponential

activates RssB, acetyl phosphate has been proposed as a potential phosphate donor to RssB (Bouche et al., 1998). Cells deficient in the synthesis of acetyl-phosphate exhibits increased RpoS stability during exponential phase (Bouche et al., 1998). Phosphotransfer to aspartic residue D58 of RssB by radiolabelled-^{32}P acetyl-phosphate further demonstrated that this molecule may act as phosphate donor to activated RssB (Bouche et al., 1998). It is proposed that as cells become starved for a carbon source, acetyl-phosphate levels drop, leading to a reduction in RssB-mediated degradation of RpoS and thus increased RpoS stability in stationary phase (Bouche et al., 1998).

Figure 1. Regulation of RpoS at the levels of transcription, translation and protein stability. Modified from (Eisenstark et al., 1996). Additions to the figure are indicated in italics.


Environmental stimuli
UV
osmolarity
heat
cell density
starvation
Secondary metabolites
Signal Molecules
cAMP
UDP-glucose
ppGpp
HSL
Weak acids
Transcription
nlpD
rpoS
Translation
OxyS
HF-1
H-NS
DsrA
Sigma 38
Protein Stability
ClpP/ClpX
RssB (SprE)
Active RNA polymerase
See Targets on Table 1

1.5 RpoS Regulon

1.5.1 Identification of RpoS Dependent Genes: Methods

Many approaches have been employed to systematically identify members of the RpoS regulon. Initially, protein profiles of wild type and *rpoS* mutant strains were examined by two dimensional gels (2-D PAGE). These revealed that the expression of approximately 30 proteins requires a functional *rpoS* gene (Groat et al., 1986). However, the study likely underestimates the actual size of the regulon since genes expressed at low levels may be undetected and the fact that some genes may encode subunits for one protein. Although two dimensional gels may be useful to determine the approximate size of a regulon, identifying a gene from a protein product is highly laborious and time consuming. The advantage of this method however, is that it does not mutate genes of interest, contrary to the use of *lacZ* fusions.

Mutagenesis techniques are commonly employed using a random *lacZ* insertion followed by screening for related characteristic phenotypes. A study by (Weichart et al., 1993) used random transcriptional and translational promoter::*lacZ* gene fusions to identify genes that were carbon starvation inducible (*csi*). These *csi*::*lacZ* fusions exhibited stationary phase induction in rich media and were induced in response to carbon, nitrogen or phosphate starvation. RpoS- dependent expression of the fusions were subsequently identified by introducing an *rpoS*::Tn10 insertion into the strains and monitoring for reduced β-galactosidase activity in comparison to wild type. The RpoS-dependent genes identified in this study included, *osmY* (*csi*-5), *glgD* (*csi*-17) and *glgA* (*csi*-18). A similar approach was employed to identify genes associated with the Acid Tolerance Response

(ATR) in *Salmonella typhimurium* (Lee et al., 1995) and acid resistance phenotype in *Shigella flexneri* (Waterman and Small, 1996). This method has proven to be highly effective in identifying RpoS dependent genes associated with a specific stress response. However, this is also its limitation since the RpoS regulon, unlike other regulons does not have a single unifying characteristic or unique phenotype that all members respond to. Therefore, only a subset of RpoS dependent genes can be identified by this manner.

Another approach involved placing the *rpoS* gene under the control of an arabinose inducible promoter (P_{BAD}) and detecting *lacZ* expression in the presence or absence of arabinose supplementation (Fang et al., 1996). In this study, the RpoS inducible vector which is based on a P_{BAD} promoter of the *araBAD* (arabinose) operon was introduced into a MudJ transposon library carrying *lacZ* fusions. Candidate MudJ fusions which were induced (appear blue) upon supplementation with arabinose were selected. These were transduced into *rpoS*$^{+}$ (wild type) and *rpoS*$^{-}$ mutant backgrounds with subsequent expression studies performed to confirm RpoS dependence. Six RpoS dependent fusions were identified including, *otsA*, *yohF*, *o186*, and three genes of unknown functions. The advantage of this method is that expression of RpoS can be controlled and therefore experiments can be performed independent of culture conditions or growth phases. A limitation of this assay is that many insertions may be induced by the presence of arabinose rather than RpoS. The extremely high levels of RpoS expression may also induce additional genes that are not truly regulated by RpoS under *in vivo* conditions (Fang et al., 1996).

An alternative to the *lacZ* reporter gene are the *luxCDABE* genes of *Photorhabdus*

luminescens. The *lux* operon encodes for the enzyme responsible for the characteristic bioluminescence of this bacteria (Van Dyk et al., 1998). Recently, plasmid-based fusions of random *E.coli* DNA fragments to the *luxCDABE* were utilized to screen for fusions that are induced when acetolactate synthase (ALS) is inhibited by the herbicide sulfometuron methyl (SM) (Van Dyk et al., 1998). Although the goal of this study was to identify genes induced by the accumulation of α-ketoacids (caused by ALS inhibition), four new RpoS dependent genes were identified in the process: *f253a, ldcC, yciG* and *yohF*. Promoter fusions to these genes were also induced in the presence of the weak acid salicylate suggesting that acetolactate synthase inhibition causes cytoplasmic acidification. An advantage of the *lux* system is that activity of the promoter::*lux* fusions can be monitored by measuring bioluminescence in real time without cell lysis as is required for measuring β-galactosidase activity in whole cells. Furthermore, the *lux* reporter is fairly sensitive and therefore useful to detect modestly activated promoters (Van Dyk et al., 1998).

1.5.2 Members of the σ^S Regulon

Stationary phase cells are characterized by their resistance to multiple stresses. It is therefore not surprising that the RpoS regulon encompasses many genes which encode a diversity of functions (Summarized in Table1). These functions include DNA protection and repair (encoded by *katE, katG, aidB* and *xthA*), thermotolerance (*otsBA, htrE*), osmotolerance (*osmY, proP, otsBA*), glycogen synthesis (*glgS*) and virulence (*spv, csgBA*) (reviewed in (Hengge-Aronis, 1996b). While Table 1 illustrates the large size of the RpoS regulon, regulation of each gene is far more complex than a simple activation by RpoS.

First of all, some σ^s-controlled genes are induced by additional signals besides starvation or stationary phase. For example, the *hyaABCDEF* operon and the *aidB* gene are activated by anaerobiosis while *otsBA, treA and osmB* are osmotically-induced. Secondly, many genes are not entirely dependent on RpoS since they often possess additional non-RpoS dependent promoters (e.g *osmC* and *bolA*). These findings indicate that additional regulators are often involved either through binding near promoter regions, or through cascade like regulation. For example, *csiE* and *csiD* are co-regulated by RpoS and cAMP-CRP (Marschall and Hengge-Aronis 1995; Marschall, Labrousse, et al., 1998), while *osmY* (Lange, Barth et al., 1993) and *osmC* (Bouvier, Gordia et al., 1998) are co-regulated by the DNA-binding protein Lrp (leucine responsive protein). Fis (Xu and Johnson, 1995), IHF (Lange et al., 1993) and H-NS (Barth et al., 1995) have also been identified as modulators of σ^s dependent genes.

1.5.3 Recently Identified Members of the RpoS Regulon

Many RpoS-dependent genes have been identified and these have been previously summarized (L. Wei, M.Sc. Thesis, 1996). In the past two years however, several new members have been added to the RpoS regulon. Many of the genes encode proteins of unknown function and therefore more research is required before their role in the RpoS stress response is completely understood. These newly-identified genes are reviewed in the following section.

1.5.3.1 Genes involved in DNA Replication and Repair: *dnaN, recF, mutS, mutH*

The 40.6 kDa β subunit of DNA polymerase III (the major replicase) is a sliding DNA clamp responsible for tethering the polymerase to DNA and endowing it with high

processivity (reviewed in (Kelman and O'Donnell, 1995)). The gene encoding for this subunit, *dnaN* is located between *dnaA* and *recF*. The *dnaA* gene product is required for initiation of DNA replication at the bacterial chromosomal origin, *oriC* while *recF* codes for a DNA-binding protein involved in recombination, repair and resumption of replication at disrupted replication forks (Kelman and O'Donnell, 1996). Transcription of the *dnaA-dnaN-recF* operon may be initiated at any of the four promoter sites located upstream of *dnaA* or within the *dnaA* structural gene itself (Armengod et al., 1991). Using promoter::*lacZ* fusions containing different lengths of the operon, it was demonstrated that *dnaA*, *dnaN* and *recF* are transcribed primarily from a single promoter upstream of *dnaA* during exponential growth. However, as cells enter stationary phase, only *dnaN* and *recF* are co-transcribed from a *dnaN* promoter located within the *dnaA* structural gene. β-galactosidase activity from a fusion containing the *dnaN* and *recF* promoter region increased one hundred-fold while activity from the *dnaA* (*dnaA*, *dnaN*, *recF*) promoter increased only four- fold (Villarroya et al., 1998). Activity in isogenic *rpoS*$^+$ and *rpoS*$^-$ backgrounds confirmed that induction of the *dnaN* promoter was dependent on RpoS while the small induction from the *dnaA* promoter was independent of RpoS. A similar increase in activity from the *dnaN* promoter was found in cells exposed to hyperosmotic conditions. Taken together, these results indicate that RpoD directs expression from the *dnaA* promoter during exponential phase while RpoS directs transcription from the *dnaN* promoter during entry into stationary phase. This results in increased expression of *dnaN* and *recF* protein products. It is postulated that since there is more frequent stalling of DNA replication during stationary phase, a coordinate induction of *dnaN* and *recF*

(independent of *dnaA*) may be required to continue the rounds of replication already under way and thus maintain DNA integrity during periods of slow growth (Villarroya et al., 1998).

The MutS and MutH proteins are involved in several DNA repair pathways and maintenance of chromosomal stability (Modrich, 1991). The methyl-directed-mismatch (MDM) repair pathway corrects mismatched base pairs and small single-stranded loops. In the MDM pathway, MutS and MutH functions as a DNA mismatch binding protein and a d(GATC) specific endonuclease respectively (Su et al., 1988). MutS and MutH protein levels decrease approximately ten-fold in overnight cultures (Tsui et al., 1997). In an *rpoS* mutant strain, the amounts of MutS and MutH remained similar in both phases of growth, indicating that RpoS may repress expression of these proteins. It was determined that *hfq* negatively regulates *mutS* expression in exponential cells independent of RpoS, while in stationary phase, both RpoS and *hfq* are involved in the repression of *mutS* and *mutH* (Tsui et al., 1991). Though the mechanism of negative control is unclear, the authors of this study propose that the cells coordinate the amount of MutS and MutH during stationary phase.

1.5.3.2 Genes Involved in pH Homeostasis : *ldcC, hdeA, gadC*

ldcC

E. coli possesses two lysine decarboxylases which catalyze the conversion of lysine into cadaverine. The main lysine decarboxylase is encoded by *cadA* and is thought to be important for pH homeostasis (Meng and Bennett, 1992). A secondary lysine decarboxylase encoded by *ldcC* is weakly expressed but is able to replace the function of

cadA when over-expressed on a multicopy plasmid (Kikuchi, 1997). Located at 4.52 min on the *E. coli* map, *ldcC* encodes an 80 kDa protein. Comparison of amino acid sequences reveal that *ldcC* is 69.4 % homologous to *cadA* (Kikuchi et al., 1997). Despite their similarities, the genes are differentially regulated. CadA is induced under anaerobic conditions at low pH (Meng and Bennett, 1992), while *ldcC*, because of its low expression levels was presumed to be constitutively expressed (Yamamoto et al., 1997). Recently, two independent groups have reported that *ldcC* expression is in fact dependent on RpoS (Van Dyk et al., 1998; Kikuchi et al., 1998). Using Western Blot analyses, LdcC and CadA expression levels were found to increase upon entry into stationary phase of growth, however only LdcC expression is dependent on RpoS (Kikuchi et al., 1998). A study by (Van Dyk et al., 1998) identified *ldcC* as one of a series of promoter-*lux* fusions induced by cytoplasmic acidification which was eventually determined to be dependent on RpoS.

CadA maintains pH homeostasis inside the cell under mildly acidic conditions by shuttling H^+ ions into the cytosol (Meng and Bennett, 1992). Due to their similarities, it is highly plausible that RpoS induction of *ldcC* expression serves to maintain pH homeostasis during stationary phase of growth where there may be mildly acidic conditions.

hdeA and gadC* in *Shigella flexineri

Stationary phase cultures of *Shigella flexineri* have the ability to survive for several hours at pH 2.5. The ability of stationary phase cells to resist low pH has been termed "acid resistance phenotype" and requires the expression of σ^s (Small et al., 1994). To identify genes associated with acid resistance, a random Tn*phoA* and Tn*lacZ* mutagenesis

screen was performed (Waterman and Small, 1996). A total of five acid-sensitive mutants were identified to map to either *hdeA* or *gadC*. Expression studies using *lacZ* fusions demonstrated that both *hdeA* and *gadC* exhibit five to six-fold stationary phase induction which were completely abolished in an *rpoS* mutant background (Waterman and Small, 1996).

The *hdeAB* operon encodes two hydrophilic proteins of molecular mass 12 kDa and 10 kDA and have no known functions. The *hdeA* gene was previously shown to be positively regulated by σ^s along with *csgBA* which encodes for the curlin subunit protein required for fibronectin binding (Arnqvist et al., 1994). The finding that *hdeA* is important for acid resistance will likely shed new light on its function.

The *gadC* gene is located downstream from a homologue of the *gadB* gene in *E. coli*. Analysis of the *gadC* amino acid sequence suggests that it encodes a putative protein of molecular mass 55 kDa. This putative protein has significant homology to many inner membrane amino acid antiporters, especially to the CadB protein of *E. coli*. CadB is involved in the transport of cadaverine and lysine across the inner membrane and is encoded by the *cadAB* operon. Based on their homology, it is possible that *gadB* and *gadC* may be involved in maintaining pH homeostasis in a similar fashion to the *cadAB* operon in *E.coli* (Waterman and Small, 1996). In a proposed model, GadC acts as a symporter of glutamic acid, H^+ and Na^+. Inside the cell, GadB catalyzes the conversion of glutamic acid into γ-aminobutyric acid which utilizes one H^+ per reaction. The byproduct, γ-aminobutyric acid is exported out to the periplasm by GadC, thereby maintaining pH homeostasis.

1.5.3.3 Universal stress protein: *uspB*

The universal stress protein *uspB* is situated upstream of the divergently transcribed stationary phase inducible *upsA* gene located at 77 min on the *E. coli* map (Farewell et al., 1998). Though the function of this gene is unknown, the amino acid sequence indicates that *uspB* encodes a 14k-Da integral membrane protein containing two transmembrane domains. The first is at the N terminus and may be a signal peptide with a cleavage site located between amino acids 30 and 31, and the second is at the C terminus (Farewell et al., 1998). Expression studies using *uspB::lacZ* fusions demonstrate that *uspB* is induced approximately fifty-fold as cells enter stationary phase. In an otherwise isogenic *rpoS* mutant background, β-galactosidase induction is significantly reduced. Thus is in contrast to *uspA* , *uspB* requires RpoS for stationary phase induction. In addition to stationary phase induction, *uspB* expression is also induced by carbon, phosphate and nitrogen starvation as well as by osmotic and oxidative stress; albeit only two to three-fold. Overexpression of *uspB* causes cell death in stationary phase, whereas *uspB* mutants are sensitive to exposure to ethanol in stationary phase.. The sensitivity of *uspB* mutants to ethanol lead the authors of this study to speculate the role of *uspB* in stationary phase resistance to ethanol. High concentrations of ethanol causes cell lysis, presumably because of effects on the lipids of the inner and outer membranes. However, stationary phase cells are characteristically resistant to multiple stresses and may therefore be insensitive to low concentrations of ethanol that would normally lyse exponential-phase cells. This increased tolerance may be due to changes in lipid composition or peptidoglycan crosslinking. Since *uspB* mutants are sensitive to ethanol only during stationary phase, it is speculated that

uspB may play a role in sensing or mediating needed alterations in membrane composition during stationary phase of growth (Farewell et al., 1998). Interestingly, UspA, encoded upstream of *uspB* is also implicated in membrane function during stationary phase. Thus, these two universal stress proteins, while differentially regulated, may both be involved in alterations of membrane composition during stationary phase (Farewell et al., 1998).

1.5.3.4 Flavohemoglobin: *hmp*

The *E. coli* gene *hmp* encodes for the flavohemoglobin Hmp. In vertebrates, hemoglobin is essential for oxygen diffusion, but the role of globins in bacteria remains unclear. Purified Hmp can bind to oxygen and form an oxygenated complex (Poole, 1994). Expression studies using *lacZ* fusions to *hmp* demonstrated that this gene is induced two fold at the onset of stationary phase in rich medium. This activity is abolished in an *rpoS* mutant strain (Membrillo-Hernandez et al., 1997). The *hmp* gene is also induced by NO, NO_2^- and NO_3^-, and is repressed by Fnr under anaerobic growth conditions (Poole et al., 1996). Though a physiological role for Hmp has yet to be determined, these findings suggest a possible role in anaerobic metabolism of small nitrogen compounds (Membrillo-Hernandez et al., 1997).

1.5.3.5 *azurin* in *Pseudomonas aeruginosa*

The *azu* gene encodes the blue copper protein azurin, a periplasmic redox protein (Wood, 1978). Azurin expression increases during the transitions from exponential to stationary phase in aerobic cultures and is highly expressed under anaerobic conditions (Vijgenboom et al., 1997). Expression studies using *lacZ* fusions to the two *azu* promoters demonstrated that induction of both promoters are dependent on RpoS. Loss

of *rpoS* results in a 50 % reduction in activity. Azurin is regulated by ANR, an Fnr analogue in *P. aeruginosa*. Originally, Azurin was thought to be involved in dissimilatory nitrate reduction since it can donate electrons to nitrite reductase *in vitro* (Zannoni, 1989). This theory was disproved when it was demonstrated that *azu* mutant strains were able to grow as well as wild-type strains under anaerobic conditions in either the presence or absence of nitrate (Vijgenboom et al., 1997). Instead, it was observed that *azu* mutant strains are sensitive to hydrogen peroxide and the oxygen radical generator paraquat. The authors of this study postulate that the physiological role of azurin involves electron transfer during times of stress, such as during the transition into stationary phase (Vijgenboom et al., 1997).

1.5.3.6 Genes of Unknown Functions

The gene *yohF* (b2137) was reported to be RpoS dependent by two independent groups (Fang et al., 1996; Van Dyk et al., 1998). Located at 48 min on the *E. coli* map, *yohF* is induced by cytoplasmic acidification (Van Dyk et al., 1998). The predicted peptide sequence of *yohF* (b2137) suggests that it belongs to a family of oxidoreductases.

The two genes *yciG* (b1259) and *f253a* (ybhP, b0790) were identified in the previously mentioned mutagenesis screen by (Van Dyk et al., 1998). Located at 32 min on the *E. coli* map, *yciG* is downstream of the RpoS-dependent *yciE* region which suggests that they constitute a single transcript (Van Dyk et al., 1998).

The open reading frame *o186* (b4339) is located at 69 min on the *E. coli* map and encodes a putative protein of unknown function (Fang et al., 1996).

A bacterial lipoprotein encoded by *blc* was observed to be induced three-fold in late

stationary phase (24 hours) that was abolished in a *rpoS* mutant strain (Bishop et al., 1995). This putative lipoprotein is located between *ampC* and *sugE* at 94.5 min on the *E. coli* map. Although the function of this gene is unknown, the amino acid sequence suggests that it is highly homologous to the apolipoprotein gene *apoD* in eukaryotic cells. Based on this homology, the function of *blc* may involve binding hydrophobic ligands in *E. coli*.

The genes *o464* (b243), *sohB*, *sdaA* and *o169* (b3287) were identified in a genetic screen where putative promoter regions were cloned into expression vectors and tested for activity in an *rpoS*$^+$ and *rpoS*$^-$ background (Talukder et al., 1996). Located at 28.5 min, *sohB* encodes a periplasmic protein while *sdaA* encodes L-serine deaminase. In an *rpoS* mutant background, all four clones were induced up to six-fold in late stationary phase (24 hours). Thus, in a wild type background, these genes represent a small subset of the regulon repressed by RpoS (Talukder et al., 1996).

Table 1. Members of the RpoS Regulon in *E. coli* and *S. typhimurium*

Gene/ Operon	Map (min)	Gene Product	Physiological Function	Environmental Inducers	References
aidB	95.2	?	Involved in leucine metabolism Defends against DNA methylated damage	Oxygen limitation, DNA damage	(Landini et al., 1994; Volkert et al., 1994)
appY	13	Regulatory protein	Controls expression of *hya* and *cyxAB-appA*		(Atlung et al., 1994)
blc	**94.5**	**Lipoprotein**	**?**	**Late stationary phase**	**(Bishop et al., 1995)**
bolA	10	Regulatory protein	Confers round morphology, controls synthesis of PBP6		(Aldea et al., 1988; Aldea et al., 1989)
cbpA	?	DnaJ like protein	Chaperone, curved DNA-binding protein	Phosphate limitation	(Yamashino et al., 1994)
cfa	36.5	Cyclopropane fatty acid synthase	?		(Wang et al., 1994)
csgBA/csgDE	23.1	*csgBA* encodes curli subunit proteins *csgD* encodes transcriptional regulator	Production of curli fimbriae for fibronectin binding		(Arnqvist et al., 1994)
csiD	57.6	?	?	Carbon starvation inducible	(Weichart et al., 1993)
csiE	54.7	?	?	Carbon starvation inducible	(Weichart et al., 1993)
csiF (csi-32)	8.8	?	?	Carbon starvation inducbile	(Weichart et al., 1993)
cyxAB(appCE)	22.4	Hydrogenase I	Oxidation of Hydrogen	Oxygen limitation	(Atlung et al., 1994; Dassa et al., 1992)

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aidB	95.2	?	Involved in leucine metabolism Defends against DNA methylated damage	Oxygen limitation, DNA damage	(Landini et al., 1994; Volkert et al., 1994)
appY	13	Regulatory protein	Controls expression of *hya* and *cyxAB-appA*		(Atlung et al., 1994)
blc	**94.5**	**Lipoprotein**	**?**	**Late stationary phase**	**(Bishop et al., 1995)**
bolA	10	Regulatory protein	Confers round morphology, controls synthesis of PBP6		(Aldea et al., 1988; Aldea et al., 1989)
cbpA	?	DnaJ like protein	Chaperone, curved DNA-binding protein	Phosphate limitation	(Yamashino et al., 1994)
cfa	36.5	Cyclopropane fatty acid synthase	?		(Wang et al., 1994)
csgBA/csgDE	23.1	*csgBA* encodes curli subunit proteins *csgD* encodes transcriptional regulator	Production of curli fimbriae for fibronectin binding		(Arnqvist et al., 1994)
csiD	57.6	?	?	Carbon starvation inducible	(Weichart et al., 1993)
csiE	54.7	?	?	Carbon starvation inducible	(Weichart et al., 1993)
csiF (csi-32)	8.8	?	?	Carbon starvation inducbile	(Weichart et al., 1993)

Gene/ Operon	Map (min.)	Gene Product	Physiological Function	Environmental Inducers	References
hmp	**55**	**Flavohemoglobin**	**anaerobic metabolism of nitrogen ?**	**Nitrate, anaerobiosis**	**(Membrillo-Hernandez et al., 1997)**
hyaABCDEF	22.4	Hydrogenase I	Oxidation of hydrogen	anaerobiosis	(Brondsted and Atlung, 1994)
katE	37.8	Catalase HPII	Protection against H_2O_2	TCA cycle intermediates and acetate	(Sak et al., 1989; Schellhorn and Stones, 1992)
katG	89.2	Catalase HPI	Protection against H_2O_2	Oxidative stress	(Ivanova et al., 1994; Mukhopadhyay and Schellhorn, 1994)
ldcC	**4.52**	**Lysine decarboxylase**	**Synthesize cadaverine from lysine Maintains pH homeostasis ?**	**Acid stess**	**(Kikuchi et al., 1998; Van Dyk et al., 1998)**
mutS, mutH	**58.8**	***mutS*: DNA mismatch binding protein *mutH*: d(GATC) specific endonuclease**	**DNA repair (Repressed by RpoS)**		**(Tsui et al., 1997)**
mcc	plasmid	7 proteins	Synthesis and secretion of microcin C7		(Diaz-Guerra et al., 1989)
osmB	28	Outer membrane lipoprotein	Cell agregation ?	Osmotic stress	(Hengge-Aronis et al., 1991)

osmY	99.3	Periplasmic protein	?	Osmotic stress	(Lange and Hengge-Aronis, 1993)
otsBA	41.6	*otsB*: trehalose phosphate phosphatase, *otsA* : trehalose-6-phosphate synthasse	Trehalose synthesis, osmoprotection, thermotolerance	Osmotic stress, heat shock	(Kaasen et al., 1992; Hengge-Aronis et al., 1991)
poxB	18.7	Pyruvate oxidase	Synthesis of acetate		(Chang et al., 1994)
proP	93	Permease for glycine betaine and proline	Osmoprotection	Osmotic stress	(Manna et al., 1994)
proU	57.7	Glycine betaine and proline transport system	Osmoprotection		(Mellies et al., 1995)
sdaA	**?**	**L-Serine deaminase**	**? (Repressed by RpoS)**	**Late stationary phase**	**(Talukder et al., 1996)**
sohB	**28.5**	**Periplasmic protease**	**? (Repressed by RpoS)**	**Late stationary phase**	**(Talukder et al., 1996)**
spvR-ABCD	Plasmid	SpvR: regulatory protein SpvABCD: 4 polypeptides	Virulence, aids in intracellular growth		(Fang et al., 1992; Kowarz et al., 1994)
stiABC	30	?	Multiple nutrient starvation - inducible genes	Carbon, Nitrogen, Phosphorous starvation	(O'Neal et al., 1994)
treA	26	Periplasmic trehalase	Growth on trehalose in high-osmolarity medium	Osmotic stress	(Hengge-Aronis et al., 1991)
xthA	38	Exonuclease III	DNA repair, Hydrogen peroxide resistance	Near UV	(Sak et al., 1989)

uspA	77	**? Integral membrane protein**	**? May be involved in mediating alteration in membrane composition.and stationary phase resistance to ethanol**	**Carbon, Nitrogen, Phosphorous starvation. Osmotic and oxidative stress.**	**(Farewell et al., 1998)**
xylF	80	D-xylose binding protein	? (Repressed by RpoS)		
yciG	**28.3**	**?**	**?**	**Acid stress**	**(Van Dyk et al., 1998)**
yohF	**48**	**?**	**?**	**Acid stress**	**(Fang et al., 1996; Van Dyk et al., 1998)**
o169	**68**	**?**	**? (Repressed by RpoS)**		**(Talukder et al., 1996)**
o186	**69**	**?**	**?**		**(Talukder et al., 1996)**
o464	**?**	**?**	**? (Repressed by RpoS)**		**(Talukder et al., 1996)**

Adapted from Hengge-Aronis (1996b)
Genes in bold represent newly identified members of the regulon since 1996.
Unless otherwise stated, genes/operons are positively regulated by RpoS

1.6 Project Outline and Objectives

Given the importance of RpoS in stress adaptation, many methods have been employed to identify and enumerate members of the RpoS regulon. The RpoS regulon, unlike other regulons, does not have a single unifying characteristic or differentiating phenotype that all members share. These factors in addition to its large size, have delayed complete characterization of the regulon. To avoid these problems, an alternative approach was taken to identify new members of the operon. An *rpoS* null allele was introduced into a previously constructed bank of random promoter-*lacZ* fusions and tested for RpoS dependency in comparison to a wild type strain. The advantage of this method is that it does not rely on a specific phenotype (e.g carbon starvation) and should therefore reflect the true size of the regulon. One hundred and five putative RpoS-dependent fusions were identified by (Linda I-C. Wei. M.Sc. Thesis).

As a continuation of this project, the objective of this study was to determine the identity of these RpoS-dependent fusions. This involved employing a sequencing strategy (Roy et al., 1995) and streamlining a DNA isolation protocol such that large amounts of DNA could be easily isolated for sequencing. Following this, sequence analyses were performed using the BLAST sequence homology search program. The top ten RpoS dependent genes are described here.

While identifying the remainder of the *rsd* fusions, we found that three mutants mapped to *narY*, a member of the *narZYWV* operon encoding for a secondary nitrate reductase Z (NRZ). This operon was investigated further since NRZ has been reported to be constitutively expressed (Bonnefoy et al., 1987). Characterization of this operon and its proposed role in the RpoS stress response is addressed.

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Chapter 2

Preface

The following article, entitled "Identification of Conserved, RpoS-dependent Stationary Phase Genes of *Escherichia coli*", was published in *The Journal of Bacteriology* (vol. *180*, No. 23 p. 6283-6291).

L. Chang isolated the DNA of the *rsd* fusions and was involved in the editing of the manuscript.

Lily Chang B.Sc. (Hons.)
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Identification of Conserved, RpoS-Dependent Stationary-Phase Genes of *Escherichia coli*

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During entry into stationary phase, many free-living, gram-negative bacteria express genes that impart cellular resistance to environmental stresses, such as oxidative stress and osmotic stress. Many genes that are required for stationary-phase adaptation are controlled by RpoS, a conserved alternative sigma factor, whose expression is, in turn, controlled by many factors. To better understand the numbers and types of genes dependent upon RpoS, we employed a genetic screen to isolate more than 100 independent RpoS-dependent gene fusions from a bank of several thousand mutants harboring random, independent promoter-*lacZ* operon fusion mutations. Dependence on RpoS varied from 2-fold to over 100-fold. The expression of all fusion mutations was normal in an *rpoS*/*rpoS*$^+$ merodiploid (*rpoS* background transformed with an *rpoS*-containing plasmid). Surprisingly, the expression of many RpoS-dependent genes was growth phase dependent, albeit at lower levels, even in an *rpoS* background, suggesting that other growth-phase-dependent regulatory mechanisms, in addition to RpoS, may control postexponential gene expression. These results are consistent with the idea that many growth-phase-regulated functions in *Escherichia coli* do not require RpoS for expression. The identities of the 10 most highly RpoS-dependent fusions identified in this study were determined by DNA sequence analysis. Three of the mutations mapped to *otsA*, *katE*, *ecnB*, and *osmY*—genes that have been previously shown by others to be highly RpoS dependent. The six remaining highly-RpoS-dependent fusion mutations were located in other genes, namely, *gabP*, *yhiUV*, *o371*, *o381*, *f186*, and *o215*.

Like many other free-living bacteria, *Escherichia coli* lives in environments that may change rapidly with respect to both nutrients and physical conditions. To survive stresses associated with starvation, *E. coli* expresses many stationary-phase-specific genes whose expression depends largely on an alternative sigma factor, σ^S, encoded by *rpoS* (27, 30). Inactivation of this gene renders the cell sensitive to heat shock (25, 29), oxidative stress (25, 29), osmotic challenge (29), and near-UV light (40). Proteins that depend on RpoS include catalase HPII (33, 39, 42) and catalase HPI (32), exonuclease III (39), penicillin-binding proteins (15), and osmoprotective proteins (21, 22, 53). RpoS is required for virulence (17) and acid tolerance (6) in *Salmonella typhimurium*. Although the signal(s) giving rise to increased expression of RpoS itself is not completely understood, homoserine lactone (23), UDP-6-glucose (10), and weak acids, such as acetate (42), have been shown to be inducers of RpoS.

Several approaches have been used to enumerate and identify RpoS-regulated functions. Many of these genes, however, are probably still unidentified. Two-dimensional gel electrophoresis studies of proteins expressed in wild-type and *rpoS* strains have revealed that the RpoS regulon is quite large (30). Mutagenesis with random *lacZ* (16, 51) or *lux* insertions (46), coupled with screening for RpoS-related characteristic phenotypes, has also been successfully employed to identify new RpoS-regulated genes (51). However, unlike other regulons, the RpoS regulon does not have a single unifying characteristic or differentiating phenotype that all members share. These factors, in addition to its suspected large size, have delayed complete characterization of the regulon. To circumvent the problems associated with the characteristics described above, we have employed a mutant identification scheme in which an *rpoS* null allele is introduced into strains containing random promoter-*lacZ* fusions to directly identify RpoS dependency. Since this procedure does not rely on a phenotype specific for the regulon (e.g., carbon starvation), this method should be of general use in the identification of members of any regulon for which a null allele of a positive-acting regulator is available.

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MATERIALS AND METHODS

Bacterial strains, phage, and plasmid. The bacterial strains, phage, and plasmid used in this study are listed in Table 1.

Chemicals and media. All chemicals were supplied by either Fisher Scientific, Ltd. (Toronto, Ontario, Canada), Sigma Chemical Co. (St. Louis, Mo.), or Gibco BRL (Burlington, Ontario, Canada). Antibiotics and other nonautoclavable stock solutions were filter sterilized with Gelman Sciences (Ann Arbor, Mich.) Acrodisc sterile filters (pore size, 0.45 μm). Liquid and solid media were prepared as described by Miller (31). Cultures were routinely grown in Luria-Bertani (LB) rich broth. The concentrations of antibiotics used were as follows: kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; tetracycline, 15 μg/ml; and ampicillin, 100 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 50 μg/ml.

Growth conditions. All growth and survival assays were performed with GC4468 derivatives. Cultures were grown overnight in LB medium containing the appropriate antibiotics. Cell growth was monitored spectrophotometrically (UV-VIS spectrophotometer, model UV-1201; Shimadzu Corporation, Kyoto, Japan) by optical density at 600 nm (OD_{600}). For expression studies, bacterial cultures were maintained in the early exponential phase (OD_{600} of <0.2) for at least 8 generations prior to the start of the experiment. Cultures were grown in flasks at 37°C at 200 rpm, sampled, and assayed for β-galactosidase activity at the times indicated.

For survival assays, bacterial cultures were incubated for 10 days in LB broth at 37°C in sealed microtiter plates (to minimize evaporation). Following this period, cultures were diluted in M9 salts buffer, plated on LB medium, and enumerated after 24 h of incubation.

Enzyme assays. β-Galactosidase activity was assayed as described by Miller (31). Units of activity were calculated as $[1{,}000 \times OD_{420}]/[\text{time of incubation (min)} \times \text{volume (ml)} \times OD_{600}]$ and were expressed as Miller units. Catalase activity was measured spectrophotometrically by monitoring hydrogen peroxide decomposition at OD_{240} as described previously (42).

Genetic methods. The phenotypic screen for RpoS dependence is based on the observation that introduction, by transduction, of *rpoS*::Tn*10* into a strain containing a *katE*::*lacZ* fusion abolishes β-galactosidase activity (41). Since conju-

TABLE 1. *E. coli* strains, plasmid, and bacteriophage used in this study

Strain, plasmid, or bacteriophage	Genotype	Source or reference
Strains		
MC4100	*Δ(argF-lacZ)205 araD139 flbB5301 relA1 rpsL150 thi ptsF25*	42
GC4468	*ΔlacU169 rpsL*	42
KL16	Hfr (PO45) *relA1 spoT1 thi-1*	K. B. Low
NC4468	As GC4468, but Φ(*katE::lacZ*$^+$)131	41
NC122	As NC4468, but *rpoS13*::Tn*10*	41
HS180	Like KL16, but *rpoS13*::Tn*10*	P1$_{vir}$ (NC122) × KL16→Tet^r
GC122	As GC4468, but Φ(*rpoS*13::Tn*10*)	41
GC202	As GC4468, but Φ(*katG*::Tn*10*)	42
HS1001–HS1105	As GC4468, but carrying *RpoS d*ependent promoter-*lacZ* fusions	This study
HS1001T–HS1105T	As HS1001-HS1105, but *rpoS*13::Tn*10*	This study
13C10	As GC4468, but carrying a growth-phase-dependent, RpoS-independent promoter-*lacZ* fusion	This study
Plasmid; pMM*katF3*	Carries *rpoS* (*katF*) gene	33
Bacteriophage; P1$_{vir}$	Generalized transducing phage	Laboratory collection

gation is much more efficient and amenable to large numbers of transfers than P1-mediated transduction, we reasoned that the use of an appropriate Hfr donor containing a null mutation in the *rpoS* gene close to the point of origin could facilitate simultaneous testing of several thousand colonies for dependence on RpoS. Since the point of origin of transfer in the Hfr strain KL16 is located at 64 min and DNA is transferred during mating in a counterclockwise direction, *rpoS*, located at 62 min, should be transferred shortly after initiation of conjugation. The *rpoS13*::Tn*10* mutation was introduced into Hfr KL16 from strain NC122 (*rpoS13*::Tn*10*; *katE*::*lacZ*) by P1-mediated transduction. Transductants were selected on media supplemented with tetracycline (to select against the recipient) and streptomycin (to select against the donor). Transductants were flooded with 30% hydrogen peroxide to confirm transfer of the *rpoS13*::Tn*10* mutation (*E. coli* colonies normally evolve gas bubbles when flooded with hydrogen peroxide because of the activity of catalase HPII, the catalase encoded by *katE*). This Hfr donor was confirmed to be HPII negative and was designated HS180. The Hfr transfer capability of HS180 was tested with the control strains NC4468 (*katE*::*lacZ*) and MC4100. All transconjugants exhibited reduced catalase levels, and as expected, transconjugants produced by mating HS180 with NC4468 also exhibited reduced β-galactosidase activity.

Plasmid transformations were performed with the TSS (transformation and storage solution) method of transforming recipient cells (13). To select for transformation of the pMM*katF3* plasmid, LB agar plates containing kanamycin, streptomycin, and ampicillin were used. Transformed *rpoS* transconjugants were selected on the same medium supplemented with tetracycline.

Identification of RpoS-dependent fusions. To isolate promoter-*lacZ* fusions that depend on RpoS, we used a previously constructed collection of 5,000 independent transcriptional *lacZ*$^+$ mutants as F$^-$ recipients (42). These LacZ$^+$ mutants, in an MC4100 background, harbor randomly inserted λ*plac*Mu53 phage that also confer kanamycin resistance (12). The donor strain (HS180) was grown to the exponential phase (OD$_{600}$ = 0.3) in LB broth, and 200-μl samples of culture were placed into microtiter plate wells. Recipient strains were grown to saturation in microtiter plate wells containing 200 μl of LB broth. Aliquots (20 μl) of strain HS180 were mated with each F$^-$ recipient directly in microtiter wells for 30 min and replica plated onto selective plates containing streptomycin, kanamycin, tetracycline, and X-Gal. Transfer of the *rpoS* allele was confirmed by testing the resulting transconjugants for catalase activity (see above). Putative RpoS-dependent (*rsd*) fusions were identified by comparing the levels of β-galactosidase activity of the fusions in *rpoS*$^+$ and *rpoS* strains on LB plates containing X-Gal. Recipients were then purified, and one clone from each was tested for RpoS dependency. Complementation tests were done by transforming each transconjugant with pMM*katF3*, a plasmid containing the *rpoS* gene (33). To ensure that strains contained single-copy chromosomal *lacZ* insertions, the fusions were transduced into GC4468 and retested for RpoS dependence.

Induction of λ lysogens. Because the phage used to generate the mutant bank was a lambda derivative (12), bacterial DNA proximal to the introduced promoter-*lacZ* mutation can be isolated by UV induction of the lambdoid prophage from the bacterial mutants (38). A single clone was inoculated into LB medium containing streptomycin and kanamycin and grown overnight at 37°C. Cells were subcultured into 50 ml of fresh medium (1/10) the next morning, grown to an OD$_{600}$ of 0.4, centrifuged, and resuspended in 10 ml of 10 mM $MgSO_4$. Induction of the lambdoid prophage was performed by irradiating the culture at 25 mW for 7 s (approximately 35 J/m^2). Five milliliters of 3xLL (38) medium was added, and the irradiated culture was shaken vigorously in a petri plate until lysis was observed (3 to 5 h). The lysate was transferred to a 30-ml glass Corex tube. Chloroform was added, the phage lysate was mixed vigorously, and cell debris was removed by centrifugation at 10,000 × *g* for 20 min. DNase (10-μg/ml final concentration) was added to remove traces of chromosomal DNA.

Sequencing of bacterial DNA proximal to the λ fusion junction. Preparation and sequencing of DNA from UV-induced lysates were performed as previously described (38) with a 25-mer primer (5'-CCCGAATAATCCAATGTCCTCCC GG-3') located 30 nucleotides from the Mu c end boundary. DNA sequencing was performed by the MOBIX central facility at McMaster University. The amount of DNA used in each sequencing reaction was approximately 0.5 to 1.0 μg. Sequences were compared to those in the GenBank database by using the BLASTN alignment algorithm (1). Bacterial homologs of identified *E. coli* RpoS-dependent genes were determined by using the gapped BLASTX alignment algorithim (2).

RNA extraction and Northern blot analysis. Cultures were grown as described above, and aliquots were removed from exponential- and stationary-phase cultures. RNA was extracted with an RNeasy Midi Kit (Qiagen, Inc., Valencia, Calif.). Northern analysis was performed with equal amounts of RNA from the different samples by standard methods (43). Total RNA was blotted onto BIO-TRANS nylon membranes (ICN, Montreal, PQ, Canada) as described in reference 43 and fixed by baking at 80°C for 2 h. Prehybridization and hybridization were performed at 42°C with gentle agitation. When necessary, blots were stripped by boiling in 0.1 to 0.5% sodium dodecyl sulfate according to the manufacturer's (ICN) instructions for reprobing.

Oligonucleotide primers were synthesized and used in PCRs to generate specific probes to five identified *rsd* genes and to a control non-*rsd* gene control (*rrnA*): *katE*, forward (with respect to the open reading frame [ORF]), 5'-CAAAGCG GATTTCCTCTCAGATC-3', and reverse, 5'-TGTCAAATGGCGTCTGACTT AG-3'; *osmY*, forward, 5'-CTGCTGGCTGTAATGTTGACCTC-3', and reverse, 5'-CATCTACCGCTTTGGCGATACTT-3'; *gabD*, forward, 5'-GAAAGGCGA AATCAGCTACGC-3', and reverse, 5'-CTTCGATGCCATACTTCGAACCT-3'; *gabP*, forward, 5'-CCATCTGGTTATTTTCCCTCG-3', and reverse, 5'-GG TAATAAAGCCGATGACTAGCCAG-3'; and *rrnA*, forward, 5'-GTGCCCAG ATGGGATTAGCTAGTAG-3', and reverse, 5'-GTCGAGTTGCAGACTCCA ATCC-3'. Each PCR tube contained 1× PCR buffer (500 mM KCl, 200 mM Tris [pH 8.4]), 50 pmol of each of the forward and reverse primers, 0.4 mM each of the four deoxynucleoside triphosphates, 4 mM $MgCl_2$, ~50 ng of *E. coli* DNA, and ~10 U of *Taq* polymerase in a final volume of 50 μl. Reactions were run for 25 cycles under the following conditions: (i) 96°C for 30 s, (ii) 61°C for 60 s; and (iii) 72°C for 90 s. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Inc.) and radiolabelled with [α-^{32}P]dCTP (NEN Life Science Products, Inc., Boston, Mass.) by random priming. The identity of all PCR products was confirmed by DNA sequencing.

RESULTS

Isolation of σ^S-dependent fusion mutants. A diagrammatic representation of the screening procedure for the isolation of σ^S-dependent promoter-*lacZ* fusions is shown in Fig. 1. Putative σ^S-dependent fusions were identified by comparing the level of β-galactosidase activity of wild-type (with respect to *rpoS*) recipients to that of *rpoS*::Tn*10* transconjugants on LB plates containing X-Gal. From this screen of 5,000 mutants, 105 *rpoS*::Tn*10* transconjugants were identified that exhibited reduced β-galactosidase activity compared with that of the wild-type recipients. Putative RpoS-dependent (*rsd*) transcriptional fusions were transduced into GC4468 and retested for σ^S

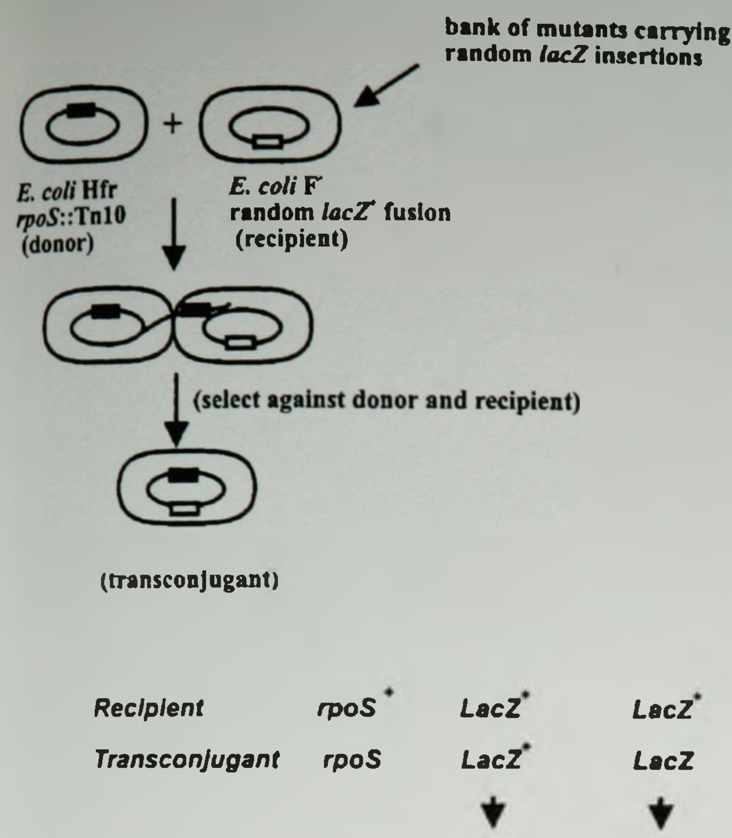


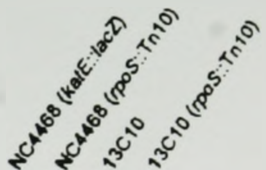
FIG. 1. Schematic representation of the strategy used to identify transconjugants harboring RpoS (σ^s)-dependent promoter-*lacZ* fusions.

dependency. A σ^s-dependent *katE*::*lacZ* fusion strain, NC4468, served as a positive control, and a strain carrying a σ^s-independent fusion, 13C10, was used as a negative control in subsequent mating procedures. The *lacZ* expression of all 105 transductants was RpoS dependent (Fig. 2).

To confirm that the lower β-galactosidase activity of the transconjugants was due to introduction of the *rpoS* null mutation and was not the result of a secondary mutation, transconjugants were transformed with pMM*katF3* containing a wild-type *rpoS* gene. Recipients were transformed in parallel, serving as controls for any variation in β-galactosidase levels due to the presence of the vector. In many cases, the transformed wild-type and *rpoS* strains exhibited higher levels of β-galactosidase activity than the nontransformed derivatives. This may be due to the increased levels of *rpoS* expression on multicopy plasmids, an observation reported by other investigators (39). As expected, all 105 mutants were efficiently complemented when transformed with plasmid-borne *rpoS* (data not shown).

Growth-phase expression of *rsd-lacZ* fusions. Many known RpoS-regulated genes are expressed at relatively low levels in the exponential phase but are induced as cells enter the stationary phase in rich medium (for review, see reference 19). We tested growth-phase induction of the *rsd* promoter-*lacZ* mutations isolated in this study. As expected, all fusions were maximally expressed in the early stationary phase or in 24-h cultures (Fig. 3). In each case, induction began before the cultures reached an OD_{600} of 0.3, suggesting that the signal(s) required for induction of these genes, whatever its nature, is present in early-exponential-phase cultures. We further examined growth-phase dependence in the other 95 fusion mutants and found that in each case, induction was initiated in the early exponential phase. We have previously observed that transcriptional induction of a single-copy *rpoS*::*lacZ* fusion occurs in the early exponential phase (42).

Identification of *rsd-lacZ* fusion junctions. λ DNA was prepared from induced lysogens as described in Materials and Methods. The Mu c end vector sequence (5'-AATACA-3') was confirmed for all sequences, and the determined DNA sequence proximal to the fusion junction was compared to published *E. coli* sequences. We have identified 50 of the 105 RpoS-dependent fusions isolated in this study by sequencing DNA prepared from UV-induced phage lysates as previously described (38). It is well established that the promoters of many RpoS-dependent genes can also be recognized by RpoD (45), the main vegetative sigma factor of *E. coli*. Since we were primarily interested in identifying genes that specifically require RpoS for expression (as opposed to those genes having promoters that can be recognized by both RpoS and RpoD [45]), we initially characterized the 10 fusions which exhibited the highest degree of RpoS dependence. Three of the 10 most-highly-RpoS-dependent mutations mapped to genes previously shown to be RpoS regulated, including *katE* (*rsd1014*), the structural gene for HPII catalase; *otsA* (*rsd1098*), which en-



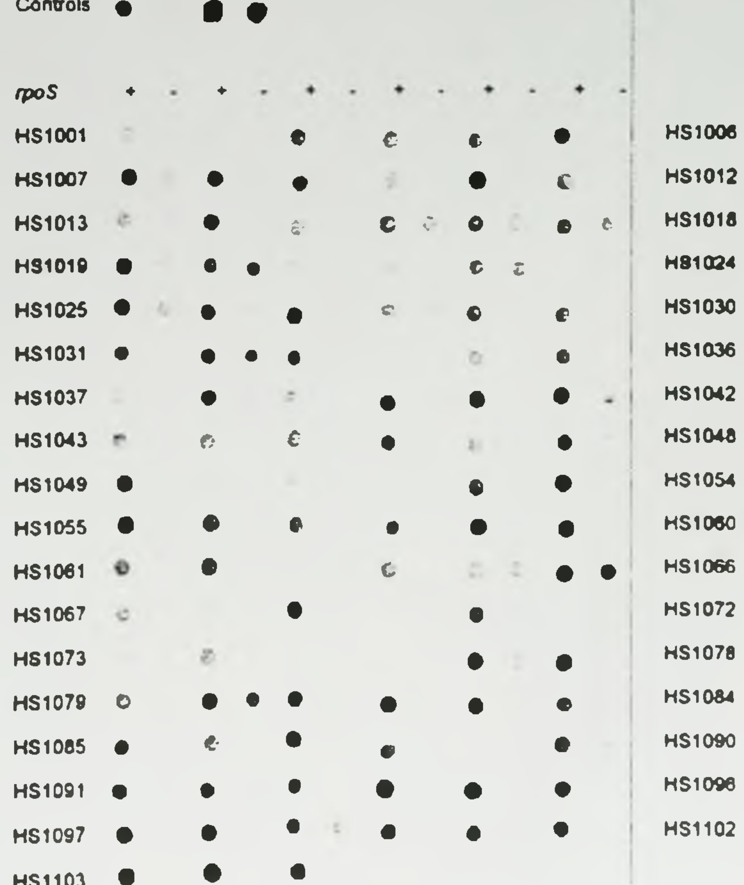


FIG. 2. The 105 recipient (*rpoS*⁺) and transconjugant (*rpoS*) pairs in a GC4468 background. Strains were plated on M9 minimal media supplemented with 0.4% glucose. The σ^s-dependent and -independent control strains NC4468 (*katE*::*lacZ*) and 13C10, respectively, were placed in the top row, with *rpoS* derivatives placed adjacent to them. *rpoS*⁺ and *rpoS* derivative pairs are adjacent to one another in rows, starting from the top left. The *rpoS* status of each column is shown on the top (+, *rpoS*⁺; −, *rpoS*).

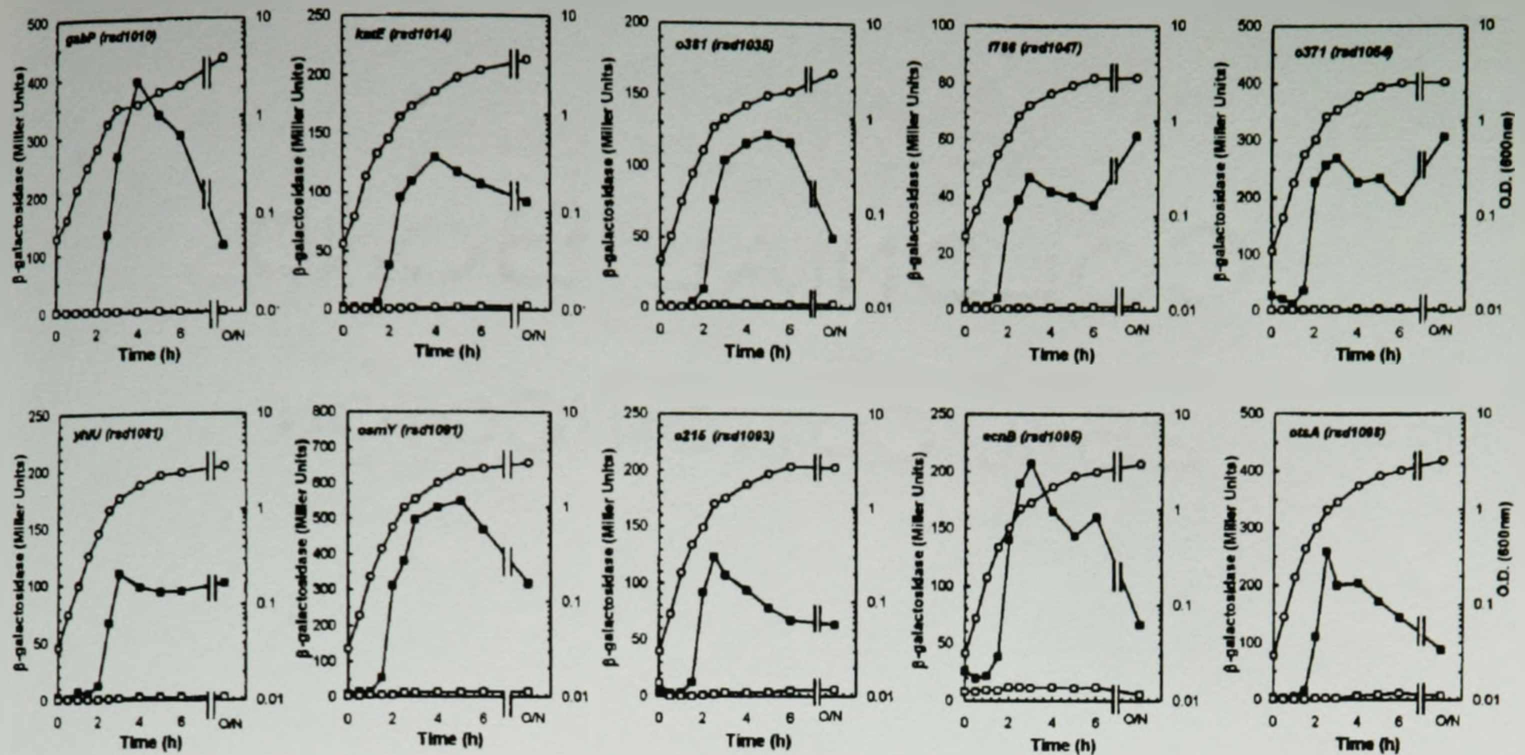


FIG. 3. Growth-phase-dependent expression of 10 highly-RpoS-dependent fusions in rich medium. Flasks containing LB broth were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. Each panel shows the growth of the culture and the β-galactosidase activity in strains carrying promoter-*lacZ* fusions to the indicated gene. The levels of growth of the wild-type strain and *rpoS* derivatives were equivalent, and thus only growth data for the wild-type strain are shown. ○, growth (OD_{600}); ■, β-galactosidase activity in the wild-type strain; □, β-galactosidase activity in the *rpoS* derivative.

codes trehalose synthase (21); and *osmY* (*rsd1091*), a probable lipoprotein of unknown function (54). The other seven mutations mapped to genes not previously known to require RpoS for expression.

The *rsd1010* mutation is located in the terminal member of the *gab* operon (35), *gabP*, encoding a γ-aminobutyric acid (GABA) permease that can also transport other amine compounds (11). Two other mutations, *rsd1057* and *rsd1058*, are in the first member of the operon, *gabD* (Fig. 4), which encodes a succinate semialdehyde dehydrogenase. The two *gabD* fusions were found to be slightly less RpoS dependent than *rsd1010-lacZ*, which may be due to a repetitive (REP) element that lies between *gabT* and *gabP*. This is consistent with the suggestion that these short DNA sequences may have a role in attenuating gene expression (5).

Strain HS1035 contains a fusion in *o381*, encoding a protein that is homologous to PotF, a periplasmic putrescine-binding protein (37). There are two known polyamine transport systems in *E. coli*. The *potABCD* and *potFGHI* operons are involved in transport of putrescine and spermidine, respectively (37). Interestingly, the ORFs downstream of *o381* (*o337*, *o313*, and *o264*) (Fig. 4) are homologous to the corresponding members of the *potABCD* and *potFGHI* operons, suggesting that *o381* is part of a third, conserved polyamine transport operon.

The *rsd1047-lacZ* mutation (strain HS1047) is located in *f786*, a gene of unknown function that is conserved in other bacteria matching hypothetical membrane proteins from *Synechocystis* sp. and *S. typhimurium*. This gene is immediately downstream of *dps* and the glutamine uptake operon, *glnHPQ* (Fig. 4), which are also induced in stationary-phase cultures. Combined with the *dps* gene (3) and the *glnHPQ* operon (52), these genes, *dps-glnHPQ-f786*, may constitute a large stationary-phase-specific operon.

The *rsd1076* mutation mapped to *o371*, a reading frame of unknown function, that is homologous to glucose dehydrogenase B from *Acinetobacter* sp. (14).

The *rsd1095* mutation mapped to the newly described *ecnB* locus, a gene coding for a bacteriolytic protein that may play a role in the selective elimination of moribund cells in stationary-phase populations (7) (Fig. 4). The expression of this gene is now known to be RpoS dependent (7). The *ecnB* gene was originally described as part of a longer gene, *sugEL*, a suppressor of GroEL chaperone function in *E. coli* (44). The *sugEL* gene was reported to have two promoters, one of which is induced in stationary-phase cells (44). An adjacent divergently transcribed reading frame encodes the Bcl lipoprotein, whose expression is also RpoS regulated (8).

One mutation (*rsd1081*) mapped to *yhiU* (Fig. 4), the first member of an operon encoding a probable two-member drug efflux pump that is homologous to AcrAB and EnvCD. A second fusion (*rsd1077*) mapped to *yhiV*, previously shown to be RpoS dependent (4). These membrane-bound complexes coordinate the energy-dependent transport of a wide variety of noxious compounds (for review, see reference 36).

Several of the other identified fusions were located in known RpoS-dependent genes, including *rsd1004*, which mapped to *ldcC*, encoding a lysine decarboxylase of *E. coli* (45, 49), and *rsd1082*, which mapped to *aidB* (49). The remaining fusions mapped to genes whose regulation was not previously known to be controlled by RpoS and will be reported elsewhere.

Expression of highly-RpoS-dependent genes in rich and minimal media. The expression of highly-RpoS-dependent fusions was examined in rich (Table 2) and in minimal (Table 3) media. The growth of the *rpoS* derivatives tested was similar to that of the *rpoS*$^+$ strains in both rich and minimal media. As expected, the expression of all promoter fusions was dependent on RpoS in the stationary phase in rich media. In the exponential phase, the expression of most fusions was severalfold higher in a wild-type strain than in an *rpoS* strain, suggesting that RpoS may be important for the expression of these genes in exponentially growing cells. This was true of cells growing in minimal medium (Table 3) as well as those grown in

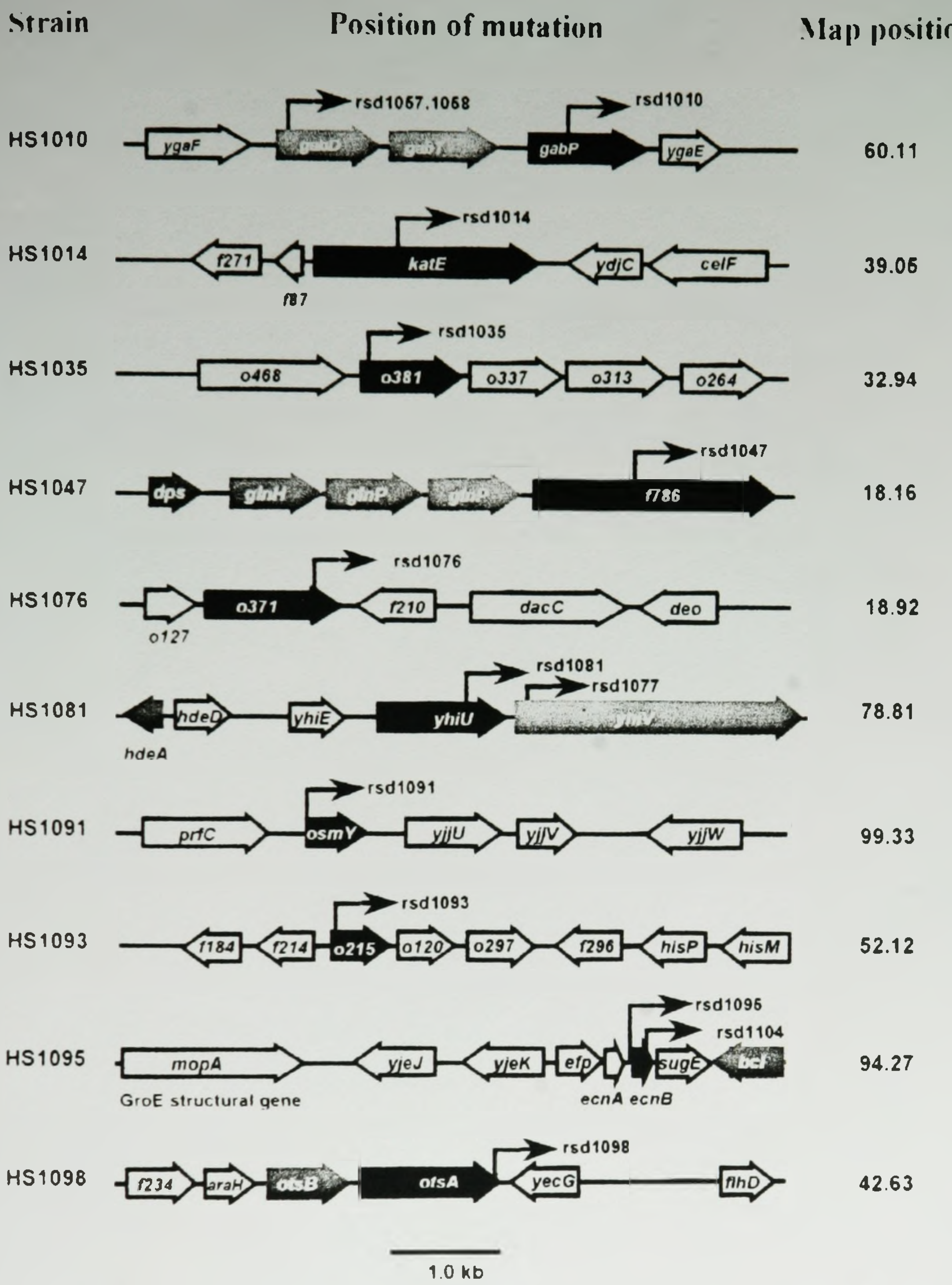


FIG. 4. Location of points of insertion of transcriptional fusions in RpoS-regulated genes identified in this study. Arrows indicate the direction of transcription of genes. ■, highly RpoS-dependent genes carrying the promoter-*lacZ* fusions identified in this study; ▨, other RpoS-dependent genes (see text); □, genes not known to require RpoS for expression.

rich medium (Table 2). The expression of *rsd1095* (*ecnB*) was moderately growth-phase dependent, even in an *rpoS* background, as previously shown (18).

Levels of mRNA of identified genes expressed in the exponential and stationary phases of growth. To confirm that the identified promoter-*lacZ* fusions accurately represented RpoS-dependent gene control in a wild-type strain, RNA was prepared from wild-type (GC4468) and *rpoS* (GC122) strains sampled in the exponential (OD_{600} = 0.2) and stationary (OD_{600} = 1.6) phases and hybridized to PCR-amplified probes specific for *gabP*, *osmY*, and *katE* (Fig. 5). *katE* and *osmY*, two well-characterized RpoS-dependent genes, were highly expressed in the stationary phase in the wild-type strain, but they were expressed at low levels in the exponential phase and were largely absent in the *rpoS* strain (Fig. 5). Similarly, the expression of *gabP* was also growth phase dependent only in the wild-type strain and was expressed at low levels in an *rpoS* strain (Fig. 5). Since *gabP* is part of an operon that includes another identified *rsd* gene, *gabD* (Fig. 4), we also performed Northern analysis with a probe specific to *gabD*. As expected, the expression pattern of this gene was similar to that of *gabP* (data not shown). The expression of the other identified highly-RpoS-dependent genes was also confirmed, by Northern analyses, to be growth phase and RpoS dependent (data not shown).

RpoS-independent, growth-phase-dependent gene expression. Surprisingly, many of the fusions were found to be growth-phase dependent even in an *rpoS* background. For ex-

TABLE 2. Growth-phase-dependent expression of highly-RpoS-dependent promoter-*lacZ* fusions in strains grown in rich medium[a]

Mutation	Identified gene	β-Galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
		Wild type	*rpoS13*::Tn*10*	RpoS dependence	Wild type	*rpoS13*::Tn*10*	RpoS dependence
rsd1010	*gabP*	6.3 ± 0.7	0.4 ± 0.1	15	116.7 ± 8.8	1.3 ± 0.0	92.0
rsd1014	*katE*	1.6 ± 0.0	0.4 ± 0.0	3.6	60.0 ± 2.0	1.2 ± 0.0	52.0
rsd1035	*o381*	6.1 ± 0.6	0.7 ± 0.0	8.2	52.6 ± 7.7	1.6 ± 0.1	33.7
rsd1047	*f786*	2.3 ± 0.1	0.3 ± 0.0	8.6	40.1 ± 1.6	0.8 ± 0.0	52.4
rsd1076	*o371*	11.0 ± 0.8	1.5 ± 0.3	7.5	137.4 ± 3.9	2.7 ± 0.1	50.9
rsd1081	*yhiU*	6.3 ± 0.5	1.0 ± 0.2	6.2	108.0 ± 0.3	2.1 ± 0.4	50.7
rsd1091	*osmY*	8.6 ± 0.9	3.0 ± 0.2	2.9	408.8 ± 27.8	6.0 ± 0.4	67.6
rsd1093	*o215*	4.5 ± 0.1	0.4 ± 0.2	10.4	60.4 ± 1.3	1.3 ± 0.3	45.0
rsd1095	*ecnB*	11.3 ± 0.9	3.1 ± 1.0	3.8	152.8 ± 12.2	7.5 ± 0.1	20.4
rsd1098	*otsA*	10.7 ± 0.4	1.2 ± 0.3	9.1	158.3 ± 8.3	1.4 ± 0.1	115.3

[a] Strains were grown overnight in LB broth, subcultured, and maintained in the exponential phase for 8 generations prior to sampling. Cultures were sampled at the exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.6).

ample, the expression of *rsd1004*, which mapped to *ldcC*, was strongly growth-phase dependent in both *rpoS*[+] and *rpoS* backgrounds (Fig. 6A). Of the 105 RpoS-dependent fusions isolated, 15 strains exhibited greater than fivefold induction of β-galactosidase as cells entered the stationary phase, suggesting that regulation by factors other than RpoS may be important in control of growth-phase-dependent gene expression. If this is true, then the expression of many of the other fusions in the mutant bank that were determined to be RpoS independent in the initial screening should exhibit growth-phase dependence even in an *rpoS* background. We found this to be the case for many mutants selected at random from our bank of transcriptional mutants. Figure 6B shows one such example of an RpoS-independent, growth-phase-dependent promoter. We then examined expression of 49 RpoS-independent fusions in both the exponential and stationary phases. Eight of the 49 fusions exhibited greater than fivefold induction, a proportion that does not differ significantly ($\chi_{1,1}$ = 0.609, ρ = 0.43) from the proportion of RpoS-dependent fusions that showed a similar degree of growth-phase induction in *rpoS* derivatives (Table 4). Taken in toto, these results suggest that a large number of nonessential genes of *E. coli* do not require RpoS for elevated stationary-phase expression and raise the intriguing possibility that RpoS-dependent genes may constitute only a small fraction of stationary-phase genes in *E. coli*.

Stationary-phase survival of *rsd* mutants. To test whether deficiency in any of the 10 identified highly-RpoS-dependent functions would impair stationary-phase survival, cultures were grown to saturation in LB broth and incubated for 10 days at 37°C. All of the mutants exhibited a 10-fold reduction in viability, about the same as that of the wild-type strain (GC4468), while the survival of an *rpoS* strain, GC122, was approximately 0.1% during this time period, consistent with results obtained by others (55).

DISCUSSION

In this paper, we describe a method for identifying members of a gene regulon by comparing expression of *lacZ* fusions in transconjugants containing a null allele of the putative regulator to that of an isogenic strain carrying the wild-type allele. We have found that this method can be used to reproducibly detect differences in expression between wild-type and *rpoS* strains that are as low as twofold. We employed this method in the study of the RpoS-controlled stationary-phase response. The probable large size of this regulon, its dependence on a single regulator, RpoS, and the fact that many of the members of this regulon may yet be undiscovered render the study of the RpoS regulon highly amenable to this type of analysis. In theory, this conjugation protocol could, however, be used to

TABLE 3. Growth-phase-dependent expression of highly-RpoS-dependent promoter-*lacZ* fusions in strains grown in glucose minimal medium[a]

Mutation	Identified gene	β-Galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
		Wild type	*rpoS13*::Tn*10*	RpoS dependence	Wild type	*rpoS13*::Tn*10*	RpoS dependence
rsd1010	*gabP*	8.5 ± 0.2	0.9 ± 0.1	9.0	43.0 ± 9.0	1.4 ± 0.0	30.5
rsd1014	*katE*	7.9 ± 1.4	0.5 ± 0.1	15.4	53.8 ± 6.8	1.3 ± 0.1	42.7
rsd1035	*o381*	14.4 ± 0.9	0.6 ± 0.0	23.2	66.4 ± 3.4	0.9 ± 0.1	74.2
rsd1047	*f786*	5.1 ± 1.1	0.5 ± 0.1	10.6	38.3 ± 3.2	0.6 ± 0.1	63.0
rsd1076	*o371*	9.8 ± 0.7	1.0 ± 0.1	9.6	72.2 ± 7.4	1.9 ± 0.1	40.1
rsd1081	*yhiU*	13.2 ± 1.2	1.6 ± 0.2	8.1	53.1 ± 0.3	3.0 ± 0.4	17.4
rsd1091	*osmY*	12.8 ± 0.1	2.1 ± 0.2	6.2	107.9 ± 7.8	2.3 ± 0.1	46.3
rsd1093	*o215*	13.6 ± 1.0	0.7 ± 0.1	19.2	79.7 ± 15.5	0.9 ± 0.1	86.4
rsd1095	*ecnB*	5.9 ± 0.3	2.0 ± 0.1	2.9	95.6 ± 7.5	4.4 ± 0.2	21.8
rsd1098	*otsA*	10.1 ± 1.6	2.0 ± 0.1	4.8	98.2 ± 3.6	2.9 ± 0.2	34.2

[a] Strains were grown overnight in minimal medium, subcultured, and maintained for 8 generations in the exponential phase prior to sampling. Cultures were sampled in the exponential phase (OD_{600} = 0.2) and stationary phase (OD_{600} = 0.9).

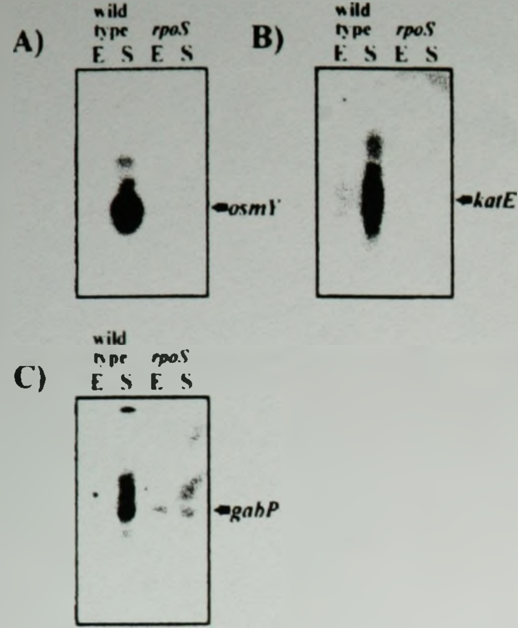


FIG. 5. RpoS- and growth-phase-dependent expression of *rsd* genes. The results of Northern analyses of total RNA isolated from exponential-phase (E) and stationary-phase (S) cultures of wild-type (GC4468) and *rpoS* (GC122) strains are shown. RNA was hybridized with probes specific for *osmY*, *katE*, and *gabP*. To confirm that equivalent amounts of RNA were extracted and loaded, control blots were probed with *rrnA*, an RpoS-independent gene (data not shown).

TABLE 4. Proportion of *E. coli* mutants whose expression is RpoS and growth-phase dependent[a]

Fusion type	No. of mutants with expression:		
	Growth-phase dependent	Growth-phase independent	Total
RpoS dependent	15	90	105
RpoS independent	8	41	49

[a] Wild-type and *rpoS* derivatives of *rsd* strains (105 total) and a randomly selected group of mutants carrying promoter-*lacZ* fusions whose expression is not RpoS dependent (49 total) were grown in LB broth and assayed in the exponential and stationary phases for β-galactosidase activity. A given promoter-*lacZ* mutation was classified as growth-phase dependent if the stationary-phase level of β-galactosidase, in an *rpoS* strain, was more than five times greater than that observed in the exponential-phase cultures.

characterize any regulon for which null-selectable alleles in a single controlling regulator exist.

The number of RpoS-regulated proteins identified has increased markedly over the past few years. Currently, more than 40 genes are known to be regulated by RpoS (for review, see reference 20). Results of in vitro transcription assays (34) indicate that many RpoS (σ^s) promoters are also recognized by RpoD (σ^{70}) (45). Although we do not yet know which promoter determinants contribute to the specificity of RpoS recognition of the fusions identified in this study, many of the fusions identified are highly dependent on RpoS for expression. As such, they should be useful in the identification of factors important in regulation by this alternative sigma factor.

A total of 105 transcriptional fusion mutants were identified in this study that are RpoS dependent. Based on our previous work isolating catalase mutants from this bank (42), we estimate there is probably a twofold redundancy in the number of isolated RpoS-dependent genes. The early-exponential-phase induction of all of the fusions identified was somewhat surprising, since RpoS, subject to complex controls at the transcriptional, translational, and posttranslational levels, is fully active only in the early stationary phase (28). However, expression of *rpoS* is induced at the transcriptional level early in the exponential phase at an OD_{600} of 0.2 (42), consistent with the idea that this is the earliest point at which induction of RpoS-dependent functions can occur. Although maximal expression was usually observed in the stationary phase, the expression of all promoter fusions isolated in this study began at an OD_{600} of 0.3, suggesting that concerted expression of RpoS-regulated genes begins well before the commencement of the stationary phase. This pattern would be consistent with the idea that adaptive proteins required for survival during periods of nutrient deprivation must be produced while the cell is capable of robust gene expression. Other identified RpoS-regulated genes exhibit a similar pattern of expression. For example, induction of *dnaN* begins in the exponential phase but is maximally expressed in stationary-phase cultures (48). Similar patterns of expression have been observed for *bolA* (9, 23), another highly-RpoS-dependent gene.

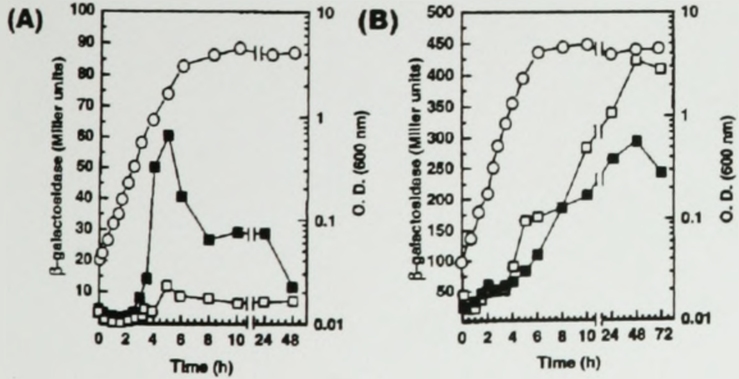


FIG. 6. Growth-phase-dependent expression of an RpoS-dependent fusion and an RpoS-independent fusion. Flasks containing LB broth were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. Each panel shows growth of the culture and β-galactosidase activity in strains carrying promoter-*lacZ* fusions to the indicated gene. The levels of growth of the wild-type strain and *rpoS* derivatives were equivalent, and thus only growth data for the wild-type strain are shown. (A) *rsd1004* (*ldcC*) (RpoS dependent). (B) 13C10 (RpoS independent). ○, growth (OD_{600}); ■, β-galactosidase activity in the *rpoS*⁺ strain; □, β-galactosidase activity in the *rpoS* derivative.

The fact that several of the genes identified were not previously known to be regulated by RpoS may be explained by several factors. First, the gene product may be masked by another compensatory functional activity with the cell. For example, the physiological function of the *ecnB*/*sugE* gene product is probably masked in cells that produce GroEL, the major chaperonin in *E. coli*. A second possible explanation is that some proteins are expressed at levels too low to measure—LdcC, a second lysine decarboxylase in *E. coli*, is detectable only when expressed on a multicopy plasmid (24). Finally, the gene of interest may be one of the many ORFs (currently more than half of all ORFs) in *E. coli* that have not been assigned any function and thus not been previously studied (e.g., *o371*).

The fact that a large proportion of fusions in the mutant bank were found to be growth-phase regulated (both RpoS dependent and RpoS independent) cannot readily be explained by current models of growth-phase regulation. We estimate that almost 20% of the mutants in the bank (~1,000) carry growth-phase-inducible fusions, a relatively small fraction (105/1,000 [~10%]) of which are RpoS dependent. This suggests that a large proportion of the bacterium's genetic repertoire is involved in adaptation to nutrient deprivation or to some other growth-phase-related stimulus. The non-RpoS-

dependent component of this response has thus far received little attention, but its characterization is undoubtedly critical in understanding how bacteria adapt to suboptimal conditions. There are probably other transcriptional factors besides RpoS that lead to increased expression of certain genes during the stationary phase. Transcriptional control of "gearbox" promoters (9, 47) is tightly coupled to growth rate, and one of these promoters is known to be RpoS independent (9, 26). Additional sequence analysis and primer extension studies are required to determine if the promoters of the *rsd-lacZ* fusions are homologous to the proposed gearbox consensus promoter sequence (47). Factors affecting posttranslational stationary-phase expression have been described and include alterations in ribosome assembly (50) and differential protein degradation (28). These are, however, unlikely to be involved in the regulation of the fusions isolated in this study, since the mutagen employed (λp*lac*Mu) generates transcriptional promoter fusions (12).

The characterization of other fusion mutations isolated in this study should aid in the identification of genes that are expressed specifically in the stationary phase and may provide additional clues regarding the regulation and physiological function of the RpoS regulon.

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CHAPTER 3

Preface

The following article, entitled "Expression of the *Escherichia coli* NRZ Nitrate Reductase, is highly growth-phase dependent and is controlled by RpoS, the alternative vegetative sigma factor" was submitted for publication in Molecular Microbiology. Characterization of NRZ mutants in section 3.3 of this chapter was not included in the submitted paper.

The work presented in this study was performed by L. Chang except for DNA isolation of *rsd* 1022 which was performed by J.P Audia. This paper was written by L. Chang.

Preceding this paper is an extended literature review on Nitrate Respiration in *Escherichia coli.*

Lily Chang B. Sc. (Hons.)
December 1998

3.1 Overview of Nitrate Respiration

Nitrate can be metabolized through assimilative or dissimilative pathways. In the assimilatory pathway, nitrate is converted to nitrite by soluble nitrate reductase enzymes. Nitrite, is further reduced to ammonia which can be incorporated into various organic compounds. This process requires NADPH as an electron donor and is widespread among bacteria, algae and fungi. In dissimilative nitrate reduction, nitrate replaces oxygen as the terminal electron acceptor during anaerobic respiration. Although *E. coli* can employ several alternative terminal electron acceptors including fumarate, trimethylamine-N-oxide (TMAO) or lactate, nitrate is the preferred alternative electron acceptor because it possesses the highest redox potential next to oxygen (reviewed in (Stewart, 1988)). The presence of nitrate in the cell will induce the synthesis of enzymes involved in nitrate respiration and repress other respiratory pathways (Stewart 1993).

During nitrate respiration, a membrane-bound nitrate reductase accepts electrons from substrate oxidation in the electron transport chain and reduces nitrate to nitrite. Much of the resultant energy is conserved as proton motive force, which is used for the generation of ATP (Stewart, 1988). The overall chemical reaction in aerobic and nitrate respiration are compared below.

Nitrate Respiration: glucose + 12 NO_3 $\rightarrow$ 6 CO_2 + 6 H_2O + 12 NO_2 (-422 kCal)

Aerobic Respiration: glucose + 6 O_2 $\rightarrow$ 6 CO_2 + 6 H_2O (-686 kCal)

The product of nitrate respiration is nitrite which can be reduced to ammonium by one of two nitrite reductases; one detoxifies nitrite and regenerates NAD^+, while the other may serve to conserve energy as proton motive force (Stewart, 1988). In some cases, bacteria are able to further reduce nitrite to nitrous oxide or nitrogen gas through denitrification however enteric bacteria usually do not perform denitrification.

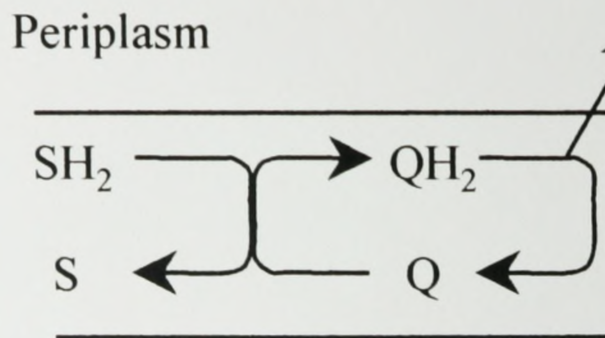
3.1.1 Nitrate Reductase

During nitrate respiration, nitrate reductase replaces the cytochrome oxidase as the terminal component in the electron transport chain. It is a membrane bound complex composed of three subunits, α, β, and γ (Chaudhry and MacGregor, 1983). The α subunit (M_r 150 000) carries the catalytic site for nitrate reduction. It requires the element molybdenum which is bound to the subunit by a pterin cofactor. The β subunit (M_r 60 000) contains four iron sulfur centres and along with the α subunit comprises the active site of the enzyme. The γ subunit (M_r 20 000) is a *b*-type cytochrome that is essential for the assembly of nitrate reductase into the cytoplasmic membrane. These subunits are present in a 2α:2β:4γ ratio when bound to the membrane (Chaudhry and MacGregor, 1983).

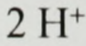
A schematic representation of the nitrate respiratory chain is described in Figure A. First, reduced substrates such as formate, NADH or lactate are oxidized by a specific dehydrogenase, which transfers the electrons to a quinone pool. Quinone is subsequently reduced and transfers electrons to nitrate reductase. Electrons are transferred from the heme of the γ subunit through the iron-sulfur center of the β subunit, to the molybdenum cofactor in the α subunit where nitrate reduction occurs (Stewart, 1988).

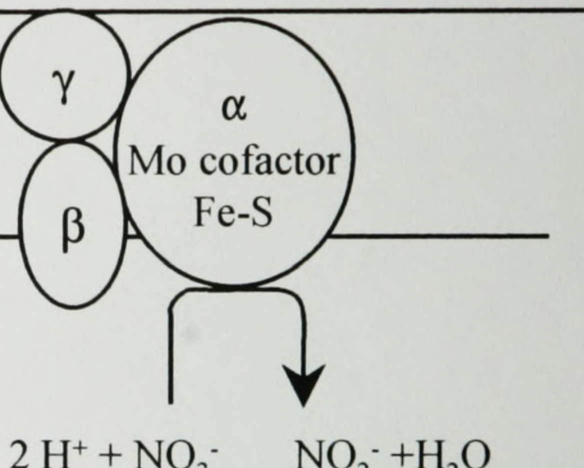
E. coli possesses two homologous membrane bound nitrate reductases and a third nitrate reductase located in the periplasm (Bonnefoy and DeMoss, 1994). The major nitrate reductase (NRA) constitutes most of the nitrate reductase activity in the cell and is induced by anaerobiosis and nitrate (Showe and DeMoss, 1968). Little is known about the secondary nitrate reductase (NRZ) (Bonnefoy et al., 1987) but it has been postulated to aid the cell in coping with stresses associated with semi-anaerobic conditions (Bonnefoy and DeMoss, 1994; Cole, 1996).

Figure A. Schematic representation of nitrate reductase.Reduced substrates (SH_2; e.g. formate, NADH, lactate) are oxidized by specific dehydrogenases, coupled to reduction of quinol (Q; ubiquinol or menaquinol) to quinone (QH_2). Quinone reduction is in turn coupled to nitrate reduction. Adapted from Stewart, 1988.



Cytoplasm


2 H+


γ
α
Mo cofactor
Fe-S
β
2 H+ + NO3-
NO2- +H2O

3.1.1.1 Nitrate Reductase A (NRA)

The α, β, and γ subunits of the major nitrate reductase A are encoded by the *narG*, *narH* and *narI* genes, respectively. These genes comprise a single transcriptional unit along with *narJ*, which encodes a chaperone required for molybdenum cofactor assembly. The *narGHJI* operon is located at 27 min on the *E. coli* map and is part of the *chlC* locus that comprises several genes involved in nitrate metabolism (Stewart, 1988). This locus encompasses the *narXL* operon, the *narK* gene and *narGHJI* operon (Figure B). The *narXL* operon is a two component regulatory system which responds to nitrate (Stewart, 1993), while *narK* encodes a nitrate/nitrite antiporter (DeMoss and Hsu, 1991). Transcription of *narK* and *narGHJI* are clockwise, while transcription of *narX* and *narL* are counter clockwise (Stewart, 1988). As mentioned, synthesis of the *narGHJI* operon is induced by anaerobiosis and nitrate (Showe and DeMoss, 1968), and regulation is at the transcriptional level mediated by two trans-acting factors, Fnr and NarL (Stewart, 1993).

3.1.1.2 Nitrate Reductase Z (NRZ)

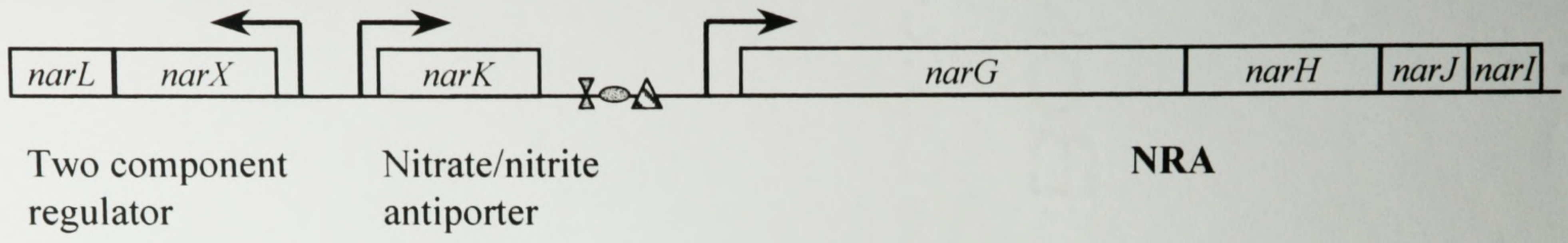
During the screening of a genomic library of *E. coli*, a plasmid-encoded clone was able to complement the *Nar*⁻ phenotype of a *narG* mutant recipient strain (Bonnefoy et al., 1987). This clone was determined to carry a gene encoding for a novel secondary nitrate reductase designated NRZ. The *chlZ* locus, comprising the genes encoding NRZ, is located at 32.5 min on the *E. coli* map (Blasco et al., 1990). The three subunits of NRZ are encoded by *narZ*, *narY*, and *narV* respectively, and are organized in a single transcriptional unit *narZYWV* (Blasco et al., 1990) (Figure B). The *narGHJI* and *narZYWV* operons are approximately 73% homologous at the DNA level (Blasco et al.,

1990). Upstream of the *narZYWV* operon is the *narU* gene which is highly homologous to *narK*. Attempts to isolate a homologous *narXL* sequence in the *chlZ* locus were unsuccessful (Blasco et al., 1990).

Although the coding regions between *chlC* and *chlZ* locus are very homologous, the regulatory regions are very different. The intergenic region between *narU* and *narZYWV* is 80 bp apart while *narK* and *narGHJI* are 500 bp apart. There are no typical *rho* independent transcription termination sites between *narU* and *narZYWV* such as those found at the 3' end of the *narK* gene. However, there is an repetitive extragenic palindromic (REP) element downstream of *narU* which may play a role in transcription termination (Bonnefoy and DeMoss, 1994).

Expression of the homologous operons are also very different. Nitrate reductase activity of *E. coli* strains devoid of NRA possess 1-3% activity of fully induced wild type strains in exponential cultures. This residual activity is believed to be due to NRZ (Iobbi et al., 1987). In strains carrying a deletion of the *narGHJI* operon and transformed with a multicopoy plasmid encoding the *narZYWV* operon, NRZ is constitutively expressed (Iobbi et al., 1987). Thus, in contrast to NRA, NRZ is not induced by nitrate, anaerobiosis or the transcriptional regulator Fnr (Iobbi et al., 1987). We have however found that NRZ expression is regulated by RpoS and is induced during stationary phase of growth under aerobic conditions. These observations are discussed in the remainder of this chapter.

chlC locus (27 min)



chlZ locus (32.5 min)

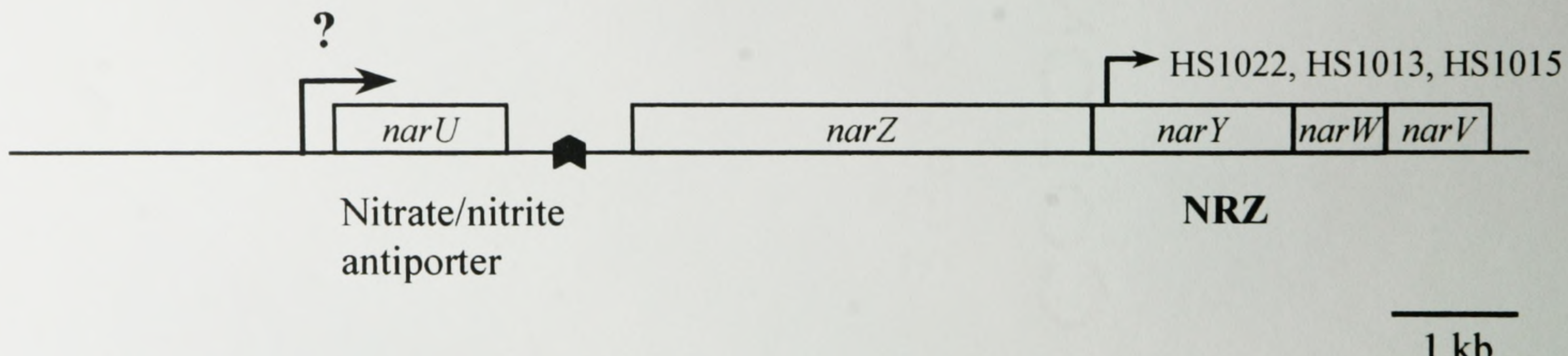


Figure B. Genetic Organization of the *chlC* and *chlZ* loci. Adapted from Bonnefoy and DeMoss, 1994.

REP element   NarL binding site   Fnr- Binding site   IHF binding site

3.1.2 Nitrate Reductase Assay

The Nitrate Reductase Assay is a commonly used colorimetric assay that quantitates nitrate reductase activity in a given substrate. This simple assay is based on the ability of nitrate reductase to convert nitrate to nitrite when a suitable reducing agent such as reduced methyl viologen is supplied (Lowe and Evans, 1964). The product nitrite, reacts with a chromogen reagent to produce a pink chromophore that can be measured colorimetrically at OD_{540}. The resulting colour intensity is proportional to the amount of active enzyme present in a given sample. The limit of detection for this assay has been reported to be 2.5μM nitrite in distilled water (Promega Corporation). Since nitrate reductase is susceptible to oxidation, reducing conditions must be maintained by the addition of a strong reducing agent such as sodium dithionite. In this environment, nitrate reductase is fairly stable and the assay can be performed at room temperature. Figure C describes the chemical reaction involved in the assay.

The method employed in this study was adapted from (Stewart et al., 1988). Crude cell extracts resuspended in 0.32 M phosphate buffer in a final volume of 0.8 ml is mixed with 0.1 ml of the electron donor methyl viologen (2 mM). The reaction is initiated by the addition of 0.1ml of solution containing 500 mM $NaNO_3$, 100 mM of $NaHCO_3$ and 50 mM of $Na_2S_2O_4$. Sodium dithionite ($Na_2S_2O_4$) maintains the reducing agent allowing for the assay to be conducted under aerobic conditions, while nitrate serves as the terminal electron acceptor for nitrate reductase. The reaction is mixed until the solution is uniformly blue. The blue colour indicates that the methyl viologen is completely reduced. The solution is incubated at room temperature for 10 min after which it is mixed

which it is mixed vigorously to oxidize the sodium dithionite, thereby stopping the reaction. To measure the amount of nitrite produced, 1 ml of 1% sulfanilic acid in 20% HCl and 1 ml of 5mM N-1-naphthylethylenediamine dihydrochloride (NED) are subsequently added. The nitrite produced in the sample reacts with the chromogens to produce a pink coloured azo compound. This can be measured spectrophotometrically at OD_{540}. Activity is calculated as follows: 100*(OD_{540}) / [time of reaction (min) x volume of cells assayed (ml) x protein concentration (mg)]. One unit of nitrate reductase activity represents the amount of enzyme catalyzing the production of 1 μmol nitrite min^{-1} mg^{-1} of protein.

Figure C. The nitrate reductase assay chemical reaction 1.) Methyl viologen serves as the electron donor for the reduction of nitrate to nitrite by nitrate reductase. 2.) In a reducing environment, nitrite reacts with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) to produce a "pink" azo compound which can be measured spectrophotometrically at OD_{540}.

1.

Methyl Viologen (reduced) → Methyl Viologen (oxidized)

Nitrate Reductase

$NO_3 + 2\ H^+$ → $NO_2 + H_2O$

2.

$NO_2 + H_2NO_2S-C_6H_4-NH_2$ (Sulfanilamide) → ($-H_2O$) → $H_2NO_2S-C_6H_4-N_2^+$

NED: $C_{10}H_7-NH-CH_2CH_2-NH_2$

$H_2NO_2S-C_6H_4-N{=}N-C_{10}H_6-NH-CH_2CH_2-NH_2$

Azo Compound
(Pink)

Expression of the *Escherichia coli* NRZ Nitrate Reductase, is highly growth-phase dependent and is controlled by RpoS, the alternative vegetative sigma factor.

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ABSTRACT

In the absence of oxygen, *Escherichia coli* can preferentially use nitrate as the terminal electron acceptor for anaerobic respiration. *E. coli* possesses two differentially-regulated nitrate reductases. While the physiological basis for this genetic redundancy is not completely understood, NRA (the main enzyme), is primarily expressed under anaerobic conditions and is induced by nitrate while NRZ is thought to be constitutively-expressed at low levels under both aerobic and anaerobic conditions. In the course of identifying genes controlled by the stationary phase regulatory factor RpoS (σ^s), we have found that the expression of NRZ is largely dependent on this alternative sigma factor, and is induced during entry into stationary phase. Expression studies, using promoter-*lacZ* fusions and nitrate reductase assays, reveal that the NRZ operon is controlled mainly at the level of transcription and is induced 10 fold at the onset of stationary phase in rich media and up to 20 fold in the presence of nitrate. We also observed a five fold stationary phase induction of NRZ in a *rpoS* background indicating that other regulatory factors, may be involved in transcriptional control of NRZ. Using a PCR-amplified probe specific for the NRZ operon, the RpoS-dependence of NRZ expression was confirmed by Northern analyses of RNA extracted from wild type and *rpoS* strains sampled in exponential and stationary phase. *In toto*, these data indicate that RpoS-mediated regulation of NRZ may be an important physiological adaptation during the transition into stationary phase.

INTRODUCTION

To survive in diverse environments *Escherichia coli* must employ efficient regulatory mechanisms to sense and react to dramatic changes in nutrient levels and osmolarity as well as altered oxygen levels. *E. coli* is able to respond to reduced oxygen levels by repressing expression of the cytochrome oxidase pathway and instead, utilizing alternative respiratory pathways. The enzymes that are induced and expressed depend on the availability/source of potential electron acceptors and the energy yield that can be generated. In the absence of oxygen, the most energetically-favorable pathway is nitrate respiration. In this pathway, nitrate (which serves as the final electron acceptor) is converted by nitrate reductase to nitrite, which can be further reduced to ammonium (Stewart, 1988).

E. coli possesses two membrane-bound nitrate reductase enzymes, Nitrate Reductase A (NRA) and a secondary enzyme, Nitrate Reductase Z (NRZ) (for review see (Stewart, 1988; Bonnefoy and DeMoss, 1994). The NRA and NRZ enzyme complexes are composed α, β, and γ subunits encoded by the *narGHJI* and *narZYWV* operons, respectively, located at 27 min and 32.5 min on the *E. coli* map (Bachmann, 1990; Bonnefoy et al., 1987). Sequence analysis reveals that they are 73% homologous at the DNA level (Blasco et al., 1990) a reflection of their common evolutionary origin.

Despite their structural similarities and common evolutionary origin, the two operons are differentially-regulated. NRA is expressed mainly under anaerobic conditions and is induced by nitrate (Showe and DeMoss, 1968). Its expression is under the control

of Fnr, a transcriptional regulator of anaerobic enzymes, and NarXL, a two-component regulator which responds to nitrate and nitrite levels (reviewed in (Stewart, 1993)). In contrast, little is known about the regulation of NRZ, though it is thought to be constitutively-expressed under both aerobic and anaerobic conditions (Iobbi et al., 1987).

Using chromosomal promoter-*lacZ* fusions to identify stationary phase genes that require RpoS for expression (Schellhorn et al., 1998), we identified three RpoS-dependent fusions that map to *narY*, a member of the *narZYWV* operon. This result strongly suggested that expression of the Nitrate Reductase Z is dependent on RpoS, an alternative sigma factor (Hengge-Aronis, 1996). In this report, we have re-examined the regulation of nitrate reductase Z using single (*narG*⁻ and *narY*⁻) and double mutants, promoter-*lacZ* expression studies and Northern analyses. In contrast to previous reports, we found that expression of NRZ is highly growth phase-dependent and is largely dependent on RpoS. These results indicate that RpoS may have a role in controlling stationary phase respiratory metabolism in addition to its well-recognized role in stress adaptation.

MATERIALS AND METHODS

Bacterial strains

All bacterial strains and phage are listed in Table 1.

Chemicals and media.

All chemicals were supplied by either Fisher Scientific Ltd. (Toronto, ON), Sigma Chemical Co. (St. Louis, Mo), or Gibco BRL (Burlington, ON). Antibiotics and non-autoclavable stock solutions were filter-sterilized. Liquid and solid media were prepared as described by Miller (Miller, 1992). Cultures were routinely grown in Luria-Bertani (LB) rich broth. Liquid media for anaerobic cultures were based on a MOPS (3-[N-morpholino] propane sulfonic acid) modified media as described by Stewart (Stewart and Parales, 1988). Where indicated, media was supplemented with 40 mM $NaNO_3$. The concentrations of antibiotics used were as follows, kanamycin (50 µg/ml), streptomycin (50 µg/ml), tetracycline (12.5 µg/ml), ampicillin (100 µg/ml). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 50 µg/ml.

Growth conditions.

Cell growth was monitored spectrophotometrically (UV-VIS Spectrophotometer, model UV-1201, Shimadzu Corporation, Kyoto, Japan) at 600 nm (OD_{600}). Cultures were grown overnight in LB media containing appropriate antibiotics. For expression studies, bacterial cultures were maintained in early exponential phase ($OD_{600} < 0.2$) for at least eight generations prior to the start of each growth experiment. This ensured that measured β-galactosidase activity in early exponential phase cultures was due to *de novo* enzyme synthesis rather than being due to carry over from overnight stationary phase

cultures. Cultures were grown in flasks at a culture/flask volume ratio of 1 to 5 at 37°C and 200 rpm to maintain proper aeration. Cultures were sampled and assayed for β-galactosidase or nitrate reductase activity at the times indicated.

Anaerobic cultures were grown in culture tubes filled to the top with media, sealed and incubated in Brewer Anaerobic Jars (Becton, Dickinson and Company, Cockeysville, MD) at 37°C (Stewart and Parales, 1988). Cultures were sampled at $OD_{600} = 0.2$ for exponential phase and $OD_{600} = 0.8$ for stationary phase (12 hours later). The generation time under anaerobic conditions was approximately 90 min.

Enzyme assays.

β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate and activity is expressed in Miller units (Miller, 1992).

Nitrate reductase activity was measured by monitoring the reduction of nitrate to nitrite using methyl viologen as the electron donor (MacGregor et al., 1974). Activity was calculated as [Average OD_{540}]/ [μmoles of nitrite/OD_{540} x time of incubation (min) x sample volume (ml) x Protein Concentration (mg/ml)]. One unit of nitrate reductase activity represents the amount catalysing the production of 1 μmol of nitrite min^{-1} mg $protein^{-1}$. A nitrite standard curve was employed to convert OD_{540} to μmole of nitrite. Nitrate reductase assays were performed in triplicate.

Crude extracts for the nitrate reductase assay were prepared by rupturing cells in a French pressure cell (Stewart and MacGregor, 1982). Extracts were subsequently centrifuged at 3000 x *g* for 15 min to remove unbroken cells (Stewart and MacGregor, 1982). Samples were stored on ice until assayed.

Protein Concentration Determination

To solubilize membrane bound protein (including nitrate reductase), crude extracts were boiled in 0.5M of NaOH for 15 min and cooled on ice. Protein concentrations were determined using the Bradford assay (Bradford, M., 1976; Bio-Rad Laboratories, Hercules, CA)(the final concentration of NaOH in the assayed samples was 0.05M). After five min, samples were measured at OD_{595}. All protein assays were performed in duplicate. A standard plot was employed to convert OD_{595} to µg of protein using bovine serum albumin protein suspended in 0.05M NaOH. We observed only a small difference in spectrophotometric readings between samples containing 0.05M NaOH and control samples containing water. Protein concentration was calculated as: [Average OD_{595}]/[Volume assayed(µl) x µg of protein / OD_{595}].

Northern probe preparation.

To measure expression of the paralogous *narGHJI* and *narZYWV* operons in wild type and *rpoS* mutant strains, we assayed exponential and stationary phase cultures by Northern analyses. DNA probes were synthesized by PCR using primers specific for *narH* and *narY*. To ensure probe specificity (i.e. to minimize the potential for cross-hybridization between these two conserved genes), regions of low homology relative to the open reading frames between *narH* and *narY* were chosen for PCR amplification: ***narY*** forward 5' TTCGACTACGAACATTTGCATAG-3', reverse 5' GTTCGGACAGTAAATTCAACCTGTTC-3', ***narH*** forward 5'-GTAACCGTGCCATGCTGCTG-3' reverse 5'-CGCTGGAAGAGGTCGGTCTGA-3', ***rrnA*** (RpoS-independent control gene) forward 5'-

GTGCCCAGATGGGATTAGCTAGTAG-3' reverse 5'-GTCGAGTTGCAGACTCCAATCC-3'. Each PCR tube contained 1X PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 50 pmol of each of the forward and reverse primers, 0.4 mM of each of the 4 nucleotide triphosphates, 4 mM $MgCl_2$, ~50 ng of *E. coli* DNA and ~50 U of Taq polymerase in a final volume of 50 µl. Reactions were run for 25 cycles under the following conditions: (1) 96 °C for 30 s; (2) 61 °C for 60 s; (3) 72 °C for 90 s. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc. Valencia, CA) and radiolabelled with [α-^{32}P]-dCTP (NEN Life Science Products, Inc., Boston, MA,) by random priming (Boehringer Mannheim, Montreal, PQ,). The identity of all PCR-amplified products was confirmed by DNA sequencing.

RNA extraction and Northern analysis.

Cultures were grown as described above and aliquots were removed from exponential and stationary phase cultures. Total bacterial RNA was extracted using an RNeasy Midi Kit (Qiagen Inc., Valencia, CA,) and was quantified spectrophotometrically at OD_{260}. Total RNA was transferred onto BIOTRANS Nylon Membranes (ICN, Montreal, PQ,) as described (Seldon, 1989) and fixed by baking at 80 °C for 2 h. Pre-hybridization and hybridization were performed at 42 °C with gentle agitation. When necessary, blots were stripped by boiling in 0.1-0.5 % SDS according to the manufacturer's instructions for re-probing.

RESULTS

Identification of RpoS dependent (*rsd*) Fusions that Map to the *narZYWV* operon

RpoS dependent genes were identified using a phenotype-independent protocol as described (Schellhorn et al., 1998). In this screening method, an *rpoS* null allele was introduced into a bank of strains carrying random promoter-*lacZ* fusions (λp*lac*Mu53) by conjugation. The resulting transconjugants were scored for a reduction in β-galactosidase activity in comparison to RpoS$^+$ strains on LB plates containing X-gal (Schellhorn et al. 1998). One hundred and five **R**po**S** **d**ependent (*rsd*) fusions were identified. The identity of the rsd genes were determined by sequencing DNA proximal to the point of insertion of the fusion as described previously (Roy et al., 1995). Three of the *rsd* fusions (*rsd*1013, *rsd*1015 and *rsd*1022) mapped to *narY*, a member of the *narZYWV* operon.

RpoS dependent regulation of *narY*

Since the expression of many RpoS-dependent genes is higher in stationary phase than exponential phase, we examined the growth phase dependent regulation of *narY*. Isogenic wild type and *rpoS* strains carrying a *narY::lacZ* reporter gene fusion (HS1022 and HS1022T) were assayed for growth phase dependent expression. In rich media, β-galactosidase activity was very low in exponential phase and was induced approximately ten fold upon entry into stationary phase (Figure 1 (A)). In an *rpoS* strain, while total activity was greatly reduced in both phases, we observed a five fold induction in stationary phase suggesting that independent regulatory factors, other than RpoS, are involved in growth phase-dependent induction of NRZ.

Since the addition of nitrate to growth media is known to induce total nitrate

reductase activity in *E. coli* (Showe and DeMoss, 1968), we tested whether the expression of *narZYWV* was specifically induced by this alternative electron acceptor. In nitrate-supplemented media, *narY* expression was induced twenty fold upon entry into stationary phase which was somewhat higher than what was found in unsupplemented cultures (Compare Figure 1(A) and 1(B)). In the *rpoS* strain, expression was not stimulated by nitrate (Figure 1(B)). These results demonstrate that the *narZYWV* operon is only slightly induced by nitrate but is highly dependent on RpoS.

Expression of *narY* and *narH* in wild type and *rpoS* strains

To exclude the possibility that the determined RpoS dependence of the *narZYWV* was not due to some artifact associated with use of the promoter-*lacZ* fusions, we further examined *narY* regulation in a wild type and *rpoS*- strains by Northern analysis. Total bacterial RNA was prepared from cultures of wild type (GC4468), *rpoS* (HS143) and *narG* (HS1610) strains grown to exponential (OD_{600}=0.3) and stationary (OD_{600} =1.6) phase and was hybridized to a PCR-amplified probe specific to *narY* (Figure 2). Consistent with results observed using promoter-*lacZ* fusions, *narY* expression was higher in stationary phase in both the wild type strain and *narG*- strain and was reduced in an *rpoS* mutant (Figure 2).

Since *narH* is highly homologous to *narY* (Blasco et al., 1990), it is possible that a probe designed to detect one gene (e.g. *narY*) may to hybridize to the mRNA of the paralogous gene (in this case, *narH*). To test the specificity of the *narY* probe, a *narH* probe (containing the corresponding region to the *narY* probe used) was synthesized and hybridized to RNA extracted from strain HS1610. This strain carries a *narG*::Tn*10*

insertion and thus does not express the *narGHJI* structural genes. We observed no detectable cross-hybridization confirming specificity of the probes used (data not shown). Further, the expression of *narY* was also found to be growth phase dependent in a strain containing a *narG*::Tn*10* mutation which should have a polar effect on the expression of *narH*, the conserved *narY* paralog (Figure 2).

To confirm the point of induction of NRZ expression, RNA prepared from a wild type strain (GC4468) sampled from early exponential to late stationary phase was hybridized to a *narY* specific probe (Figure 3). The results obtained were in good agreement with the β-galactosidase expression studies indicating that expression of *narY*, like that of many RpoS regulated genes is growth phase dependent (compare Figure 1 and Figure 3).

NRZ expression under aerobic conditions

To confirm that the transcriptional regulation of NRZ affects enzyme activity, we assayed nitrate reductase activity in a wild type (GC4468) and an isogenic mutant strain that expresses only NRZ (HS1610). Initially, we employed whole cell assays (Stewart and Parales, 1988) but found that this method was not sensitive enough to detect the relatively low levels of enzyme activity found in strains defective in the main nitrate reductase A. We observed that nitrate reductase assays using crude extracts were sufficiently sensitive to allow reproducible measurements of NRZ activity.

A wild type strain (possessing both NRA and NRZ) exhibited high induction of nitrate reductase activity in early stationary phase and decreased activity in late stationary phase (Figure 4 (A)). This marked increase in activity may be due to induction by residual

nitrate or other factors present in the rich media. In nitrate-supplemented cultures, the wild type strain (GC4468) exhibited higher activity in stationary phase than in unsupplemented cultures confirming that enzyme activity is induced by nitrate (compare Figure 4 (C) to 4 (A)). Unexpectedly, nitrate reductase activity of the *rpoS* strain was much less than that of the wild type strain, even in cultures supplemented with nitrate, which may indicate a possible role for RpoS in NRA regulation (Figure 4 (A) and (C)).

As expected, a NRA-deficient mutant strain (HS1610), exhibited much lower nitrate reductase activity than the wild type strain (compare Figure 4 (B) to 4(A)). The observed residual induction, which is due to NRZ activity, paralleled that of a *narY::lacZ* transcriptional fusion and was growth phase dependent. Activity was induced two fold in early stationary phase and was slightly higher in nitrate supplemented cultures (Figure 4 ((D)). In an *rpoS* background, NRZ activity could not be detected in supplemented (Figure 4 (D)) or unsupplemented (Figure 4 (B)) cultures. *In toto*, these results indicate that growth phase-dependent, transcriptional regulation of NRZ is dependent on RpoS.

NRZ expression under Anaerobic conditions

The main nitrate reductase (NRA) is known to be mainly expressed under anaerobic conditions and is induced by nitrate (Showe and DeMoss, 1968). We therefore examined if NRZ is similarly regulated under anaerobic conditions (Table 2). Wild type cultures (possessing both NRA and NRZ) exhibited three times higher activity in stationary phase than exponential phase cultures (Table 2). In nitrate-supplemented cultures, both exponential and stationary phase cultures exhibited twenty fold higher activity than unsupplemented cultures. A similar result was observed for a strain

possessing only NRA (HS1022) which was expected since NRA probably contributes to the majority of activity observed under anaerobic conditions. In a strain possessing only NRZ (HS1610), activity of both exponential and stationary phase samples remained very low (<0.007μmol nitrite min^{-1} mg^{-1}) in both nitrate supplemented and control (no added nitrate) cultures (Table 2). These results demonstrate that NRZ, in contrast to NRA, is not induced under anaerobic conditions and does not exhibit growth phase induction under conditions of anaerobiosis. Since NRZ activity was not detectable in an *rpoS* background under aerobic conditions (Figure 4), we did not test this strain (HS1611) under anaerobic conditions.

DISCUSSION

The alternative vegetative sigma factor RpoS (σ^s) is a master regulator that controls a large regulon that is expressed during the transition to stationary phase growth and under various stress-related conditions (Hengge-Aronis, 1996; Loewen and Hengge-Aronis, 1994). Currently, over forty genes are known to be RpoS regulated, including *katE* encoding catalase HPII (Sak et al., 1989; Schellhorn and Hassan, 1988), *xth*A encoding exonuclease III (Sak et al., 1989) and the plasmid-borne *spv* virulence genes of *S. typhimurium* (Chen et al., 1995). To systematically identify additional members of the RpoS regulon, we previously employed a phenotype-independent protocol to isolate mutants containing promoter-*lacZ* fusions that specifically require RpoS for expression (Schellhorn et al, in press). One of the many new members identified was *narY* coding for a subunit of nitrate reductase Z (NRZ) respiratory complex. Expression of this gene, which is a member of the *narZYWV* operon, was found to increase in stationary phase cultures and was highly RpoS-dependent in both rich and minimal media (this study).

The identification of *narY* as an RpoS-dependent gene was somewhat surprising since expression of the *narZYWV* operon had been previously-reported to be constitutive (Iobbi et al., 1987), an observation that led to the idea that NRZ may serve an adaptive role by allowing cells to more readily survive the transition from aerobiosis to anaerobiosis (Bonnefoy and DeMoss, 1994; Cole, 1996). Under the latter condition, NRA, the main nitrate reductase, which is known to be induced by anaerobiosis and nitrate (Showe and DeMoss, 1968), functions as the main reductase. The findings reported in this study, while consistent with idea of an adaptive role for NRZ, suggests that the cell can active

modulate NRZ levels.

It is not clear why expression of NRZ was found to be constitutive in previous studies, but differences in *E. coli* strains used may be partially responsible. It is well-known that many laboratory stocks of *E. coli* have acquired independent mutations in RpoS leading to lower, attenuated levels of RpoS (Jishage and Ishihama, 1997). This can lead to differences in expression of RpoS-dependent genes between "wildtype" stocks of the same strain (Jishage and Ishihama, 1997) which will almost certainly compromise comparisons of studies examining regulation of RpoS-dependent genes. The wild type strain used in this study, GC4468, produces high levels of RpoS (determined by Western blotting-unpublished data) and is thus well-suited to the study of RpoS-regulated gene expression.

The results of β-galactosidase expression studies of a representative *narY* promoter-*lacZ* fusion clearly demonstrate that *narY* expression is dependent on RpoS. Northern analyses confirm that regulation occurs mainly at the transcriptional level in a wild type background (this study). However, the observed growth phase dependent NRZ expression, even in the *rpoS* background, suggests that other, as yet-undefined, regulatory factors may also control NRZ levels.

Consistent with the idea that transcriptional control is the main factor in regulating NRZ levels, changes in cellular NRZ activity closely paralleled changes in NRZ message (this study). As cellular NRZ activity is much lower than NRA activity it is difficult to reliably measure the changes in specific activity for this enzyme. However, a clear increase in NRZ expression was observed as cultures entered stationary phase (this study).

Although changes in NRZ enzyme activity were somewhat less than the ten fold induction observed for *narY::lacZ* expression studies, this may be due to the much greater sensitivity of the β-galactosidase assay relative to the nitrate reductase assay. Nonetheless, the reduction of nitrate reductase activity in an *rpoS* strain supports the main finding that RpoS is an important regulator of *narZYWV* transcription. This regulation appears to be primarily operant under aerobic conditions since no growth phase dependent increase in activity was observed in anaerobic cultures (this study). Expression of NRA was also growth phase dependent, however, this may be due to reduced oxygen levels sensed by Fnr as cultures became more dense. Reduced oxygen levels can induce Nitrate reductase A activity which becomes maximally expressed under anaerobic conditions (Showe and DeMoss, 1968; Tseng et al., 1996).

Why are the cellular levels of NRA and NRZ activity so different? To determine if the differential expression observed can be explained by differences in translation efficiency, we examined the codon usage pattern within the two paralogous operons. It is well-known that there is a positive correlation between codon usage and protein levels in *E. coli* (Gouy and Gautier, 1982; Sharp and Li, 1987). That is, the codon usage of genes encoding highly-expressed proteins is biased to those codons recognized by abundant tRNAs, while those of poorly-expressed proteins are less-biased. The Codon Adaptation Index (CAI) is a numerical measure that quantifies the degree of codon usage bias of a given gene relative to that of a standard set of highly-expressed genes (Sharp and Li, 1987). Consistent with the observed difference in the contribution of the two paralogous reductases to the total cellular nitrate reductase activity, the calculated CAI of each

paralogous member of the NRA operon is much higher than corresponding paralogs of the NRZ operon (Figure 5). A similar results was obtained with another, recently-identified RpoS-dependent gene, *ldcC* gene, which encodes a second lysine decarboxylase that is highly homologous to *cadA* (Kikuchi et al., 1997; Yamamoto et al., 1997). Like NRZ, *ldcC* was thought to be constitutively-expressed at very low (almost undetectable) levels but has recently been found to RpoS dependent (Van Dyk et al., 1998). Consistent with the idea that poorly-expressed genes have low CAI values, the calculated CAI of *ldcC*, is much lower than that of *cadA*, the main lysine decarboxylase
(0.301 vs. 0.562, respectively).

Why has *E. coli* maintained two apparently redundant nitrate reductase enzymes? While the two operons may be the result of gene duplication (Iobbi et al., 1987), NRZ must have an important conserved physiological function or it would have been eliminated by evolutionary selective pressures (Bonnefoy and DeMoss, 1994; Cole, 1996). It has been suggested that the constitutive expression of NRZ may serve to facilitate rapid adaption of low oxygen conditions pending the synthesis of the major respiratory nitrate reductase (Bonnefoy and DeMoss, 1994; Cole, 1996). Our findings extend this basic idea by showing that NRZ is a member of the RpoS regulon, a complex adaptive cellular response.

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Figure Legends

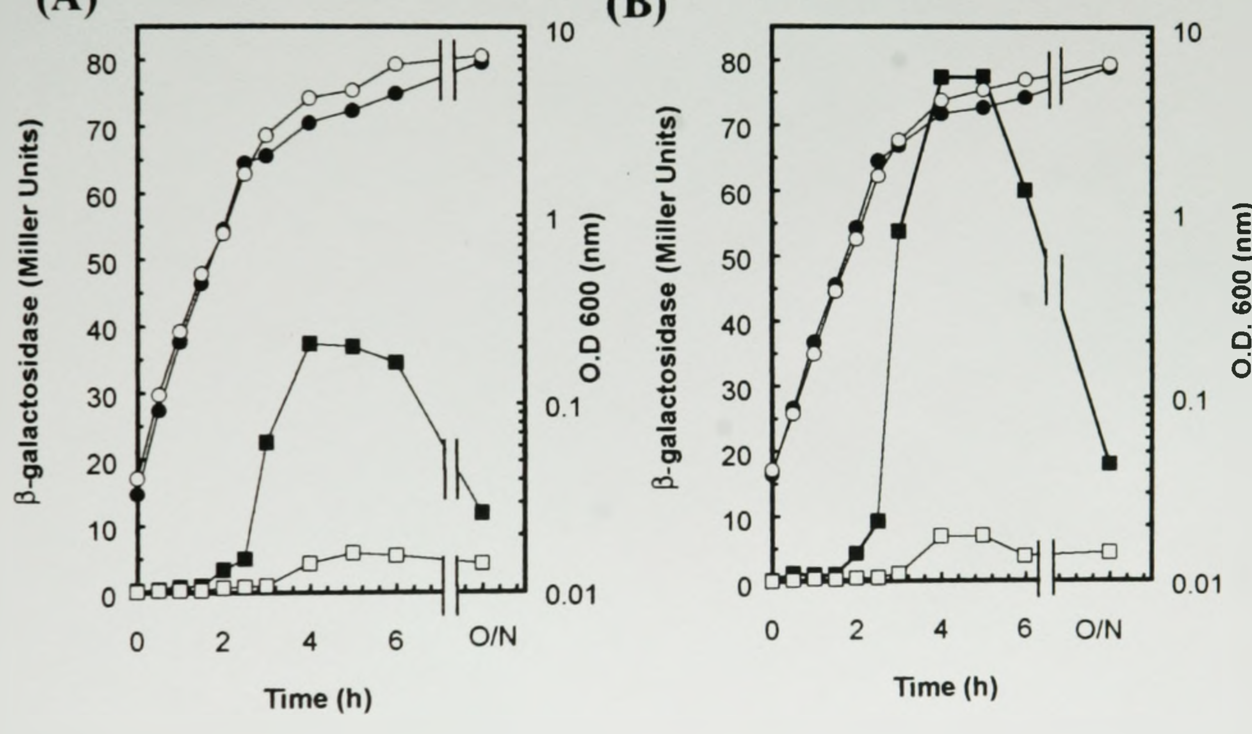
Figure1. Expression of a *narY::lacZ* fusion in isogenic wild type and *rpoS*$^-$ backgrounds grown in (A) rich media and (B) rich media supplemented with 40mM nitrate. Flasks containing LB broth were inoculated with exponentially-growing cultures as described in Materials and Methods, sampled periodically and assayed for β-galactosidase activity. Symbols: Growth (expressed in OD_{600}) for wild type (●) and *rpoS*$^-$ (○) strains, β-galactosidase activity of a *narY::lacZ* fusion for wild type (■) and *rpoS*$^-$ (□) strains. O/N=overnight.

Figure 2. RpoS-dependent expression of *narY*. Northern analyses of total RNA isolated from exponential (E) phase and stationary (S) phase cultures of wild type (GC4468), *rpoS* (HS143) and *narG*$^-$ (HS1610) strains. Probes (labeled with [α^{32}P]-dCTP) specific to *narY* were hybridized to total bacterial RNA. To confirm that equivalent amounts of RNA were extracted and loaded, blots were stripped and re-probed with a PCR-amplified DNA probe to *rrnA*, an RpoS-independent gene.

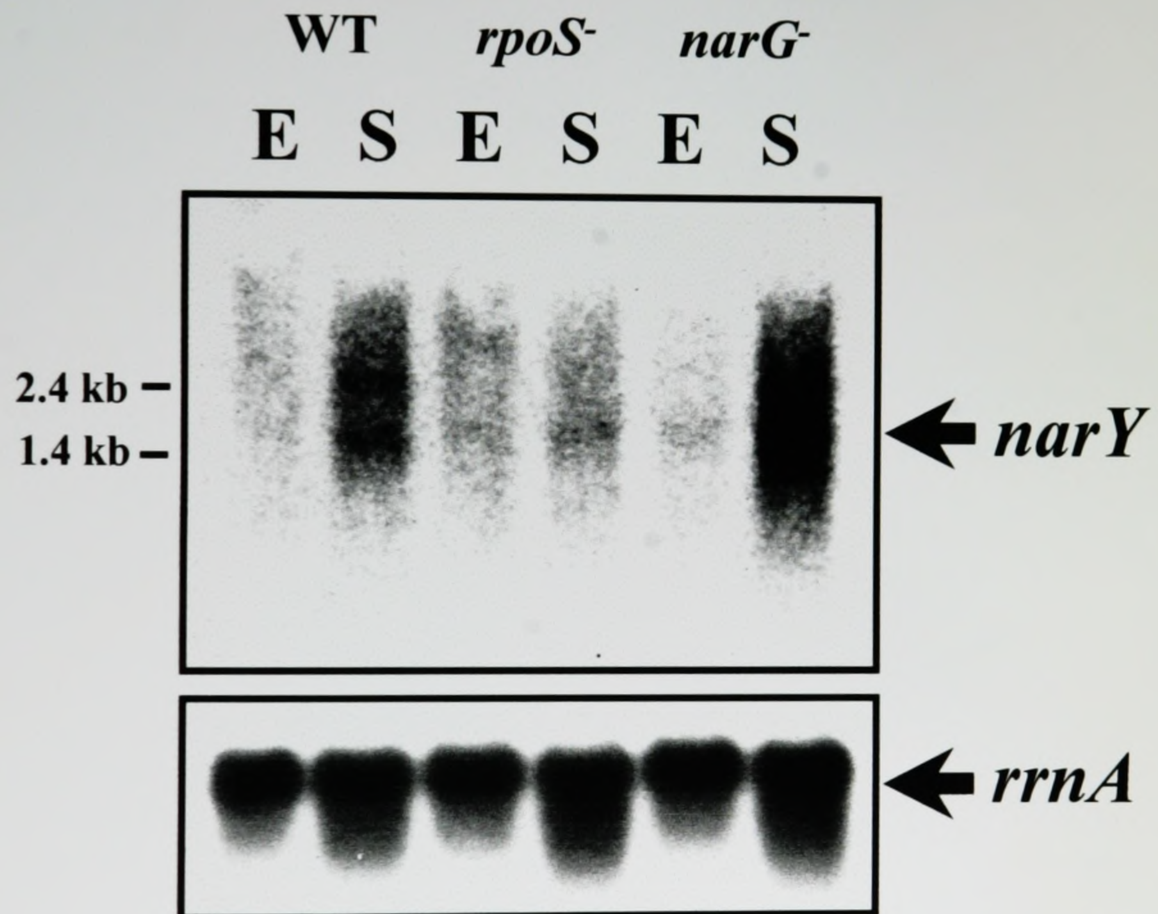
Figure 3. Northern analyses of total RNA isolated from wild type (GC4468) culture sampled throughout growth. Rich media was inoculated with exponentially-growing cells as described in Materials and Method. Samples were taken as indicated and total bacterial RNA was isolated. Probes (labeled with [α^{32}P]-dCTP) specific to *narY* were hybridized to the isolated RNA. To confirm that equivalent amounts of RNA were extracted and loaded, blots were stripped re-probed with a PCR-amplified DNA probe to *rrnA*, an RpoS-independent gene.

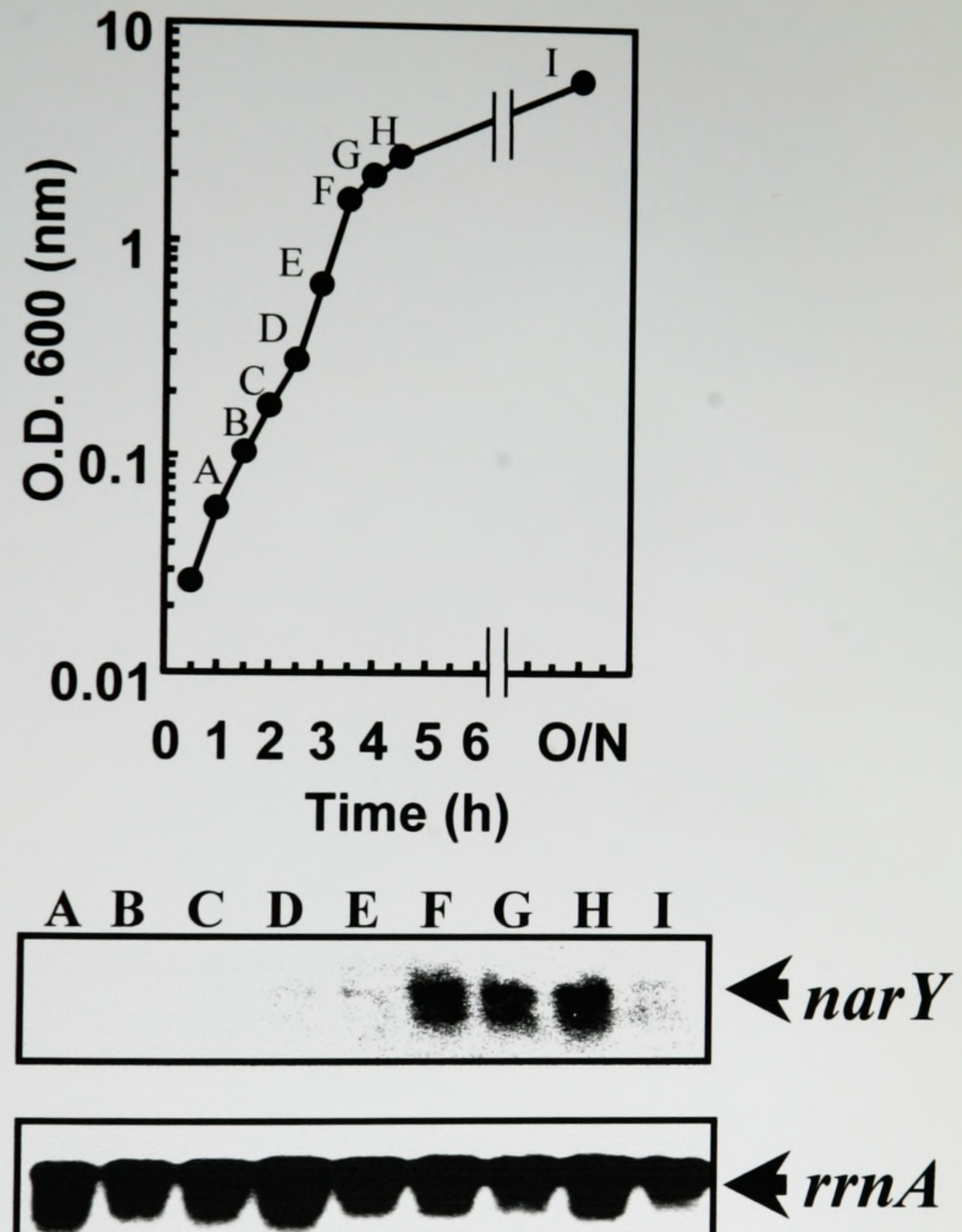
Figure 4. RpoS- and nitrate-dependence of nitrate reductase activity in strains producing NRA and NRZ. Flasks containing LB broth were inoculated with exponentially-growing cells as described in Materials and Methods and sampled periodically as indicated. (A) GC4468 (NRA$^+$ NRZ$^+$ *rpoS*$^+$) and GC122 (NRA$^+$ NRZ$^+$ *rpoS*$^-$) grown in LB, no added nitrate (B) HS1610 (NRZ$^+$ *rpoS*$^+$) and HS1610T (NRZ$^+$ *rpoS*$^-$) grown in LB, no added nitrate (C) GC4468 (NRA$^+$ NRZ$^+$ *rpoS*$^+$) and GC122 (NRA$^+$ NRZ$^+$ *rpoS*$^-$) grown in + 40mM nitrate (D) HS1610 (NRZ$^+$ *rpoS*$^+$) and HS1610T (NRZ$^+$ *rpoS*$^-$) grown in LB + 40mM nitrate. Symbols: Growth (expressed as O.D. (600)) for wild type (●) and *rpoS* (○) strains, nitrate reductase activity for wild type (■) and *rpoS* (□) strains. O/N=overnight.

Figure 5. Codon Adaptation Indices (CAIs) of the *narGHJI* and *narZYWV* operons.

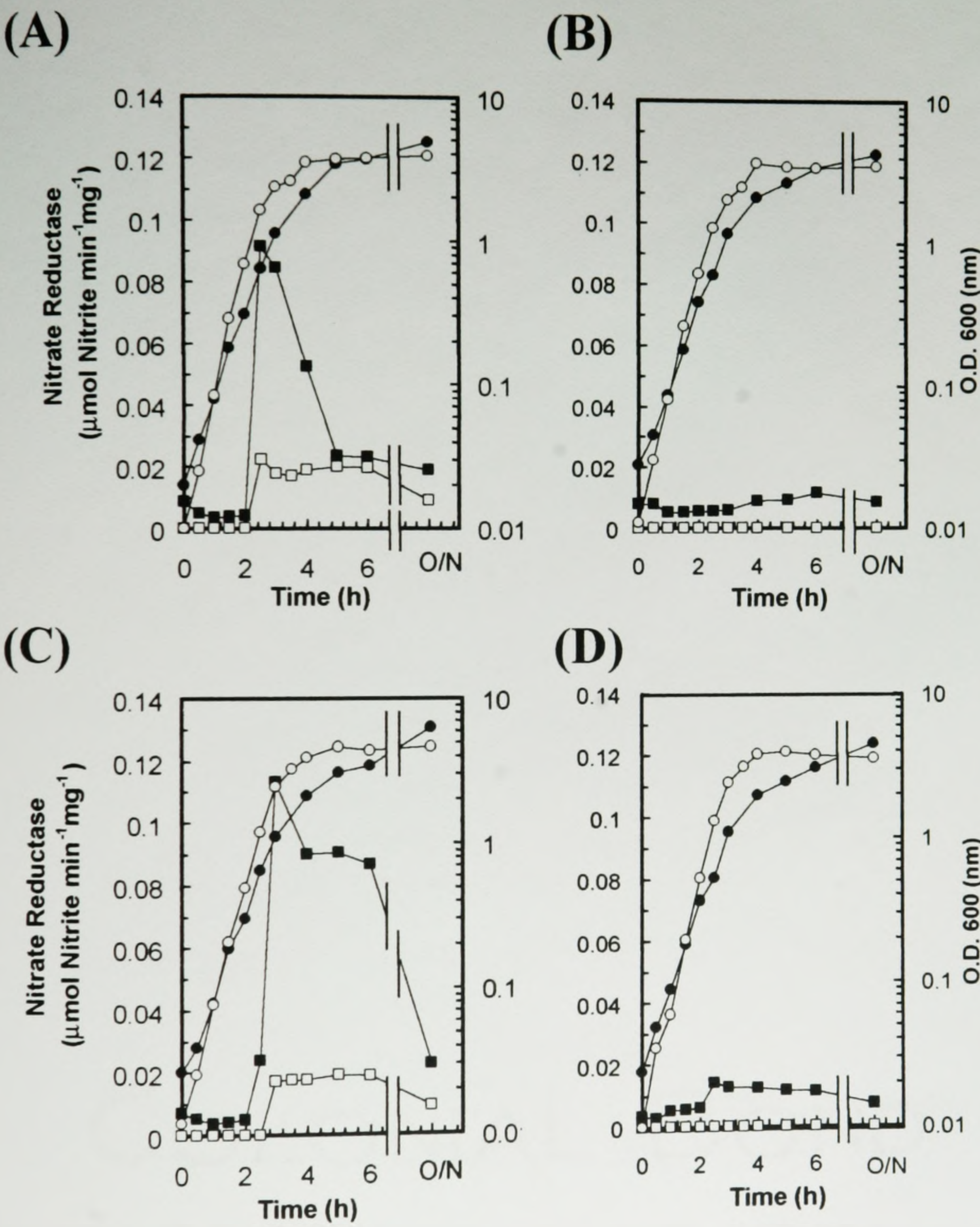


(A)
β-galactosidase (Miller Units)
O.D 600 (nm)
Time (h)
O/N
(B)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N

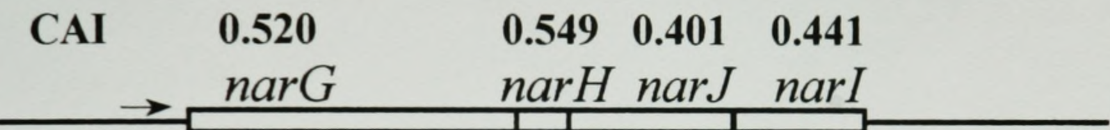

WT
rpoS-
narG-
E S E S E S
2.4 kb
1.4 kb
narY
rrnA



10
1
0.1
0.01
O.D. 600 (nm)
0 1 2 3 4 5 6 O/N
Time (h)
A
B
C
D
E
F
G
H
I
A B C D E F G H I
narY
rrnA


(A)
(B)
(C)
(D)
Nitrate Reductase
(μmol Nitrite min-1mg-1)
O.D. 600 (nm)
Time (h)
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.01
0.1
1
10
0
2
4
6
O/N

NRA Main nitrate reductase of *E. coli*, induced by nitrate, anaerobiosis



NRZ Second nitrate reductase of *E. coli*, induced in stationary-phase

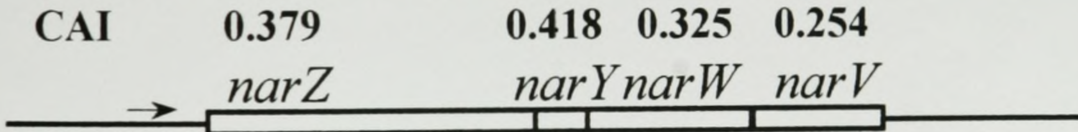


Table 1. *E. coli* strains used in this study.

Strains	Genotype	Source/Reference
A) Strains		
GC4468	Δ*lacU169 rpsL*	(Schellhorn and Stones, 1992)
HS143	as GC4468 but φ(*rpoS::lacZ*$^{+}$)143	(Schellhorn and Stones, 1992)
HS1022	as GC4468 but φ(*narY1::lacZ*$^{+}$)	(Schellhorn et al., in press)
HS1022T	as HS1022 but *rpoS13*::Tn*10*	(Schellhorn et al., in press)
HS1610	as GC4468 but φ(*narG205*::Tn*10*)	P1(RK5268)xGC4468→tet^{R}
HS1611	as HS143 but φ(*narG205*::Tn*10*)	P1(RK5268)xHS143→tet^{R}
HS1612	as HS1022 but φ(*narG205*::Tn*10*)	P1(RK5268)xHS1022→tet^{R}
RK5268	*araD139 Δ(argF-lacU169) deoC1 flhD5301 gyrA non-9 ptsF25 relA1 rpsL150 narG205*::Tn*10*	(Stewart and MacGregor, 1982)
B) Phage		
P1*vir*	generalized transducing phage	laboratory stock

Table 2. Nitrate Reductase Activity of Strains Grown under Anaerobic Conditions.

			Nitrate Reductase[a] (μmol of NO_2^- $min^{-1}mg^{-1}$)			
			$-NO_3$		$+NO_3$	
Strain	**Class**	**Active Enzyme**	**Exponential Phase**	**Stationary Phase**	**Exponential Phase**	**Stationary Phase**
GC4468	Wild type	NRA, NRZ	0.042± 0.007[b]	0.117± 0.007	0.701± 0.080	0.972± 0.032
HS1022	*narY*⁻	NRA	0.034± 0.005	0.116± 0.013	0.705± 0.103	0.701± 0.004
HS1610	*narG*⁻	NRZ	0.004± 0.000	0.00049± 0.00023	0.007± 0.001	0.008± 0.002
HS1612	*narG*⁻ *narY*⁻	none	N.D.[c]	N.D.	N.D.	N.D.

[a] Crude extracts from exponential phase (OD_{600}=0.2) and stationary phase (OD_{600}=0.8) samples were assayed for Nitrate reductase activity as described in Materials and Methods.

[b] Values represent the average determined from three independent samples

3.2.1 Growth study of NRZ

To determine whether NRZ contributes to overall cell growth, a strain possessing only NRZ (HS1610) and a strain deficient in both nitrate reductases (HS1612) were grown aerobically in rich and 0.2% glucose minimal liquid cultures (Figure 6). No significant difference in growth rate was observed between the two strains in cultures supplemented with or without nitrate. HS1610 exhibited slightly higher growth only in late stationary phase (24 hours). This therefore, suggests that the secondary nitrate reductase is not essential for growth.

3.2.2 Phenotypic characterization of wild type and nitrate reductase mutant strains

To determine whether NRZ mutants exhibit a discernable phenotype, six isolates of each strain used in this study were replica plated onto LB media, 0.2% glucose minimal plates and 1% glycerol (non-fermentable carbon source) minimal plates. Plates were supplemented with either 10 mM or 50 mM nitrate and incubated under both aerobic and anaerobic conditions (Table 3 and 4). Wild type and mutant colonies incubated under aerobic conditions exhibited no differences in growth on rich or minimal plates (Table 3). Maximal growth was observed on plates supplemented with 50mM nitrate. On glycerol minimal plates, no growth was observed for *rpoS* mutant strains (HS143, HS1611, HS1022T).

Under anaerobic conditions, mutant strains carrying a *narG*::Tn10 insertion (HS1610, HS1611, HS 1612) grew better than their *narG*$^{+}$ counterparts (GC4468, HS143, HS1022) on unsupplemented rich and glucose minimal plates (Table 4). However, on nitrate supplemented media, the phenotypes were reversed. Strains carrying a

narG::Tn10 insertion exhibited less growth than the *narG⁺* strains. On glycerol minimal plates, no growth was observed for *narG⁻* mutant strains while *narG⁺* strains exhibited very little growth. Surprisingly, HS1022T (*rpoS⁻narY⁻*) did not grow on glucose or glycerol minimal media in presence or absence of nitrate under anaerobic conditions.

Discussion

The growth studies demonstrate that NRZ is not essential for growth. This was not surprising since *E. coli* is highly adaptable and can utilize several alternative respiratory pathways if one is impaired. A strain possessing NRZ grew only slightly better than a strain lacking both NRZ and NRA after 25 hours. Perhaps if growth was monitored for several more days, a greater difference in growth may have been observed.

Consistent with the results of the growth studies, NRZ mutant strains did not exhibit a discernable phenotype on rich or minimal plates incubated under aerobic conditions, confirming that NRZ is not essential for growth. Under anaerobic conditions, several interesting phenotypes were observed. First, differences in growth was attributed mainly to a *narG⁻* mutation. This was most evident on glycerol minimal media, where *narG⁻* mutant strains did not grow regardless of the presence or absence of a functional NRZ. Second, *narG⁻* mutants colonies were larger than their *narG⁺* counterparts on unsupplemented rich media and glucose minimal media plates. Although, we do not know the reason for this, it is possible that the residual nitrate reductases present in wild type strains may either repress or compete with alternative respiratory pathways for electrons. Since there is only residual nitrate (electron acceptor) present in the media, most of the electrons shunted away by nitrate reductase are not utilized and hence

wasted. The result of this is less energy generated and thus reduced growth. A *narG::Tn10* strain however lack a functional nitrate reductase and thus electrons are efficiently directed to alternative respiratory pathways resulting in more energy for cell growth. On nitrate-supplemented media, *narG*$^+$ strains grew better than *narG*$^-$ strains. This was expected since nitrate induces the synthesis of nitrate reductase which under anaerobic conditions is the most energetically favorable respiratory pathway

The most surprising phenotype is HS1022T (*rpoS*$^-$, *narZ*$^-$) which did not grow on either supplemented or unsupplemented media. This was evident only in minimal media plates indicating that a component of rich media is required for growth.

Figure 6. Growth of NRA$^-$NRZ$^+$ strain and NRA$^-$NRZ$^-$ strain in (A) rich media and (B) rich media supplemented with 40mM nitrate (C) 0.5% glucose minimal media and (D) 0.5% glucose minimal media supplemented with 40mM nitrate. Flasks containing LB broth and 0.5% glucose minimal media were inoculated with exponentially-growing cells as described in Materials and Methods and measured for growth at OD_{600} as indicated. Symbols: HS1610 (NRA-NRZ+) (●) and HS1022T (NRA-NRZ-) (O). O/N= overnight.

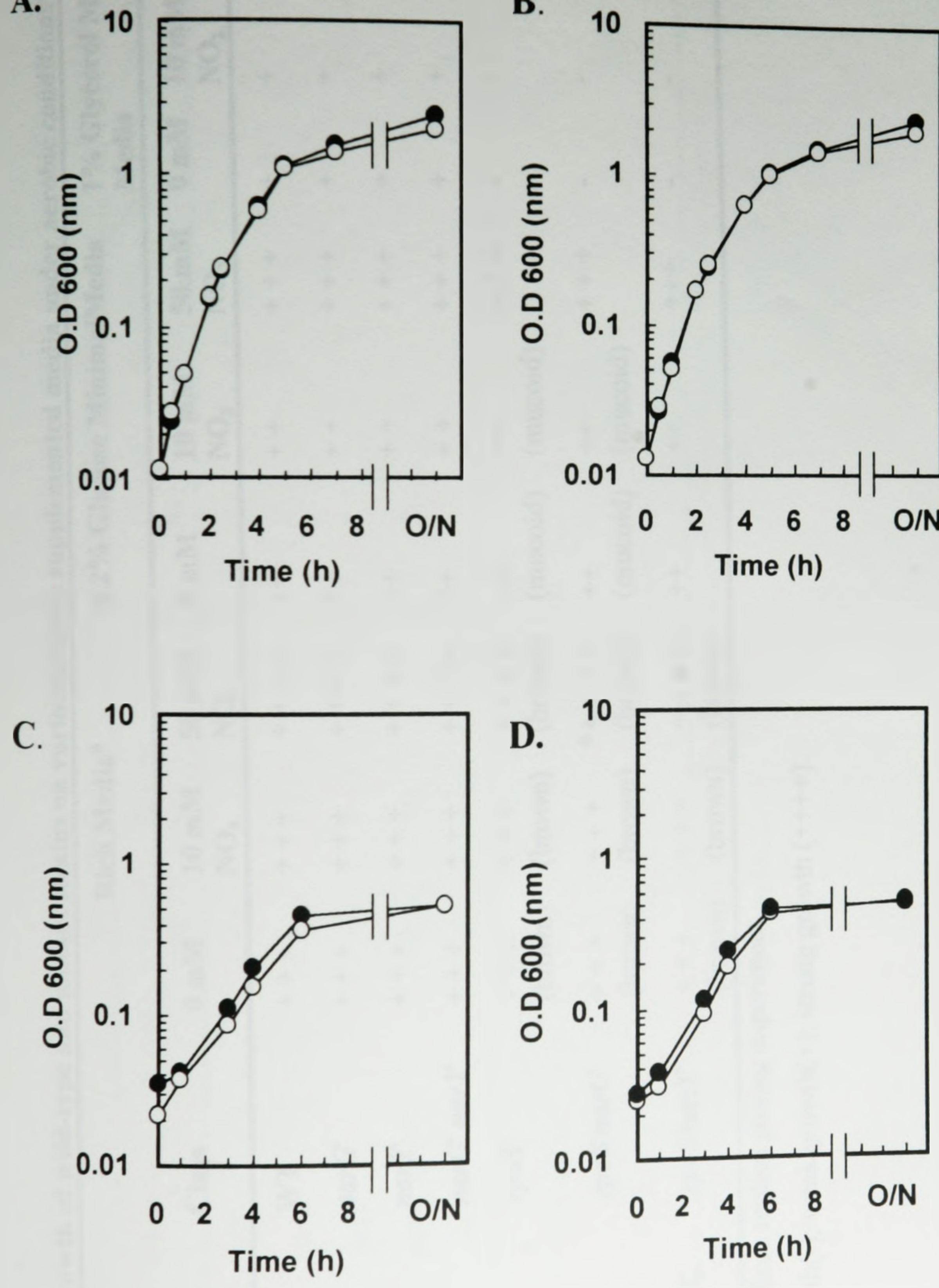

A.
10
1
0.1
0.01
O.D 600 (nm)
0 2 4 6 8 O/N
Time (h)
B.
10
1
0.1
0.01
O.D 600 (nm)
0 2 4 6 8 O/N
Time (h)
C.
10
1
0.1
0.01
O.D 600 (nm)
0 2 4 6 8 O/N
Time (h)
D.
10
1
0.1
0.01
O.D 600 (nm)
0 2 4 6 8 O/N
Time (h)

Table 3. Growth of wild-type and mutant strains on various nitrate supplemented media under aerobic conditions[a]

Strain	Class	Rich Media[b]			0.2% Glucose MinimalMedia			1% Glycerol Minimal Media		
		0 mM	10 mM NO_3	50 mM NO_3	0 mM	10 mM NO_3	50 mM NO_3	0 mM	10 mM NO_3	50 mM NO_3
GC4468	WT	+ + +	+ + +	+ + + +	++	+ +	+ + +	+	+	+
HS1610	*narG*$^-$	+ + +	+ + +	+ + + +	++	+ +	+ + +	+	+	+
HS1022	*narY*$^-$	+ + +	+ + +	+ + + +	++	+ +	+ + +	+	+	+
HS1612	*narG*$^-$ *narY*$^-$	+ + +	+ + +	+ + + +	++	+ +	+ + +	+	+	+
HS143	*rpoS*$^-$	+ + + (brown)	+ + + (brown)	+ + + + (brown)	++ (mucoid)	++ (mucoid)	+ + +	-	-	-
HS1611	*rpoS*$^-$*narG*$^-$	+ + + (brown)	+ + + (brown)	+ + + + (brown)	++ (mucoid)	++ (mucoid)	+ + +	-	-	-
HS1022T	*rpoS*$^-$*narY*$^-$	+ + + (brown)	+ + + (brown)	+ + + + (brown)	++	++	+++	-	-	-

[a] Results from replica plating experiment.

[b] no growth(-); weak growth(+); strong growth (++++)

Table 4. Growth of wild-type and mutant strains on various nitrate supplemented media under anaerobic conditions[a]

Strain	Class	Rich Media[b]			0.2% Glucose Minimal Media			1% Glycerol Minimal Media		
		0 mM	10 mM NO_3	50 mM NO_3	0 mM	10 mM NO_3	50 mM NO_3	0 mM	10 mM NO_3	50 mM NO_3
GC4468	WT	+	+ + +	+ + +	-	+ +	+ +	-	+	++
HS1610	*narG*$^-$	+ +	+ +	+ +	+	+	+	-	-	-
HS1022	*narY*$^-$	+	+ + +	+ + +	-	+ +	+ +	-	+	++
HS1612	*narG*$^-$ *narY*$^-$	+ +	+ +	+ +	+	+	+	-	-	-
HS143	*rpoS*$^-$	+	+ + +	+ + +	-	+ +	+ +	-	+	+
HS1611	*rpoS*$^-$ *narG*$^-$	+ +	+ +	+ +	+	+	+	-	-	-
HS1022t	*rpoS*$^-$ *narY*$^-$	+	+ + +	+ + +	-	-	-	-	-	-

[a] Results from replica plating experiment. Plates were incubated in Anaerobic Brewer jars at 37°C for 24 hours

[b] no growth (-); weak growth (+); strong growth (+++)

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Chapter 4

4.1 Summary

Enteric bacteria are able to survive prolonged periods of starvation because of complex physiological and morphological alterations. The identification of RpoS as the central regulator for these processes has significantly stimulated interest in this field particularly since many stationary phase genes are potential virulence factors in pathogenic bacteria (Fang et al., 1992). This study employed a unique phenotype-independent screen to identify several new members of the RpoS regulon. The effectiveness of this protocol may in theory be applied to the characterization of other regulons controlled by specific trans-acting regulatory factor. Like other mutagenic screens however, it is limited to the detection of non-essential genes.

The majority of genes identified in this study represent new members of the RpoS regulon (Schellhorn et al., 1998). While it is surprising that so many genes have evaded detection, this can be reasoned by several factors. First, as stated, previous screens were limited to the identification of genes associated to specific phenotype. Secondly, many of the identified fusions map to open reading frames of unknown reading frames and thus have not been previously studied such as *o371* (b1527) or *o381* (b1440). Finally, some proteins are expressed at low levels which may have gone undetected (e.g NRZ).

Nitrate reductase Z (NRZ) was previously thought to be constitutively expressed

at low levels and its function was to serve as an auxiliary nitrate reductase during sudden transitions from aerobic to anaerobic growth while the major respiratory nitrate reductase is induced (Bonnefoy and DeMoss, 1994; Cole, 1996). The observation that NRZ expression is dependent upon RpoS supports the adaptive role of NRZ but demonstrates that the cell can actively modulates NRZ levels as opposed to being passively expressed. In addition to NRZ, several other secondary or redundant genes have been identified to be RpoS-regulated (e.g *ldcC, talB).* A recent study reported that RpoS is induced during a diauxic shift from a rich carbon source to a poor carbon source (Fischer et al., 1998). This implied that induction of RpoS and the stress regulon may serve as an rapidly activated emergency system, which eventually shuts off as soon as cells are able to cope with the stress situation by utilizing a more specific and more economical system (Fischer et al., 1998). In light of this, it is possible that many RpoS-dependent redundant genes (e.g *narZ, ldcC*) may be part of this emergency system which facilitates adaption to a continuously changing environment.

Overall, several points can be concluded from this study. First, the identification of novel RpoS-dependent genes in this study indicates that the RpoS regulon may be much larger and diverse than previously believed. Second, several fusions are also induced in early exponential phase cultures suggesting that the signal(s) required for the expression of the RpoS and its regulon is present in early exponential phase. Finally, many of the fusions exhibit RpoS-independent stationary phase induction indicating that additional regulators are involved. Given the complexity of RpoS-dependent stationary phase regulation, future work will likely involve studying the effects of co- regulators (e.g IHF and N-NS) and determining their interaction with RpoS.

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Appendix 1

REP-PCR Amplification of *rsd* fusions

Introduction

Repetitive extragenic palindromic (REP) elements are 35 bp sequences that are believed to comprise 1% of the *E. coli* genome (Stern et al., 1984). These elements have been identified in the intercistronic regions of numerous operons and the characteristic stem loop structure is proposed to modulate gene expression by decreasing expression of downstream genes, while stabilizing transcripts of upstream sequences (Stern et al., 1984). During the course of identifying *rsd* fusions using the BLAST sequence alignment program, it was observed that three fusions (HS1054, HS1085, HS1049) partially mapped to the *lac* operon. Interestingly, the *lac* operon contains a REP element located between the *lacY* and *lacA* gene. Since the λp*lac*Mu phage contains a portion of the *lacYZ* gene, an illegitimate recombination may have occurred between the REP element found near the *lacY* gene and a REP element in the chromosomal DNA where excision took place. For example, *rsd*1049 partially maps to the *yjbE* gene and the *lacY* gene. A REP element is located upstream of the *yjbE* gene where recombination probably took place resulting in the *lacY* read-through observed in the *yjbE* sequence. Similarly, *rsd*1054 and *rsd*1085 map to the gene *yebF*. Although no REP elements have been reported in this region, a short sequence similar to the REP sequence was found 300 bases upstream of the fusion

junction where recombination could have occurred.

REP elements have been utilized as primers to PCR amplify genomic DNA (REP-PCR) (Subramanian et al., 1992; Woods et al., 1993). Since REP elements are dispersed throughout the bacterial genome, REP-PCR will generate a DNA fingerprint that can be used to discriminate bacteria strains or species (Woods et al., 1993). Taking advantage of this system, we proposed amplifying our isolated DNA using primers specific to the Mu c end of the λphage and REP elements. Theoretically, PCR amplified products should contain unique sequences that lie between the Mu c end and adjacent REP site as well as between adjacent REP sequences themselves. By comparing the profiles of DNA amplified using Mu c-REP primers and REP primers alone, a unique Mu c-REP PCR product should be readily identified. Since whole cell samples have been used directly as DNA template for REP-PCR (Woods et al., 1993), success of this method would abolish the need to perform time consuming DNA isolation and purification.

REP-PCR

Amplification of *rsd*1049, *rsd*1085, and *rsd*1054 DNA was performed using a Mu c primer and three REP primers previously described (Subramanian et al., 1992). The sequence for each primer are:

(AB10690)	Rep1R-1	5'-IIIICGICGICATCIGCC-3'	left side of the stem of the Rep palindrome.
(AB10691)	Rep 1R-1a	5'-IIIICGICGCATCIGGC-3'	same as Rep1R-1 with one less inosine
(AB10692)	Rep2-1	5'-ICGICTTATCIGGCCTAC-3'	right side of the stem loop of the palindrome
	Mu c	5'-CCCGAATAATCCATGTCCTCCCGG-3'	Mu c end of λp*lac*Mu phage

Inosine contains the purine base, hypoxanthine and is capable of base pairing with A,G,C or T. A total of 10 possible primer pair combinations were used for PCR reactions in each sample. Each PCR tube contained 1x PCR buffer, 50 ng of template DNA, 50 pmol of each primer, 0.4 mM of each of the 4 dNTPs, ~10 U of Taq polymerase, and 4 mM $MgCl_2$ in a final volume of 50 µl. PCR amplification was performed in an automated thermal cycler with an initial denaturation step at 95 °C for 3.5 min followed by 30 cycles of (1) 96 °C for 15 s, (2) 58 °C for 30 s (3) 72 °C 2.5 min with a final extension at 72°C for 5 min.

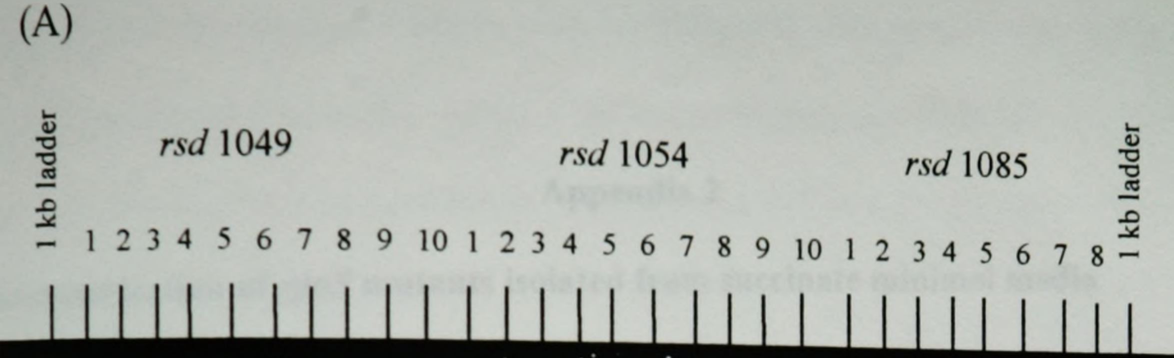
PCR products were separated on 0.7 % agarose gels containing 1 x TAE buffer and 0.5 µg/µl of ethidium bromide. PCR products were purified using the Qiagen PCR Purification kit (QIAGEN Inc., Valencia, CA, USA) as specified by the manufacturer.

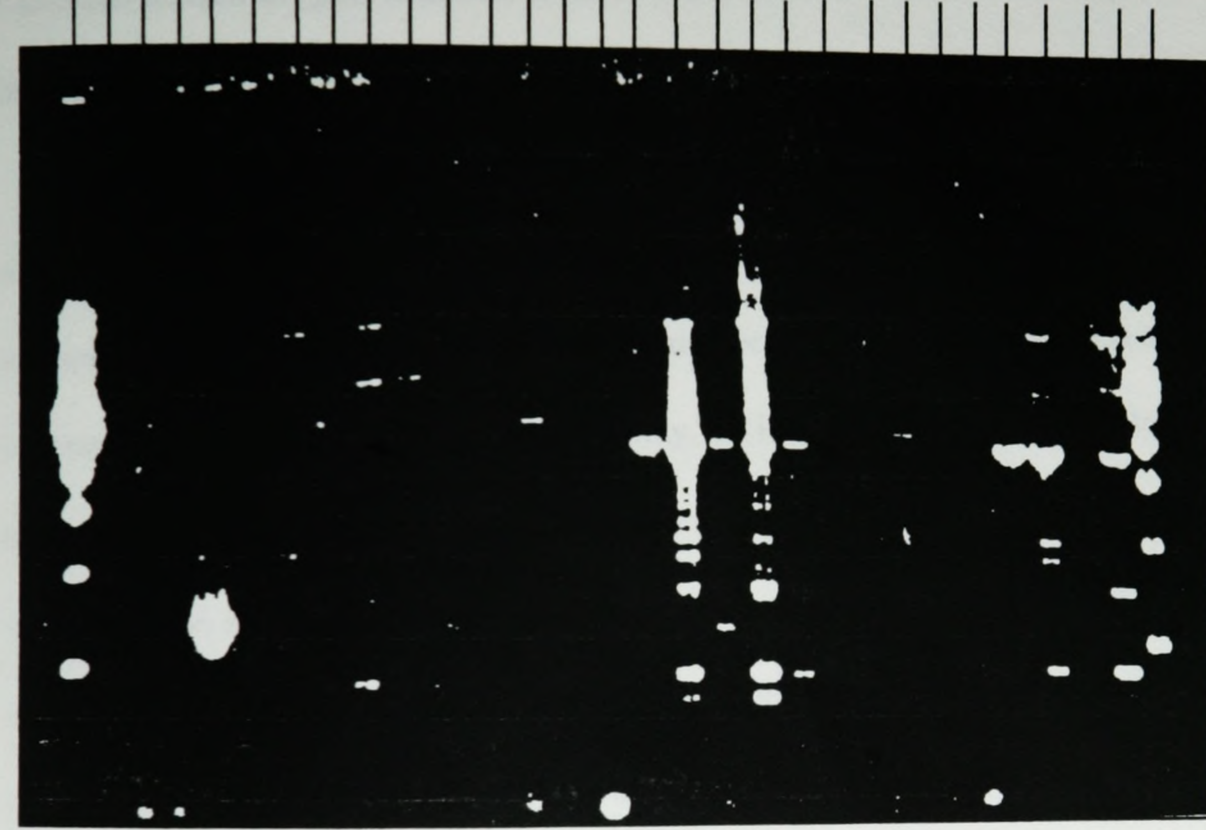
Results and Discussion

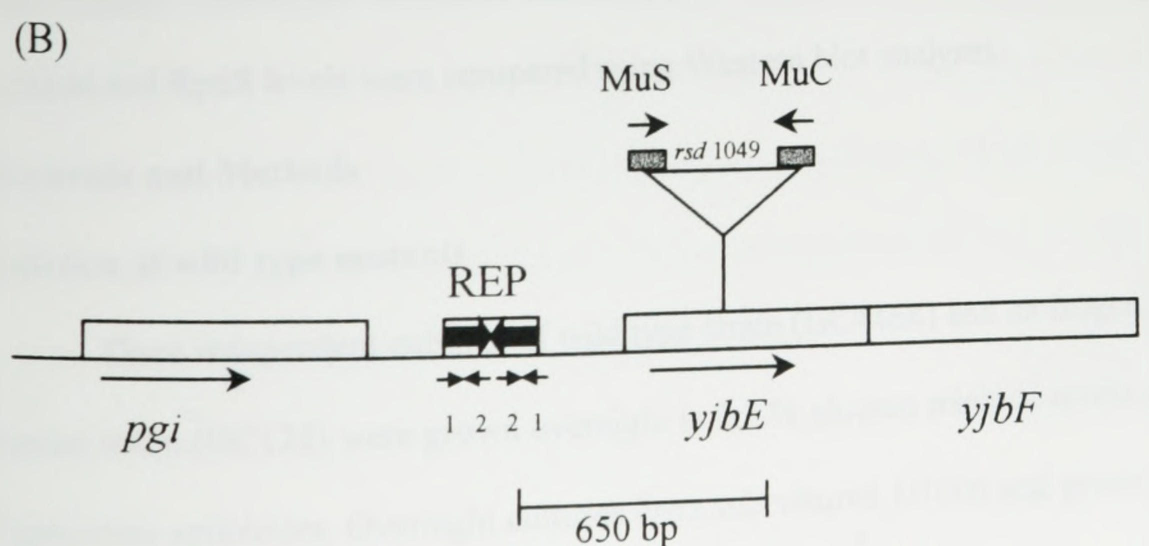
Using different combinations of REP and Mu c primers, PCR amplification was performed on DNA from *rsd*1049, *rsd*1054 and *rsd*1085 templates (Figure 1). Only *rsd*1049 produced a unique PCR product 650 bp in size using the REP-2 and Mu c primer combination. No unique products were observed in the other two reactions. Based on our sequence analysis, a 350 bp and 115 bp PCR product were predicted for *rsd*1054 and *rsd*1085. However, it is possible that a smaller product was amplified that was simply not visible on our agarose gel. Sequence analysis of the *rsd*1049 PCR product confirmed the results produced from sequencing the phage DNA directly (data not shown). PCR amplification of the top ten *rsd* fusions was also attempted using these primer pair combinations. However, after numerous failed attempts, this work was eventually abandoned. The failure to obtain Mu c- REP products may be due to the inability to obtain

PCR conditions favorable to Mu c-REP amplification. Alternatively, if Mu c-REP products were generated, they were too small to visualize on 0.7% agarose gels. If this is the case, sequence of these PCR products is unlikely to have generated useful information.

Figure 1. REP-PCR amplification of *rsd* 1049, *rsd*1054. *rsd* 1085. (A) DNA from each strain were PCR amplified using ten different primer-pair combinations: **1.** MuC only **2.** MuC and REP1R-1 **3.** MuC and REP1R-1 **4**. MuC and REP2-1 **5**. REP1R-1 only **6.** REP1R-1 and REP1R-1a **7.** REP1R-1 and REP2-1 **8.** REP1R-1a only **9.** REP1R-1a and REP2-1 **10.** REP 2-1 only. (B) Schematic representation of the REP-PCR amplification of *rsd* 1049. REP arrows indicate direction of REP 1R-1 primer and REP-2 primer respectively.


(A)
1 kb ladder
rsd 1049
rsd 1054
rsd 1085
1 kb ladder
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8




(B)
MuS
MuC
rsd 1049
REP
1 2 2 1
pgi
yjbE
yjbF
650 bp

Appendix 2

Characterization of *rpoS* mutants isolated from succinate minimal media

Introduction

As discussed in Chapter 2, *rsd*1010 map to *gabD*, while *rsd*1057 and *rsd*1058 maps to *gabP*. These genes encode enzymes in the γ-aminobutyrate (GABA) degradation pathway. GABA is the product of the glutamate decarboxylase reaction and is an intermediate in the arginine utilization pathway. To identify phenotypic characteristics, wild type and GABA mutants were replica plated onto minimal media supplemented with various carbon sources. While characterizing these mutants, it was observed that $rpoS^-$ mutant colonies were twice as large as $rpoS^+$ wild type colonies on 1% succinate minimal plates. $rpoS^-$ mutants were also characteristically brown in colour. This suggested that enhanced growth was related to the loss of RpoS. To test this, several spontaneous wild type "mutant" strains that exhibited enhanced growth on succinate minimal plates were isolated and RpoS levels were compared using Western blot analyses.

Materials and Methods

Isolation of wild type mutants

Three independent cultures of wild type strain (GC4468) and an isogenic $rpoS^-$ mutant strain (GC122) were grown overnight in 0.2 % glucose minimal media containing appropriate antibiotics. Overnight cultures were subcultured 1/1000 and grown to an

OD_{600} of 0.2 before plating. Cultures were centrifuged at 3000 rpm and the pellets were resuspended in M9 salts buffer. Samples were appropriately diluted in M9 salts buffer and plated on 1% succinate minimal media in triplicate as well as 0.2% glucose minimal media (control) and enumerated after 24h.

Wild type colonies which appeared larger than other colonies (comparable to GC122 colonies) were selected (usually from the 10^{-3} and 10^{-4} plates). Colonies were re-streaked onto both LB plates and succinate minimal media plates three times in order to purify the "mutant" colonies and to ensure that the phenotype was stable.

To assay RpoS activity, putative wild type mutants were tested for catalase activity by flooding the colonies with 30% hydrogen peroxide and monitored for bubbling in comparison to wild type.

Western Blot Analysis

Cultures for Western blot analysis were grown overnight in LB media. Overnight cultures were diluted 1/1000 into fresh pre-warmed LB media and grown to an OD_{600} ~1.3. Samples of 0.25 to 0.5 ml were taken from cultures, centrifuged and the pellet was resuspended in 50µl of lysis buffer (1 ml H_2O, 0.2 ml glycerol (80 %), 0.4ml SDS (10 %), 0.1 ml β-Mercaptoethanol and bromphenol blue). Approximately 15 µg of each sample was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel and run for 45 min at 200 V. Proteins were transferred onto nitrocellulose membranes. For gel electrophoresis and blotting, the modules of a Bio-Rad Mini-Protean device were used in accordance with the instructions of the manufacturer. For immunostaining, a polyclonal antibody against σ^s and a secondary antibody conjugated to horseradish peroxidase were

used.

Results and Discussion

Five wild type Mutants (M1, M2, M3, M4, M 5) were selected. These mutants were phenotypically larger than other wild type colonies and characteristically brown on succinate minimal plates (See table 2). Mutant strains were tested for catalase activity and all but one (M1) exhibited reduced catalase activity in comparison to wild type. Since the gene responsible for catalase activity (*katE*) is regulated by RpoS, this was the first indication that mutant strains may have also lost a functional RpoS protein.

Western blot analyses were in good agreement with the catalase activity of each mutant (Figure 1). As expected, the wild type strain (GC4468) contained high levels of RpoS protein while an *rpoS* mutant (GC122) and an *rpoS* amber mutant strain (AB1157) contained low levels of protein. RpoS levels in M1 were comparable to wild type. M2 and M3 expressed reduced RpoS levels while M4 appeared to lack RpoS altogether. Interestingly, the RpoS band for M5 was slightly lower than the other RpoS bands. This observation suggests that M5 may express an altered or attenuated form of RpoS. Thus, reduced expression of RpoS in these mutants demonstrate that the increased growth on succinate minimal media is directly related to the loss of RpoS. It is tempting to conclude that under wild type conditions, RpoS represses some factor which is required for growth on a poor carbon source such as succinate. However, the true explanation is likely far more complex.

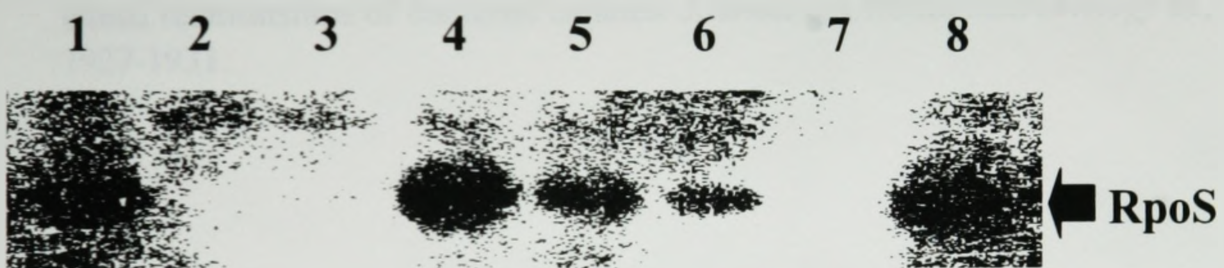
It has been well documented that *rpoS* is prone to mutations (See section 1.3) under certain conditions. Since *rpoS* mutants exhibits a distinct phenotype on succinate

minimal media, this method may be utilized in future research to study the rate of spontaneous *rpoS* mutant formation.

Table 1. Characterization of wild type mutants on 1 % succinate minimal media

Strain	Phenotype	Catalase Activity
GC4468	small, white colonies	+++
GC122	large, brown colonies	+
AB1157	large, white colonies	+
M1	intermediate, brown size	++
M2	large, brown colonies	+
M3	intermediate size, brown colonies	++
M4	large, brown colonies	+
M5	large, brown colonies	+

Figure 1. The levels of RpoS protein are reduced in wild type mutant strains isolated on 1% succinate minimal media plates. Cell extracts were prepared from stationary phase cultures (OD_{600} ~1.3) and probed with anti-RpoS antisera. Lane 1, wild type (GC4468); lane 2, RpoS mutant (GC122); lane 3, RpoS amber mutant (AB1157); lanes 4-8, wild type mutants (M1-M5).


1 2 3 4 5 6 7 8
RpoS

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Thode
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