MODULATION OF RPOS EXPRESSION BY

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AN INDUCIBLE RPOS SENSE AND ANTISENSE

ON A HIGH-COPY PLASMID

AND

AS A SINGLE COPY

IN THE ESCHERICHIA COLI CHROMOSOME

By

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TITLE: Modulation of RpoS Expression by an Inducible *rpoS* Sense and Antisense
     on a High-Copy Plamid and as a Single Copy in the *Escherichia coli*
     chromosome

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Abstract

Escherichia coli and several other bacteria express a stationary phase sigma factor, RpoS, for RNA polymerase that is responsible for inducing the expression of stress response genes. *rpoS* expression is induced during early exponential phase and the concentration of RpoS dramatically increases during the transition from log phase to stationary phase. The goal of this study was to test whether *rpoS* expression could be modulated using an inducible *rpoS* antisense and *rpoS* sense.

In the first part of this study, a *rpoS* antisense under the control of an IPTG-inducible promoter was tested for its efficiency for modulating the expression of the RpoS regulon. RpoS-dependent and RpoS-independent *lacZ* fusions were utilized to quantify the effect of *rpoS* antisense expression on *rpoS* translation. Unlike an earlier study, the results of this study suggest that the *rpoS* antisense was not induced and/or that it was not inhibiting the expression of RpoS-dependent genes.

In the second part of this study, an IPTG-inducible *rpoS* sense was used to test whether expression of certain members of the RpoS regulon were solely dependent on RpoS and not additional factors present in stationary phase. Thus, their expression could be induced in exponential phase. The *rpoS* sense was integrated into the *E. coli* chromosome at the *ebg* locus by homologous recombination utilizing a one-step PCR method. Some putative *rpoS* sense recombinants showed an increase in catalase expression, which is known to be RpoS-dependent. However, upon further verification, the 5kb PCR fragment encoding the inducible *rpoS* sense did not appear to have integrated to the intended site.

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Finally, I am grateful to Allah for giving me the strength to endure all the difficult times life has thrown my way and overcoming them all with my head held high.

Dedication

To my kid brother, your sister loves you very much.

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List of Abbreviations

bp	base pair
ddH$_2$O	deionized distilled water
°C	Degrees Celsius
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
g	gram
hr	hour(s)
IPTG	isopropylthio-β-D-galactoside
kb	kilobase pair
kg	kilogram
kV	kilovolt
λ	Lambda
LB	Luria-Bertani
mRNA	messanger RNA
μg	microgram
μl	microlitre
max	maximum
mg	milligram
ml	millilitre
mM	millimolar
min	minute(s)
M	molar
ng	nanogram
nm	nanometer
nt	nucleotide(s)
OD 420	Optical Density at 420nm
OD 600	Optical Density at 600nm
O/N	overnight
ONPG	o-nitrophenyl-β-D-galactopyranoside
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
sec	second(s)
SDS	Sodium Dodecyl Sulfate
rpm	revolutions per minute
Rnase	ribonuclease
RNA	ribonucleic ccid
UV	ultraviolet
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: Introduction

Microorganisms encounter many types of stresses in their local environment. For long-term survival under environmental stresses bacteria undergo numerous physiological changes (Kolter *et al.*, 1993). Bacteria enter a state referred to as stationary phase; a period during which cultures no longer show a net growth (Kolter *et al.*, 1993). Only metabolic processes essential for survival are functioning during this phase. In *Escherichia coli*, the cell morphology changes from rod-shaped to spherical (Lange and Hengge-Aronis, 1991a), largely due to the action of the BolA morphoprotein (Lange and Hengge-Aronis, 1991a), but the physiological advantage to having a spherical shape in stationary phase is unclear. Additionally, the chromosomal DNA becomes relaxed (Balke and Gralla, 1987), however, the physiological function of this is unknown but it may play a role in regulating gene expression. In several bacteria, these physiological changes are regulated by an alternative sigma factor for RNA polymerase (Loewen and Hengge-Aronis, 1994).

1.1 RpoS, the stationary phase sigma factor for RNA polymerase

RpoS, also called σ^S and σ^{38}, is one of the alternative sigma factors for RNA polymerase found in *E. coli* (Lange and Hengge-Aronis, 1991b). It was first identified as Nuv, for near-UV resistance (Tuveson and Jonas, 1979). It was also identified as KatF, a regulator for HPII (Loewen and Triggs, 1984; Sak *et al.*, 1989); AppR, a regulator of acidic phosphatase (Touati *et al.*, 1986); and Csi-2, a carbon starvation-inducible regulator (Lange and Hengge-Aronis, 1991b). All these regulators were eventually discovered to be alleles of the same gene, and the gene was subsequently renamed *rpoS*

when it was identified as a sigma factor.

Under conditions such as nutrient deprivation, high levels of hydrogen peroxide, hyperosmolarity, and/or achange in pH or temperature, σ^S replaces the housekeeping sigma factor, σ^{70}, also called RpoD, bound to the core RNA polymerase (Loewen and Hengge-Aronis, 1994). σ^{70} is the sigma factor responsible for the transcription of genes related to cell growth during exponential phase. It also has the strongest binding affinity for the core RNA polymerase (Maeda *et al.*, 2000). Other sigma factors, σ^N, σ^F, σ^E, σ^{FecI}, and σ^H, in *E. coli* have much lower binding affinities for the core enzyme when no other ancillary factors are present; σ^S has the lowest binding affinity. Nonetheless, during stationary phase, RpoS directs the transcription of its regulon that is composed of over 60 stress response genes (Hengge-Aronis, 2002).

1.1.1 Characterization of RpoS

RpoS, a 38-kDa protein, is encoded by the *rpoS* gene, which has an open reading frame (ORF) of 1026bp (Loewen and Hengge-Aronis, 1994). *rpoS* is located at 61.78 min in the *E. coli* chromosome in an operon with *nlpD* (Hengge-Aronis, 2002). *nlpD* encodes a lipoprotein (NlpD) that has a function in cell wall formation; However, its expression is not stationary phase dependent like *rpoS* (Lange and Hengge-Aronis, 1994b).

1.1.2 Regulation of RpoS expression

The expression of RpoS is controlled at four different levels: the transcriptional level, translational level, proteolysis and protein stability, and its activity (Hengge-Aronis, 2002). However, a complete understanding of the regulatory mechanisms of

RpoS has not been achieved.

Transcriptional Regulation of *rpoS*

During early exponential phase, *rpoS* transcription is induced (Schellhorn *et al.*, 1998). Factors such as promoters, cAMP-CRP, BarA, polyphosphates, and small compounds such as ppGpp, homoserine lactone and homocysteine thiolactone, and weak acids play an important role in the regulation of *rpoS* transcription.

Structure of the Promoter Region of *rpoS*

The transcription of *rpoS* occurs under the control of three promoters (Hengge-Aronis, 2002). The two promoters (P1$_{nlpD}$ and P2$_{nlpD}$) located upstream the *nlpD* gene allow for basal level transcription of *rpoS* (Figure 1). The promoter mainly responsible for *rpoS* transcription during entry into stationary phase (P3$_{rpoS}$) is located 567 nucleotides upstream of the AUG start codon of *rpoS* within the *nlpD* ORF (Takayanagi *et al.*, 1994). Thus, upon entry into stationary phase the level of *rpoS* transcription significantly increases (Loewen *et al.*, 1998).

cAMP-CRP

In an experiment conducted with Δ*cya* and Δ*crp* mutants, it was found using *rpoS-lacZ* transcriptional fusions that the level of *rpoS* transcription was relatively high during exponential phase (Lange and Hengge-Aronis, 1991b). *cya* encodes for adenylate cyclase and *crp* encodes for the cyclic AMP (cAMP) receptor protein (CRP). In the Δ*cya* mutant, the level of *rpoS* could be reduced by the addition of an exogenous supply of cAMP (Lange and Hengge-Aronis, 1994a). This appears to indicate that *rpoS*

transcription is negatively regulated by cAMP-CRP. Two putative CRP binding sites are shown to flank the *rpoS* promoter, P3$_{rpoS}$ (Takayanagi *et al.*, 1994). These binding sites may play a role in regulating *rpoS* transcription.

BarA

BarA, a histidine sensor kinase, has been proposed to be a two component regulator in *E. coli* (Nagasawa *et al.*, 1992). Recently, the response regulator for BarA was identified to be UvrY (Pernestig *et al.*, 2001). Previous to this no known function had been characterized for the protein encoded by *uvrY* (Moolenaar *et al.*, 1987). This gene received this name due to its close linkage to the *uvrC* gene that encoded a subunit of UvrABC, which plays a role in UV-light-induced DNA repair (Moolenaar *et al.*, 1987). However, a *uvrY* mutant showed no effect on this DNA repair system (Moolenaar *et al.*, 1987).

In an isolated hydrogen peroxide sensitive mutant that carried a *lacZ* fusion in the *barA* gene, the level of *katE* expression was found to be reduced (Mukhopadhyay *et al.*, 2000). Since KatE (HPII) is RpoS-dependent (Schellhorn and Hassan, 1988), it was concluded that *rpoS* expression must also be significantly lowered (Mukhopadhyay *et al.*, 2000). This was confirmed by Western and Northern analysis. This effect on *rpoS* expression was seen during exponential phase and not during stationary phase (Mukhopadhyay *et al.*, 2000). It was noted using *rpoS-lacZ* and *barA-lacZ* fusions that the expression of *barA* was induced prior to *rpoS* (Mukhopadhyay *et al.*, 2000). The results of this study suggested that BarA positively regulates *rpoS* at the transcriptional

level.

Polyphosphates

Polyphosphates are synthesized by polyphosphate kinase, encoded by *ppk*, and degraded by exopolyphosphatases, encoded by *ppx* and *gppA* (Kornberg *et al.*, 1999). In *ppk* mutants, there is a deficiency in polyphosphate concentration, which leads to increased sensitivity to stress during stationary phase (Rao and Kornberg, 1996). Overexpression of an exopolyphosphatase, from a plasmid carrying the *ppx* gene, caused cells to become more sensitive to hydrogen peroxide (Shiba *et al.*, 1997). KatE, more commonly referred to as HPII, is a catalase that is stationary phase and RpoS-dependent (Schellhorn and Hassan, 1988). Using a transcriptional *rpoS-lacZ* fusion, it was shown that *rpoS* levels decreased with a reduction of polyphosphates (Shiba *et al.*, 1997). Thus, it was concluded that the polyphosphates induce *rpoS* transcription.

ppGpp

Under starvation conditions, many amino acids become limiting, resulting in an increase in the level of uncharged transfer RNA (tRNA) (Gentry *et al.*, 1993). This increase is detected by RelA (ppGpp synthase I) or SpoT (ppGpp synthase II). As a result, the synthesis of guanosine-3',5'-bispyrophosphate (ppGpp) is significantly increased in stationary phase. In a $\Delta relA \Delta spoT$ (ppGpp⁻) background, expression of a *rpoS-lacZ* transcriptional fusions was decreased 4-fold relative to a wildtype (ppGpp⁺) strain, revealing that rpoS is positively regulated by ppGpp levels in the cell (Lange *et al.*, 1995).

Also, using promoter deletion constructs in the same background, it appears that ppGpp affects the elongation and stability of the *rpoS* transcript and not the initiation of its transcription (Lange *et al.*, 1995). A more recent study indicated that in a similar *ΔrelAspoT* (ppGpp⁻) background, *rpoS* expression was also increased during exponential phase (Hirsch and Elliott, 2002). This result seems to suggest that ppGpp affects *rpoS* expression at the basal level and not just during entry into stationary phase.

An increase in the intracellular levels of ppGpp also results in the accumulation of polyphosphates (Kuroda *et al.*, 1997), which have been shown to induce *rpoS* expression *in vivo* but not *in vitro* (Shiba *et al.*, 1997). Thus, it is yet to be determined whether ppGpp directly or indirectly affects *rpoS* expression (Hengge-Aronis, 2002).

Homoserine lactone and Homocysteine thiolactone

Homoserine lactone (HSL) is a metabolite formed by intermediates in the threonine biosynthetic pathway (Huisman and Kolter, 1994). In a *thrA metL lysC* triple mutant *E. coli* strain, which is deficient in enzymes that lead to the synthesis of lysine, methionine, threonine, and isoleucine, the cellular concentration of *rpoS* is low (Huisman and Kolter, 1994). It was determined that the addition of HSL from an external source could reverse the effect of the triple mutation. However, the HSL-dependent bioluminescence in *Vibrio fischeri* is repressed by the overproduction of RspA, a protein homologous to catabolic enzymes (Huisman and Kolter, 1994). This suggests that HSL may simply play a role in signaling for starvation.

Homocysteine thiolactone (HCTL) is another cyclic metabolite that alters *rpoS*

expression (Goodrich-Blair and Kolter, 2000). In a *metE* mutant that is deficient in an enzyme that converts homocysteine to methionine, there is an accumulation of HCTL. The elevated levels of HCTL result in an increase in *rpoS* levels. However, in an *asd* mutant that is deficient earlier in the biosynthetic pathway of methionine, homocysteine and HTCL accumulation resulting in a decrease in the level of *rpoS* transcription (Goodrich-Blair and Kolter, 2000). It was determined that *rpoS* levels could increase with exogenous HCTL. By monitoring HCTL synthesis, it was determined that HCTL accumulates during entry into stationary phase, more so than that in exponential phase (Goodrich-Blair and Kolter, 2000). Thus, it was concluded that HCTL does regulate *rpoS* expression at the onset of stationary phase.

Weak acids

It was shown in an earlier study that benzoate, a weak acid, induces the transcription of *rpoS* (Mulvey *et al.*, 1990). In another study using *katE-lacZ* and *rpoS(katF)-lacZ* transcriptional and translational fusions, it was noted that weak acids, including acetate, propionate, and benzoate, present in the supernatant of the bacterial culture induced *katE* and *rpoS(katF)* during entry into stationary phase (Schellhorn and Stones, 1992). The same effect was noted when exponential phase cells were treated with weak acids (Schellhorn and Stones, 1992).

<u>**Translational Regulation of *rpoS***</u>

Once *rpoS* has been transcribed, there are several regulatory mechanisms in place to accelerate or inhibit the translation of *rpoS* by ribosomes. The secondary structure of

rpoS mRNA and other factors such as Hfq (HF-I), HU, H-NS, small antisense RNAs,

LeuO, and UDP-Glucose help regulate the translation of rpoS.

Secondary Structure of rpoS

rpoS mRNA has a large untranslated region at the 5' end of the transcript

(Takayanagi et al., 1994). Deleting different lengths of sequence from the 5' region of the

rpoS mRNA exhibited varying effects on rpoS translation (Cunning et al., 1998). Thus, it

was concluded that the leader sequence plays an important role in rpoS expression. The

secondary structure of the rpoS transcript is predicted to be composed of several large

hairpins that give it a cruciform-like appearance (Hengge-Aronis, 2002). It has been

indicated that the sequence of the ribosome binding site (RBS) is base paired to an

"internal antisense" region that prevents ribosomes from translating the rpoS mRNA

(Cunning et al., 1998; Hengge-Aronis, 2002). However, the RBS can be released with the

aid of small regulatory RNA molecules that will be discussed below.

Hfq

Hfq, also known as HF-I, is encoded by the hfq gene and was first identified to be

a host factor required for phage Qβ RNA replication (Franze de Fernandez et al., 1972).

However, more recently, a role for Hfq in rpoS expression has been revealed. In an hfq⁻

mutant, the level of rpoS translation was lowered. This was determined using an rpoS-

lacZ gene fusion and a pulse-chasing experiment (Muffler et al., 1996a). A similar

conclusion was reached in a study using Salmonella typhimurium (Brown and Elliott,

1996). In a later study, it appeared that Hfq may act as a global regulator since it also

alters the expression of RpoS-independent genes (Muffler *et al.*, 1997a).

The exact mechanism of Hfq action still remains unclear. It has been noted that that Hfq binds to several locations in the 5' untranslated region of the *rpoS* mRNA (Cunning *et al.*, 1998), suggesting that it some how stimulated the translation of *rpoS*. However, single point mutations in this region that would disrupt the secondary structure of the *rpoS* transcript showed less of a dependence on Hfq for *rpoS* translation (Brown and Elliott, 1997). It is possible that Hfq attaches to the *rpoS* mRNA and recruits other factors (such as HU and small regulatory RNA molecules [see below]) that play a role in translational regulation of *rpoS*.

HU

In *E. coli,* HU is a histone-like protein that is composed of two homologous subunits, HUα and HUβ and is found in the bacterial nucleiod (Rouviere-Yaniv *et al.*, 1979). It has been characterized to negatively supercoil relaxed circular DNA. The heterodimeric form, HU$\alpha\beta$, is induced just prior to stationary phase (Claret and Rouvière-Yaniv, 1997). Thus, HU also appears to be required for long-term survival in starvation conditions.

In a HU-deficient mutant, it was found that there was a significant reduction in levels of RpoS (Balandina *et al.*, 2001). *In vitro*, HU binds to regions in the 5' untranslated region of *rpoS* mRNA (Balandina *et al.*, 2001). An earlier study indicated HU binds to nicked and cruciform DNA (Kamashev *et al.*, 1999). Thus, HU probably binds to the secondary structure of the *rpoS* transcript. These findings suggested that HU

plays a role in the up-regulation of *rpoS* translation.

H-NS

H-NS is another histone-like protein associated with nucleoid organization like HU (Williams and Rimsky, 1997). It is also composed of two homologous subunits similar to HUα and HUβ. In H-NS mutants, the level of RpoS was increased in exponential phase to levels similar to that observed in stationary phase (Barth *et al.*, 1995; Yamashino *et al.*, 1995). The rate of *rpoS* translation increased as RpoS degradation decreased (Yamashino *et al.*, 1995). This elevation in RpoS level resulted in slower growth and genetic instability in the H-NS mutants (Barth *et al.*, 1995). Thus, it was concluded that H-NS down-regulates *rpoS* translation. However, the mechanism of action is not yet clear.

In an *hfq* background that is also deficient in H-NS, the level of *rpoS* translation is unaffected (Muffler *et al.*, 1996a). This suggests that H-NS may inhibit *rpoS* translation by preventing Hfq from initiating the translation of *rpoS*. A recent study suggests that H-NS may alter the stability of the *rpoS* mRNA and make it more susceptible to degradation (Brescia *et al.*, 2004). Thus, as a result, possibly affecting the translation of the *rpoS* mRNA.

Small Antisense RNAs

In *E. coli*, there are many small, untranslated RNA molecules that regulate gene expression (Altuvia and Wagner, 2000; Gottesman, 2002; Massé *et al.*, 2003a). *rpoS* translation appears to be affected by three such small regulatory RNAs: *dsrA*, *oxyS*, and

rprA.

dsrA

dsrA was first identified to affect transcription initiation, directly or indirectly, of *rcsA*, which encodes a regulator for capsular polysaccharide synthesis. It was located in the downstream region of *rcsA* (Sledjeski and Gottesman, 1995). Thus, it was subsequently named *dsrA*.

dsrA is a 87-nt RNA that forms a three-stem-loop structure (Lease and Belfort, 2000; Majdalani *et al.*, 1998). It consists of a region that is complementary to the 5' untranslated region of the *rpoS* transcript that is important for secondary structure formation (Lease and Belfort, 2000; Majdalani *et al.*, 1998). Thus, *dsrA* binds this region preventing the formation of the secondary structure and allowing ribosomes to translate *rpoS*.

dsrA was determined to accelerate *rpoS* translation at low temperatures (Sledjeski and Gottesman, 1996) when the transcription and stability of *dsrA* is increased (Repoila and Gottesman, 2001). The synthesis of *dsrA* at low temperatures appears to be the result of specific elements such as the sequence of the −10 promoter region and the spacer region in the *dsrA* promoter (Repoila and Gottesman, 2003).

It had been suggested that Hfq stabilizes *dsrA* and alters its structure enabling *dsrA* to efficiently bind the *rpoS* leader sequence (Sledjeski *et al.*, 2001). Recently, the binding sites for Hfq on *dsrA* were identified (Brescia *et al.*, 2003). This study concluded that the secondary structure of *dsrA* was unaffected by the binding of Hfq. Therefore,

unaltering the ability of *dsrA* to bind the *rpoS* transcript.

oxyS

oxyS, a member of the OxyR regulon, is a 109-nt regulatory RNA that also affects the expression of *rpoS* (Altuvia *et al.*, 1997). OxyR induces the expression of genes required to deal strictly with increased hydrogen peroxide concentration (Christman *et al.*, 1989). Thus, *oxyS* is induced under conditions of oxidative stress.

In an earlier study, it was indicated that *oxyS* binds the *fhlA* transcript, which encodes a transcriptional activator, and inhibits its translation (Altuvia *et al.*, 1998). It does this by forming a "kissing complex" with two sites of the *fhlA* mRNA that blocks the RBS (Argaman and Altuvia, 2000). However, this does not appear to be the same mechanism used to alter *rpoS* expression.

oxyS inhibits the translation of *rpoS* by interacting with a long A-rich region located between two hairpin loops in the *rpoS* transcript (Zhang *et al.*, 1998). This occurs in an indirect manner as there is no complementary sequence present in this region between *oxyS* and the *rpoS* mRNA. Using co-immunoprecipitation experiments it was determined that Hfq was bound to *oxyS* (Zhang *et al.*, 1998). Earlier on, Hfq had been revealed to stimulate *rpoS* transcription (Muffler *et al.*, 1996a; Brown and Elliott, 1996). It has been suggest *oxyS* sequesters Hfq; Thus, preventing Hfq from affecting the expression of *rpoS* (Zhang *et al.*, 1998). This may be to inhibit the expression of RpoS-dependent genes that deal with oxidative stress since OxyR-dependent genes can perform the same function without depleting as many resources (Hengge-Aronis, 2002).

rprA

rprA (Rpo̱S r̲egulator RNA) is a 106-nt regulatory RNA that was also discovered to affect *rpoS* expression (Majdalani *et al.*, 2001). In a *dsrA* mutant, the deletion of *rprA* resulted in lowered levels of *rpoS* translation. This effect was not observed when *dsrA* was present. Recently, a study indicated that *rprA* binds to the same 5' untranslated region as *dsrA* (Majdalani *et al.*, 2002).

However, the expression of *rprA* is not induced at low temperature. It is induced by the RcsC/RcsB phosphorelay system that triggers capsule synthesis (Majdalani *et al.*, 2002). Thus, it was suggested that *rprA* was synthesized in response to cell surface stress (Majdalani *et al.*, 2002).

LeuO

In *E. coli*, H-NS represses the expression of LeuO (Klauck *et al.*, 1997), a LysR-like regulator, which is an autoregulatory transcriptional regulator (Schell, 1993). *rpoS* translation has been shown to decrease in the presence of excess LeuO (Klauck *et al.*, 1997). This effect is particularly evident at low temperatures. It appears that *dsrA* transcription is inhibited by LeuO, which prevents *dsrA* from accelerating *rpoS* translation (Klauck *et al.*, 1997). Recently, binding sites for LeuO were located in the promoter region of *dsrA* (Repoila and Gottesman, 2001). However, the exact role of LeuO is still to be determined.

UDP-glucose

In several UDP-glucose deficient mutants, the level of RpoS was significantly

increased during exponential phase (Böhringer *et al.*, 1995). If these mutants were supplemented with glucose or galactose, then the concentration of UDP-glucose returned to normal (Böhringer *et al.*, 1995). This resulted in a decline in RpoS expression.

Recently, using transcriptional and translational *rpoS-lacZ* fusions and a pulse chase experiment to measure the amount of RpoS formed, it was determined that UDP-glucose does affect the translation of *rpoS* (Hengge-Aronis, 2002). However, the mechanism by which UDP-glucose regulates *rpoS* translation remains unclear.

Regulation of RpoS Proteolysis and Protein Stability

During stationary phase, the half-life of RpoS is increased from 1.4 min to 25 min (Lange and Hengge-Aronis, 1994a). Thus, it becomes a more stable protein. The cellular level of RpoS is maintained by controlling its proteolysis and stability by factors such as ClpXP, RssB, and DnaK.

ClpXP

ClpXP is a barrel-shaped protease composed of two parts; ClpX is an ATP-hydrolyzing, chaperone protein that targets the protein for degradation and sends it the internal component, ClpP, which performs the actual degradation (Kim *et al.*, 2000). RpoS has been shown to be degraded by ClpXP protease (Scheweder *et al.*, 1996).

In *clpX* and *clpP* mutants, RpoS levels in exponential phase increased to levels found in wild-type cells during stationary phase (Scheweder *et al.*, 1996). However, in ClpP-deficient mutants, RpoS became completely stable during stationary phase (Scheweder *et al.*, 1996). This suggested that both components were required for the

complete degradation of RpoS to occur.

In cells expressing ClpXP, RpoS appeared to become more stable during stationary phase than it was in log phase (Scheweder *et al.*, 1996). This increase in stability was determined not to be a result of a decrease in ClpXP concentration. Therefore, it was suggested that this increased stability of RpoS was due to other factors present in stationary phase (see below).

RssB

RssB is a response regulator that has been shown to de-stabilize RpoS, in *E. coli* (Muffler *et al.*, 1996b; Zhou *et al.*, 2001). In an *rssB* mutant, RpoS became more stable; therefore, levels of RpoS increased during log phase (Muffler *et al.*, 1996b). A recent study suggested that RssB was the rate-limiting factor for RpoS degradation (Pruteanu and Hengge-Aronis, 2002). It appeared that the expression of RssB was continuously being adjusted to maintain RpoS degradation as the rate of RpoS expression also increased. However, in stationary phase, RssB was not able to maintain the same rate of RpoS degradation as in log phase (Pruteanu and Hengge-Aronis, 2002), therefore dramatically increasing levels of RpoS due to its increased stability.

A sensor kinase has not yet been identified for RssB. However, a study has suggested that acetyl phosphate, a small molecule produced from acetyl coenzymeA by phosphotransacetylase, phosphorylates RssB (Bouché *et al.*, 1998). Thus, in its phosphorylated form, RssB directly interacts with RpoS (Becker *et al.*, 1999).

To determine the region of RpoS that RssB interacts with, site-specific

mutagenesis was used to substituted single amino acid residues in RpoS (Becker *et al.*, 1999). The lysine(K)-173 residue in RpoS appeares to be critical for RssB to bind to RpoS (Becker *et al.*, 1999).

Comparison of promoter sequences between RpoS and RpoD revealed significant homology except for a region located, in the middle of the RpoS amino acid sequence, between methionine(M)159 and histidine(H)187 (Becker *et al.*, 1999). With further analysis, the RpoS "turnover element" was identified to be located slightly downstream of the –10 promoter-recognizing region (Becker *et al.*, 1999). This region is predicted to form a α-helix where the K173 residue of RpoS would be available to interact with RssB (Becker *et al.*, 1999).

A recent study has revealed that RssB binds this region causing the conformation of RpoS to change exposing a site near the N-terminus of RpoS for ClpX to bind (Studemann *et al.*, 2003). Once RpoS, RssB, and ClpX(P) have formed a complex, RssB is released (and recycled) and the RpoS degradation process is initiated (Klauck *et al.*, 2001; Zhou *et al.*, 2001).

The mechanism by which RssB is released is not yet understood. It may be the result of de-phosphorylation of RssB (Klauck *et al.*, 2001) or altering the conformation of the "turnover element" of RpoS during its unfolding by ClpX at the start of the degradation process (Becker *et al.*, 1999).

DnaK

DnaK is a chaperone protein that is induced under carbon starvation (Spence *et*

al., 1990). In *E. coli*, it appears to be crucial for resisting heat, oxidation and reductive division (Rockabrand *et al.*, 1995; Rockabrand *et al.*, 1998). *dnaK* mutants show a decrease in RpoS during stationary phase (Muffler *et al.*, 1997b; Rockabrand *et al.*, 1998). The level of RpoS in these *dnaK* mutants has been determined to be similar to that found in *rpoS* mutants. This mutation can be complemented by the overproduction of DnaK from an inducible plasmid (Rockabrand *et al.*, 1998), which results in a significant increase in levels of RpoS.

It appears that a change in the cellular concentration of DnaK causes a similar change in the level of RpoS. Thus, DnaK has been suggested to stabilize RpoS during stationary phase by making it less prone to degradation (Muffler *et al.*, 1997b; Rockabrand *et al.*, 1995; Rockabrand *et al.*, 1998). However, the mechanism of action still needs to be determined.

Regulation of RpoS Activity and Relative Levels of Binding with RNA Polymerase

Along with regulation of RpoS at the transcriptional, translational, and protein degradation and stability levels, the level of transcription of the RpoS regulon is affected by σ factor competition between RpoS and other σ factors present in the cell. Factors such as Rsd, ppGpp binding, and Crl are thought to affect competition between sigma factors for the core RNA polymerase.

σ factor competition

RpoS (σ^S) competes with six other sigma factors for binding to the core RNA polymerase and inducing transcription of RpoS-dependent genes (Maeda *et al.*, 2000). As

indicated earlier, RpoS has been determined to have the lowest binding affinity for the core enzyme (Maeda *et al.*, 2000). During stationary phase, RpoS manages to replace RpoD (σ^D), the vegetative sigma factor (Loewen and Hengge-Aronis, 1994). Yet, RpoD remains in higher concentration than RpoS (Jishage and Ishihama, 1995). There are several factors that have been proposed to improve the interaction of RpoS with RNA polymerase.

Rsd

It has been suggested that Rsd, the putative anti-RpoD factor, may allow RpoS the opportunity to bind RNA polymerase (Jishage and Ishihama, 1998; Jishage and Ishihama, 1999). In stationary phase cells, it was discovered that RpoD is complexed with a protein, which was subsequently named Rsd, Regulator of sigma D (Jishage and Ishihama, 1998). It was revealed that the concentration of Rsd increased over the transition from log phase to stationary phase (Jishage and Ishihama, 1998). Using *lacZ* reporter gene fusions, it was determined that overexpression of Rsd leads to an increase in the expression of RpoS-dependent genes and a decrease in RpoD-dependent gene expression (Jishage and Ishihama, 1999). The binding site of Rsd has been located to a region downstream of the promoter region of *rpoD* (Jishage *et al.*, 2001).

It appears that Rsd bound to RpoD reduces the affinity between RpoD and the core enzyme. The level of RpoD in the cell still remains high but it is unable to associate with RNA polymerase in order to transcribe RpoD-dependent genes during stationary phase. Thus, RpoS is able to bind the core enzyme and induce the expression of the RpoS

regulon. However, this is not thought to be the only factor that increases the binding affinity of RpoS for RNA polymerase.

ppGpp binding

There is some evidence indicating that modifications to the core RNA polymerase such as ppGpp binding improves the affinity of RpoS for the core enzyme (Toulokhonov *et al.*, 2001; Jishage *et al.*, 2002). It was mentioned earlier that ppGpp is found in abundance during nutrient deprivation (Gentry *et al.*, 1993). It has been revealed that ppGpp binds to the RNA polymerase causing an allosteric conformational change near the N-terminus of the β'-subunit of the holoenzyme (Toulokhonov *et al.*, 2001). *In vitro* and *in vivo* transcription/competition assays showed that the binding of ppGpp to the core enzyme leads to a decreased binding affinity for RpoD (Jishage *et al.*, 2002). Thus, this change to the structure of RNA polymerase leads to an increase in affinity for RpoS.

Crl

Crl was first identified as an inducer of the expression of curli fimbrae surface structures, encoded by the *csgBA* operon, that are required for cell aggregation and adhesion (Olsén *et al.*, 1989). In a later study, Crl was found to have an effect on RpoS-dependent genes (Pratt and Silhavy, 1998). In a *crl* mutant, the expression of *ompF*, a negatively RpoS-dependent gene, was increased during stationary phase similar to an rpoS null mutant (Pratt and Silhavy, 1998). After observing a similar effect on other RpoS-dependent genes, it was concluded that Crl directly or indirectly stimulated RpoS activity (Pratt and Silhavy, 1998).

Recently, it has been suggested that Crl does not actually influence the binding affinity of RpoS for RNA polymerase (Bougdour *et al.*, 2004). Rather, Crl bound to the RpoS-RNA polymerase complex enhances its binding affinity for the *csgBA* promoter. However, Crl appears to play a role in preventing the degradation of RpoS by ClpXP (Bougdour *et al.*, 2004).

1.2 Antisense RNAs

1.2.1 Characteristics of Antisense RNAs

In general, antisense RNAs are highly structured, untranslatable, small regulatory RNA molecules (as reviewed in Wagner and Simons, 1994; Brantl, 2002). They are usually cis- or trans-encoded from bacterial plasmids or chromosomes but they may also be encoded from phage and transposons. Antisense RNAs contain complementary sequence with their target mRNAs, which can be either partially or completely complementary. Sometimes, these antisense RNAs may even have multiple targets. As a result, these antisense RNAs can form a stable duplex with their target RNAs by complementary base-pairing. The consequence of this interaction is usually the inhibition of target gene translation. However, a few antisense RNAs have been discovered to promote the translation of the target gene.

Antisense RNAs are usually found to be 35 to 150 nt in length and form a secondary structure consisting of one to four stem loops (as reviewed in Wagner and Simons, 1994; Brantl, 2002). The loop domains are comprised of 5-8 nt and are usually GC rich. This loop size allows for interaction with loops of the target RNA that can

efficiently regulate gene expression (Hjalt and Wagner, 1992). They usually contain a U-turn structure that allows for rapid interaction with the target mRNA (Franch *et al.*, 1999). The stem domains most often contain a small bulge that interrupts the double-stranded region and allows for the formation of a stable RNA duplex (Hjalt and Wagner. 1995a; Kolb *et al.*, 2001). This bulge also prevents degradation of the antisense RNA by RNase III (Hjalt and Wagner. 1995b).

1.2.2   Antisense RNA Regulation in Prokaryotes

Several antisense RNAs have been discovered that inhibit the translation of bacterial genes (for review see Wagner and Simons, 1994; Wassarman *et al.*, 1999; Brantl, 2002; Wassarman, 2002.   Characteristics and mechanisms of action for some representative antisense RNAs are discussed in detail below.

<u>Regulation of Plasmid Function</u>

Antisense RNAs were discovered to regulate the replication, conjugation, and segregation of several bacterial plasmids. These antisense RNAs include RNAI, CopA, and Inc RNA.

Control of Plasmid Replication

RNA I

The multi-copy plasmid ColE1 requires the synthesis of a pre-primer, RNA II, before replication can be initiated (Itoh and Tomizawa, 1980). However, RNA I, inhibits the initiation of the plasmid replication (Tomizawa *et al.*, 1981). RNA I is a 108nt antisense RNA composed of three stem loops that are complementary to the 5' end of the

RNA II molecule (Tomizawa *et al.*, 1981; Lacatena and Cesareni, 1981). In order to inhibit replication it must bind to RNAII before RNAII acquires a more stable structure, termed structure IV (Masukata and Tomizawa, 1986).

RNA I and RNA II form a kissing complex where the stem loops of RNA I interact, reversibly, with the complementary sequence in RNA II (Tomizawa, 1984). Once this complex forms, stable base-pairing between the unbound 5'end of RNA I and RNA II is triggered (Tomizawa, 1984; Tomizawa, 1990). As a result, a conformational change in RNA II occurs making it unstable (Masukata and Tomizawa, 1986); thus, it prevents the formation of the primer necessary for plasmid replication to be initiated.

CopA

A copy control antisense RNA has been identified for the low-copy-number IncFII-like plasmids (e.g. R1) found in *E. coli* and other closely related bacteria (Scott, 1984). For the IncFII R1 plasmid, a 90nt antisense RNA (termed CopA) composed of two loops was determined to inhibit the translation of *repA*, which encodes the replication initiator protein RepA (Stougaard *et al.*, 1981).

The *repA* ORF was found to contain another ORF, the *tap* ORF, located slightly downstream of the CopA binding site (Blomberg *et al.*, 1992). The RBS of *repA* was determined to overlap with the 3'end of *tap*. This region was identified to form a stem loop structure (Blomberg *et al.*, 1994). Due to the secondary structure, the *repA* RBS remained inaccessible to ribosomes until *tap* translation was initiated.

CopA was thought to directly inhibit *repA* translation by binding to a small region

of the *repA* mRNA leader sequence (termed CopT) (Light and Molin, 1983). However, it was determined that the CopA-CopT interaction prevented *tap* translation (Blomberg *et al.*, 1992). The stem loops II of CopA and CopT interacted together to form a kissing complex (Persson *et al.*, 1990). Once this was established, the single-stranded regions located between the stem loops of CopA and the complementary region in CopT base-paired to form a stable RNA duplex. As a result, *tap* translation was not initiated and the RBS binding site for *repA* remained inaccessible (Persson *et al.*, 1990). Thus, CopA indirectly inhibited the synthesis of the *repA* leader peptide needed for translation.

Inc RNA

ColIB-P9, a type of Incα plasmid, is found in a few copies in *E. coli* and other enterobacteria (Clewell and Helinski, 1970). The plasmid requires the replication activator protein, RepZ, to initiate plasmid replication (Hama *et al.*, 1990a). The translation of *repZ* requires the formation of leader peptide and pseudoknot formation to free the RBS located in a stem loop. A pseudoknot forms when base-pairing occurs between the stem of stem loop III and the loop of stem loop I (Wagner and Simons, 1994). However, *repZ* translation can be inhibited by a 70nt antisense RNA referred to as Inc RNA (Shiba and Mizobuchi, 1990).

The region of Inc RNA that interacts with *repZ* forms a large stem loop structure (Hama *et al.*, 1990; Asano *et al.*, 1998). This stem loop binds to the complementary sequence located in the stem loop II of *repZ* (Asano *et al.*, 1998). The interaction between Inc RNA and its target sequence prevents the formation of the pseudoknot; Thus,

preventing the disruption of the stem loop structure to release the *repZ* RBS to allow its translation.

Control of Transcription

RNAIII

In its gram-positive host, the replication of staphylococcal plasmid pIP501 is controlled by the expression of the RNAIII antisense RNA (Brantl *et al.*, 1993; Brantl and Wagner, 1996). This is achieved by inhibiting expression of RNAII, which is encoded by *repR* (Brantl *et al.*, 1993). RNAIII was determined to be 136nt long and comprise of four stem loop structures and a single-stranded region between loops II and III (Brantl and Wagner, 1994). Due to the stable nature of RNAIII antisense RNA, CopR regulates its expression (Brantl, 1994).

RNAIII was found to contain sequence complementary to the *repR* mRNA leader (Brantl *et al.*, 1993). It interacts with this complementary region and triggers a conformational change in the nascent *repR* mRNA (Brantl and Wagner, 1994; Brantl and Wagner, 1996). The formation of a terminator stem loop causes the premature termination of *repR* transcription (Brantl *et al.*, 1993) preventing transcript elongation and inhibiting the translation of *repR*. Therefore, this mechanism allows for earlier repression of *repR* translation (Brantl and Wagner, 1994). This antisense RNA-mediated transcriptional attenuation was also demonstrated to function in *E. coli* (Brantl and Wagner, 2002).

Control of Plasmid Conjugation

FinP

For plasmid conjugation to occur the conjugal transfer operon must be transcribed. This operon is activated by TraJ, which is translated from *traJ* mRNA (Finnegan and Willetts, 1973). However, the translation of *traJ* can be inhibited by FinP, a 72nt antisense RNA (Koraimann *et al.*, 1996).

FinP is composed of a short single-stranded region followed by two stem loops (van Biesen et al., 1993). It has exact sequence complementarity to the 5' untranslated region of *traJ*, which includes the *traJ* RBS (Koraimann *et al.*, 1991). The central nucleotides of the two stem loops of FinP form a kissing complex with the stem loops of *traJ* (Koraimann *et al.*, 1996). Further base-pairing of *traJ* and FinP (van Biesen and Frost, 1994) and the stability of the *traJ*-FinP complex is promoted by the FinO protein (van Biesen and Frost, 1994; Jerome *et al.*, 1999), which results in the blockage of the *traJ* RBS, inhibiting *traJ* translation.

Control of Killer Gene Expression

Sok

For plasmid maintenance, several bacterial plasmids utilize host-killing systems such as the *hok/mok/sok* system of plasmid R1 (Gerdes *et al.*, 1997). Hok RNA (host killer) encodes a killer protein that damages the cell membrane and eventually causes cell death (Gerdes *et al.*, 1986). However, *hok* expression requires the translation of *mok* (modulation of killing), which overlaps the ORF of *hok* (Thisted and Gerdes, 1992). To

form a translationally active structure, the 3' and 5' end of the Hok RNA forms a *fbi*

(fold-back-inhibition) structure by base-pairing (Thisted and Gerdes, 1992). As a result,

this truncated form of *hok* kills the cells (Gerdes *et al.*, 1992). Translation of the host-

killing gene, *hok*, can be inhibited by the Sok (suppressor of killer) antisense RNA

(Thisted and Gerdes, 1992).

Sok is a 67nt antisense RNA (Gerdes *et al.*, 1990) that is composed of a hairpin

loop with a stable stem preceded by a single-stranded region of 11 nucleotides at the

5'end (Thisted *et al.*, 1994). The 5'end of Sok interacts with the target loop in the

truncated *hok* mRNA (Thisted *et al.*, 1994). It binds to the *mok* RBS (the *hok* mRNA

leader sequence) preventing its translation (Gerdes *et al.*, 1986; Gerdes *et al.*, 1990;

Gerdes *et al.*, 1992), preventing the expression of the Hok killer-toxin.

Regulation of Transposition

RNA-OUT

The transposable genetic element, IS10, expresses *tnp*, a transposase function

(Foster *et al.*, 1981). Transposition occurs when *tnp* acts at the ends of IS10. RNA-OUT

is a 70nt IS10 antisense RNA that is composed of one stem loop that is mostly a stem

domain (Kittle *et al.*, 1989). It shares complementarity with the 5'end of the *tnp* mRNA,

termed RNA-IN (Kittle *et al.*, 1989).

RNA-OUT binds to a 36 nt region (Simons and Kleckner, 1983) that spans the

loop domain and down one side of the stem domain (Kittle *et al.*, 1989). Upon binding,

RNA-OUT and RNA-IN immediately form a stable RNA duplex (Ma and Simons, 1990)

that may be cleaved by RNase III (Case *et al.*, 1990). As a result, the *tnp* RBS becomes blocked, preventing *tnp* translation and transposition from occurring. This antisense control increases proportionally, as the rate of *tnp* expression and IS10 transposition increases (Simons and Kleckner, 1983). However, transposition may also be inhibited by the formation of a *fbi* (fold-back-inhibition) structure (Kittle *et al.*, 1989).

Regulation of Phage Development

Bacteriophage use antisense RNAs to assist in the maintenance of lysogeny or occurrence of lysis. Two examples of such antisense RNAs are OOP RNA and Sar RNA.

OOP RNA

In bacteriophage λ, expression of the *cII* gene, encoding the CII repressor protein, allowing the phage to enter the lysogenic pathway (Echols, 1986). The 77nt OOP antisense RNA was identified to negatively regulate *cII* expression, directing the phage into the lytic pathway (Hayes and Szybalski, 1973, Krinke and Wulff, 1987). Unlike other antisense RNAs, OOP RNA showed complementarity to the 3'end of the *cII* ORF (Hayes and Szybalski, 1973). Since this region was distant from the *cII* RBS, it was thought that there must be another mechanism for inhibiting *cII* expression.

OOP RNA is transcribed in the opposite direction of the *cII* gene, starting in the *O* gene ORF and extending beyond the RBS of the *O* gene into the 3'end of the *cII* ORF (Hayes and Szybalski, 1973). Following post-transcriptional modification, it forms a stem loop structure consisting of a large stem and loop domain (Hayes and Szybalski, 1973). This loop domain forms base-pairs with its target site in the 5' end of the *cII* mRNA,

blocking the *cII* RBS (Hayes and Szybalski, 1973; Krinke and Wulff, 1987). The

formation of the OOP RNA-*cII* mRNA duplex triggers cleavage by RNase III (Krinke

and Wulff, 1987). This results in the inhibition of *cII* expression, and prevention of

lysogeny. However, *oop⁻* phages were found to show significant *cII* expression (Hayes

and Szybalski, 1973), which suggested that perhaps OOP RNA only played a small role

in preventing the expression of *cII*.

Sar RNA

The bacteriophage P22 expresses an antirepressor (Ant) that prevents the C2

repressor (which promotes lysis) from binding DNA (Wagner and Simons, 1994). Ant

expression is inhibited by the 69nt Sar (small antisense regulatory) RNA (Liao *et al.*,

1987; Wu *et al.*, 1987). Sar RNA is transcribed in the opposite direction as the *ant* gene,

spanning a region that includes the end of the *arc* gene and the start of the *ant* gene (Liao

et al., 1987; Wu *et al.*, 1987). Following transcription, a two stem loop structure forms,

where the 5' stem loop has a very short stem domain and a large loop domain that makes

it unstable (Jacques and Susskind, 1991). Sar rapidly binds to its target *arc-ant* sequence,

forming a stable complex (Liao *et al.*, 1987; Wu *et al.*, 1987). As a result, the *ant* RBS is

blocked (Liao *et al.*, 1987; Wu *et al.*, 1987), preventing the translation of the *ant* gene

(Liao *et al.*, 1987; Wu *et al.*, 1987).

<u>Regulation of Bacterial Gene Expression</u>

There are many RNA transcripts in prokaryotes that are regulated by a

chromosomally, trans-encoded antisense RNA. In the previous section, DsrA, RprA, and

OxyS, the antisense RNAs that regulate *rpoS* translation were discussed in detail. However, OxyS is known to have other targets. Besides OxyS, other antisense RNAs, MicF, DicF, RNAIII, RyhB, and *Gln*A antisense RNA, have been discovered in *E. coli* that regulate other genes.

OxyS

As mentioned earlier, OxyS is a 109nt antisense RNA induced under conditions of oxidative stress (Altuvia *et al.*, 1997). Its expression is transcribed from a location at 89.6 min in the *E. coli* chromosome. Along with regulating *rpoS* translation, OxyS was identified to also inhibit the translation of *fhlA* (Altuvia *et al.*, 1998; Argaman and Altuvia, 2000), a gene that encodes the transcriptional activator of formate hydrogenlyase system (Maupin and Shanmugam, 1990; Schlensog and Böck, 1990). The *fhlA* gene product contains metal cofactors that may be deterimental under conditions of oxidative stress (Maupin and Shanmugam, 1990; Schlensog and Böck, 1990).

Two out of the three stem loop structures of OxyS showed partial complementarity to two regions on the *fhlA* gene, 41 nt apart, which included the RBS and a region of the coding sequence (Argaman and Altuvia, 2000). As a result, OxyS can interact with both regions on the *fhlA* mRNA forming a kissing complex (Argaman and Altuvia, 2000). This interaction blocks access to the *fhlA* RBS to ribosomes (Altuvia *et al.*, 1998; Argaman and Altuvia, 2000), inhibiting the translation of *fhlA*.

MicF

The first trans-encoded antisense RNA to be discovered was MicF RNA, which

was located at 49.8 min in the *E. coli* chromosome (Mizuno *et al.*, 1984). The 93nt antisense RNA showed complementarity to the region surrounding the *ompF* RBS (Mizuno *et al.*, 1984; Andersen *et al.*, 1987). The *ompF* gene encodes OmpF, which is an outer membrane porin found in *E. coli* (Hall and Silhavy, 1981). Its expression was significantly reduced during environmental stresses such as high temperature and high osmolarity. It was determined that MicF RNA expression was induced by stress factors as mentioned above (as reviewed in Delihas and Frost, 2001).

MicF RNA forms a structure consisting of a single-stranded region at the 5'end followed by two stem loops (Schmidt *et al.*, 1995). The stability of MicF RNA is regulated by the RNA chaperone protein, StpA (Deighan *et al.*, 2000). The 5' end stem loop base-pairs with the 5' untranslated region of *ompF* (Mizuno *et al.*, 1984). The interaction leads to the formation of a stable duplex, which tagged for degradation. As a result, *ompF* translation is inhibited due to the blockage of the RBS.

DicF

DicF RNA, transcribed from the *dicB* operon, was identified to inhibit *ftsZ* expression (Bouché and Bouché, 1989; Tétart and Bouché, 1992). FtsZ is required for the initiation of cell division in *E. coli* (Tétart and Bouché, 1992). The 53nt DicF antisense RNA is encoded at 35.5 min in the chromosome (Bouché and Bouché, 1989; Faubladier *et al.*, 1990). It is composed of a stem loop structure that is not completely complementary to the *ftsZ* RBS (Faubladier and Bouché, 1994). Nonetheless, a partial DicF-*ftsZ* mRNA duplex forms that is sufficient for inhibiting the translation of *ftsZ*

(Tétart and Bouché, 1992). However, the exact mechanism of DicF-*ftsZ* mRNA interaction remains unclear.

RNA III

RNAIII was identified to promote the translation of *hla* mRNA, which encodes α-toxin in *Staphylococcus aureus* (Novick *et al.*, 1993; Morfeldt *et al.*, 1995). It does this by binding to the anti-RBS of the *hla* transcript and freeing its RBS site, which is blocked due to formation of a stem loop structure (Morfeldt *et al.*, 1995). Besides DsrA and RprA, RNAIII is the only other antisense RNA known to positively regulate expression of its target gene (Novick *et al.*, 1993; Morfeldt *et al.*, 1995). Expression of the 514nt RNAIII, also termed *hld* RNA, is encoded at the *agr* locus in the opposite direction as the *agrBDCA* operon (Janzon and Arvidson, 1990) but the secondary structure of RNA has not yet been determined. However, the 5'end of RNAIII was shown to have partial (~75%) complementarity to the region surrounding the *hla* RBS (Morfeldt *et al.*, 1995).

RyhB

Recently, the 90nt RyhB RNA was discovered to regulate six genes that play a role in intracellular storage of iron or encode Fe-containing proteins (Massé and Gottesman, 2002). The expression RyhB is encoded from the *ryhB* gene located at 77 min in the *E. coli* chromosome (Massé and Gottesman, 2002). One of the Fe-containing proteins regulated by RyhB was identified as succinate dehydrogenase (*sdhCDAB*), which is required for growth on succinate in rich media (Massé and Gottesman, 2002). In Fe limiting conditions, *ryhB* expression is induced and iron storage is downregulated

(Massé and Gottesman, 2002). However, under conditions of high iron concentrations RyhB expression was inhibited by Fur (Ferric uptake repressor) (Massé and Gottesman, 2002), which inhibits iron acquisition genes (Bagg and Neilands, 1987). Therefore, Fur regulation appeared to be dependent on RyhB expression.

The RyhB secondary structure is predicted to be composed of three stem loops containing complementary sequence to regions upstream the RBS in its target genes (Massé and Gottesman, 2002). The target genes of RyhB were all noted to have long, conserved sequence in their 5' untranslated region. In the case of *sdhCDAB* operon, the complementary region was located just upstream of the second gene in the operon, *sdhD* (Massé and Gottesman, 2002). As a result, RyhB with the aid of Hfq is able to the base-pair with the target sites forming a duplex (Massé and Gottesman, 2002) inhibiting the translation of the target genes and signaling the degradation of the RNA-RNA duplex (Massé and Gottesman, 2002; Massé *et al.*, 2003b). However, the details regarding the initial interaction between RyhB and its target mRNAs are still not understood.

GlnA antisense RNA

In *Clostridium acetobutylicum*, a 43nt antisense RNA was discovered to inhibit expression of glutamine synthetase, encoded by the *glnA* gene (Fierro-Monti *et al.*, 1992). The expression of *glnA* antisense RNA was determined to be differentially regulated by nitrogen (Fierro-Monti *et al.*, 1992). Under nitrogen-rich conditions, the expression of this antisense RNA was increased as glutamine synthetase activity decreased.

glnA antisense RNA is transcribed in the opposite direction from a location

downstream of the *glnA* gene (Fierro-Monti *et al.*, 1992). It was proposed to consist of a long single-stranded region at its 5'end and a stem loop structure at the 3' end (Fierro-Monti *et al.*, 1992). The 5' end of the *glnA* antisense RNA is to the 5' untranslated region of *glnA*. As a result, complementary base-pairing allows the two RNA molecules to form a duplex (Fierro-Monti *et al.*, 1992) blocking the *glnA* RBS and inhibiting *glnA* translation.

1.2.3   Antisense RNA Technology

Antisense RNA technology has been useful in inhibiting translation of target genes. White *et al.* (1997) have successfully shown the inhibition of the translation of the multiple antibiotic resistance operon (*marORAB*) using a *marRA* antisense (White *et al.*, 1997). The *mar* operon encodes for proteins that make *Escherichia coli* more resistant to antibiotics and other antimicrobial agents. By inhibiting the expression of this operon, the microbe is made more susceptible to antimicrobial agents.

Another study has successfully used *pta* and *atkA* antisenses to block the translation of the acetate pathway enzymes in *E. coli* (Kim and Cha, 2003). By inhibiting these enzymes, production of foreign proteins can be increased. These findings are greatly beneficial to the pharmaceutical industry as they synthesize drugs to kill harmful bacteria and often use microbes to mass-produce proteins such as insulin.

1.3 Modulation of *rpoS* expression

As discussed in detail earlier, *rpoS* expression is regulated at many levels. However, several researchers have been able to successfully modulate the expression of

rpoS in a different manner.

1.3.1 Inhibition of *rpoS* using an *rpoS* antisense

An *rpoS* antisense RNA would be useful for inhibiting translation of *rpoS* and studying the effects this has on the RpoS regulon. In theory, an antisense *rpoS* RNA should be capable of efficiently inhibiting *rpoS* translation by binding to the *rpoS* transcript. Previously, Chen *et al.*, (2003) synthesized an inducible-*rpoS* antisense (to be used in this study) specific for *rpoS* that exhibits an inhibitory effect on *rpoS* expression. As a result, RpoS-dependent catalase HPII expression and glycogen synthesis were significantly inhibited following the expression of the *rpoS* antisense (Chen *et al.*, 2003).

1.3.2 Inducible-*rpoS* expression prior to exponential phase

As discussed earlier, transcription of *rpoS* is induced in exponential phase and RpoS is most stable and active upon entry into stationary phase, which subsequently induces the expression of stress response genes (Hengge-Aronis, 2002). Thus, most genes of the RpoS regulon are induced upon entry into stationary phase. However, many genes of the RpoS regulon are regulated by additional factors and it is important to distinguish which genes are solely dependent upon RpoS, and those that may require other factors present in stationary phase.

A few studies in *Salmonella typhimurium* have shown that induction of *rpoS* expression early in log phase can induce the expression of various genes from its regulon prior to entry into stationary phase. Early induction of *rpoS* was shown to increase acid shock protein expression in log phase cells and thus, trigger an early acid tolerance

response (Bearson *et al.*, 1996). Also, early induction of *rpoS* resulted in a decrease in CRP during exponential phase (Fang *et al.*, 1996). More recently, it was revealed that many RpoS-dependent genes normally expressed in stationary phase can be induced in exponential phase cells (Chen and Schellhorn, 2003).

1.4 Construction of *rpoS* sense and antisense encoding constructs

Prior to the conduction of this study, an *rpoS* antisense and an *rpoS* sense were created and cloned into a pET21 vector under a T7 RNA polymerase, lactose inducible promoter, T7*lac* (Chen *et al.,* 2003; Chen and Schellhorn, 2003) (Figure 2). By site-specific integration, a λDE3 prophage, which encodes the T7 RNA polymerase gene under an IPTG (Isopropyl-β-D-thiogalactopyranoside) inducible $P_{lac\mathrm{UV5}}$ promoter, was inserted into the *E. coli* chromosome (Novagen). The plasmid pSOPR, which encodes the *rpoS* antisense, was transformed into an RpoS[+] MC4100DE3 background and the pRPOS plasmid, which encodes the *rpoS* sense, was transformed into an RpoS[-] HS1600DE3 background. The unaltered pET21 vector was also transformed into the two backgrounds to act as controls. The LacI repressor bound to the *lac* operator upstream the T7 RNA polymerase gene and the T7*lac* promoter in the pET21 plasmid is released upon induction with IPTG (Novagen). T7 RNA polymerase is synthesized from λDE3 and can induce the transcription of the *rpoS* antisense and *rpoS* sense on pSOPR and pRPOS, respectively (Chen *et al.,* 2003; Chen and Schellhorn, 2003).

1.5 Utilization of High-Copy Plasmids for Expression

High-copy plasmids are commonly utilized to overexpress foreign proteins. Gene

expression using such plasmids can be advantageous, however, there are also some disadavantages that should be examined.

1.5.1 Advantages of high-copy plasmids

Using high-copy plasmids, such as pET vectors, has some advantages. The transcription of foreign genes in pET vectors is under the direction of a bacteriophage T7 promoter (Novagen). The native *E. coli* RNA polymerase does not recognize this promoter, so the cloned gene will not be expressed in the absence of the T7 RNA Polymerase. The gene encoding T7 RNA Polymerase is introduced into the host chromosome and its expression is induced with IPTG (Novagen). As a result, these high-copy plasmids can be used to overproduce large amounts of foreign protein in a controllable manner (Studier *et al.*, 1986) preventing any serious consequences that may arise from the continuous expression of potentially toxic foreign genes.

1.5.2 Disadvantages of high-copy plasmids

High-copy plasmids, such as pET vectors, are accompanied by several disadvantages, which in this study may be problematic. High-copy plasmids can range from 10 to 100 copies per cell and are found in varying numbers from cell to cell (Glick and Pasternak, 1998). This is a problem when doing expression studies since the number of transcripts would differ from cell to cell making it difficult to accurately quantify the modulation of expression, particularly in the case of the *rpoS* antisense, and would not be physiologically relevant. Use of high-copy plasmids allows for a high yield in gene product but the overproduction of the product from the inducible promoter can create a

metabolic load on the organism (Glick, 1995). The overexpression is induced by IPTG, which is toxic to the cells at high concentrations, and cannot be metabolized by bacteria (Donovan *et al.*, 1996; Schweder *et al.*, 2002). Together, these can lead to a significant decline in the growth rate of such cells. Additionally, the selectable marker used to maintain the plasmid is ampicillin, which could also impede growth as it inhibits the synthesis of bacterial cell walls (Rolinson *et al.*, 1977).

1.6 Integration into the *E. coli* chromosome

1.6.1 Elimination of problems associated with use of high-copy plasmids

Integrating the inducible rpoS sense and rpoS antisense constructs into the chromosome would eliminate most of the concerns associated with utilizing high-copy plasmids. Only a single copy of the *rpoS* sense and the *rpoS* antisense would be present in each cell. This would be more physiological relevant and more reliable data could be collected from performing expression studies. Additionally, the sequences would still be under controlled expression but the recombinants would not need to be selected by ampicillin as the integrated sequence/gene DNA would be stable.

1.6.2 One-Step Integration Method

Datsenko and Wanner (2000) have developed a one-step method utilizing PCR products that allows for linear DNA segments to be integrated into the chromosome. In summary, this procedure requires the use of a plasmid encoding the λRed recombinase system, pKD46, to allow for homologous recombination to occur between the desired sequence/gene and the chromosome. A linear PCR product carrying the gene/sequence of

interest is synthesized using primers with 5'end homology to the target integration site. The PCR product is transformed into cells carrying the plasmid that encodes the λRed recombinase system, which is induced by L-arabinose. The transformed cells are incubated for an hour at 37°C to ensure that antibiotic resistance marker is being expressed. The cells are plated onto the appropriate selective media and grown at 42°C to cure the temperature sensitive pKD46 plasmid and obtain the recombinants (Datsenko and Wanner, 2000).

1.6.3 Site of Integration: *ebg* operon

To integrate a gene or sequence into the *E. coli* genome a suitable site must be chosen. The integration must not disrupt any genes/operons required for the bacterium to survive. In this study, the *ebg* operon was chosen as the site of integration. The *ebg* operon is not essential for the survival and proliferation of *E. coli*.

ebg stands for evolvable beta-galactosidase (Lin 1996). This operon is composed of three genes; *ebgR*, *ebgA*, and *ebgC* (Figure 3). *ebgR* encodes for a repressor similar in structure to LacI. The alpha and beta subunits of a β-galactosidase are encoded by *ebgA* and *ebgC*, respectively (Lin 1996). *ebgR* and *ebgA* show 50% sequence homology to the repressor and β-galactosidase of the *lac* operon (Stokes *et al.*, 1985a; Stokes *et al.*, 1985b). The origin of this operon is thought to be a result of gene duplication and divergence (Stokes *et al.*, 1985b).

This operon is cryptic since the gene products made are ineffective. Several site-specific mutations are required for the EbgAC β-galactosidase to be functional (Stokes *et*

al., 1985a; Stokes *et al.*, 1985b). However, even then the level of activity would not be at the same level as the β-galactosidase encoded by *lacZ* in the *lac* operon. Therefore, disrupting this operon should have no effect on the growth and survival of the bacteria.

1.7 Goal of this Study

The overall goal of this study was to show that the *rpoS* sense and *rpoS* antisense could be utilized to modulate *rpoS* expresson.

1.7.1 Part One

In this part of the study, modulation of *rpoS* expression by the *rpoS* antisense RNA was analyzed on plates and in liquid media. Expression of a highly RpoS-dependent *osmY-lacZ* transcriptional fusion in a strain carrying the pSOPR plasmid was compared to the same strain carrying the empty pET vector as a control. Expression of the RpoS-independent *rsi001-lacZ* transcriptional fusion, characterized in this study, in the same strains was compared to ensure specificity of the antisense for *rpoS* transcripts.

1.7.2 Part Two

In the latter part of this study, the technique developed by Datsenko and Wanner (2000) was adapted to integrate the *rpoS* antisense and *rpoS* sense, from the previously mentioned constructs, into the *E. coli* chromosome. Putative recombinants were tested for appropriate antibiotic resistance and the expression of catalase HPII. The integration of the linear DNA fragments was verified by three separate methods.

CHAPTER 2: Materials and Methods

2.1 Bacterial strains and plasmids

All of the Escherichia coli strains and bacteriophage used in this study and the plasmids utilized are listed in Table 1 and Table 2, respectively.

2.2 Oligonucleotides

All oligonucleotides used to amplify PCR products and used to sequence chromosomal integration in this study are listed in Table 3.

2.3 Chemicals, enzymes, reagents, and supplies

The chemicals, media, molecular biology reagents and lab supplies used in this study were supplied by ACP Chemicals Inc. (Montreal, QC), Bio-Rad Laboratories (Mississauga), Bioshop Canada Inc. (Burlington, ON), Caledon Laboratories Ltd. (Georgetown, ON), Commercial Alcohols Inc. (Brampton, ON), Diagnostic Chemical Ltd. (Charlottetown, PEI), Diamed Lab Supplies Inc. (Mississauga, ON), EMD Chemicals Inc. (Gibbstown, NJ), Fisher Scientific Ltd. (Ottawa, ON), Invitrogen Canada Inc. (Burlington, ON), MBI Fermentas (Burlington, ON), Novagen (San Diego, CA), Qiagen Inc. Canada (Mississauga, ON), Roche Diagnostics Canada (Laval, QC), and Sigma-Aldrich Canada Ltd. (Oakville, ON).

2.4 Growth Conditions

Escherichia coli was grown in Luria-Bertani (LB) broth at 37°C (Miller, 1992). Cultures in liquid media were shaken at 200 revolutions per minute (rpm). Antibiotics were added for the correct selection of the strains. Strains carrying the pET21 plasmids and its derivative plasmids, pRPOS and pSOPR, were selected by adding 100µg/ml

ampicillin to the media. Transductants carrying *lacZ* gene fusions were selected by growing in kanamycin (50μg/ml) supplemented media. Kanamycin was also used to select cells containing pKD46, a temperature-sensitive plasmid, and its derivative, pKAN, which was created in this study. These strains needed to be grown at 30°C to sustain the plasmid and to express the λRed system.

2.5 Growth media

The growth media, listed below, were prepared using deionized distilled water (ddH$_2$O) and autoclaved at 121°C (15 lbs.sq.in.) for 20 minutes. It was stored at room temperature unless otherwise indicated.

LB broth contained, per litre: 10g tryptone, 5g yeast extract, 10g NaCl. This was dissolved in ddH$_2$O and the volume was brought up to one litre. The broth was pH 7, if not, it was adjusted.

LB Agar contained, per litre: 10g tryptone, 5g yeast extract, 10g NaCl, 15g Agar.

2.6 Antibiotics

Antibiotics were prepared in ddH$_2$O, unless otherwise indicated, and filter-sterilized using a 0.45μm pore size filter disc.

Name	Stock concentration (mg/ml)	Storage (°C)	Working concentration (μg/ml)
Streptomycin	100	-20	100
Kanamycin	50	-20	50
Tetracycline	15 (in 50% ethanol)	-20	15
Ampicillin	100	-20	100
Chloramphenicol	34	-20	25

2.7 Expression of *lacZ* reporter gene fusions in the *E. coli* chromosome

2.7.1 Preparation of *lacZ* gene fusion lysates

To transduce *lacZ* reporter gene fusions into the experimental strains, lysates carrying such gene fusions needed to be prepared. *osmY-lacZ* fusion lysate from the HS1091 strain and the lysate carrying a *rpoS* independent promoter fusion (referred to as *rsi001-lacZ* in this study until its identification) from the 13C10 strain (Schellhorn *et al.*, 1998) were obtained using a generalized transducing bacteriophage, P1$_{vir}$, that had recently been titred.

First, the strains HS1091 and 13C10 containing the desired *lacZ* gene fusions were grown overnight (O/N) in 10ml of 1xLB supplemented with 5mM calcium chloride (CaCl$_2$). The next day the O/N culture was subcultured 1:100 and grown to OD 600 = 0.3. The culture was then split in two tubes. 100µl of P1 lysate was added to one tube of culture and both tubes were incubated on a rotating well at 37°C until lysis was evident. The lysed culture was transferred to a 15ml red-capped tube and 100µl of 100% chloroform was added to remove cell debris and centrifuged at max speed for 10 mins. The supernatant was transferred to a clean tube and chloroform was added again. The lysate was stored at 4°C, indefinitely.

2.7.2 Transduction of *lacZ* gene fusions

In order to be able to measure the effect of the *rpoS* antisense on *osmY* gene expression, the strains containing the pET21 vector and its derivative, pRPOS and pSOPR, were transduced with the *osmY-lacZ and rsi001-lacZ* gene fusion.

The recipient strains (MC4100, HS1600, MC4100DE3, MC4100DE3 pET21, MC4100DE3 pSOPR, HS1600DE3, HS1600DE3 pET21, and HS1600DE3 pRPOS) were inoculated into 10ml of 1xLB containing 5mM $CaCl_2$ and grown O/N at 37°C. The next day, these O/N cultures were transferred to 15ml red-capped tubes and centrifuged for 10 mins at 4°C to pellet the cells. The pellet was resuspended into 250µl of 1xLB containing 5mM $CaCl_2$. 100µl of the lysate was added to 100µl of cells in a clean test tube, gently mixed and incubated for 15 mins at 37°C. The incubated culture was washed with 12ml of 1xLB containing 100mM sodium citrate. This wash step was repeated once more. Before the third wash, the culture was incubated in the LB/sodium citrate media for 30 mins at 37°C. The pellet obtained was resuspended in the residual media. 100µl of this mixture was spread-plated onto selective LB media and incubated O/N to obtain transductants.

2.7.3 Phenotype characterization of transductants

To test the effect of *rpoS* antisense and *rpoS* sense expression on *osmY*, the experimental *E. coli* strains carrying the *osmY-lacZ* fusion were streaked onto selective media containing X-gal. They were incubated O/N at 37°C to obtain isolated colonies. A single isolated colony was inoculated into a microtiter well, in a 96 well microtiter dish, containing 200µl of 1xLB containing the appropriate antibiotics. This was repeated twice more for each strain. The plate was incubated for 1 hour and the bacterial cultures were replica-plated onto LB Agar plates containing the appropriate antibiotic(s), X-gal, and +/- 0.3mM IPTG (final concentration) metal-pronged block. The plates were inverted and

incubated at 37°C O/N. The level of *lacZ* expression (represented by the degree of blue colouring) was recorded on the following day.

2.8 Expression studies to determine the effect and specificity of the *rpoS* antisense

2.8.1 Plate Assay

To compare the effect and specificity of *rpoS* antisense expression on *osmY* and *rsi001*, isolated colonies of the *E. coli* strains, MC4100DE3 pET21 and MC4100DE3 pSOPR, carrying the *osmY-lacZ* and *rsi001-lacZ* fusions were inoculated into a microtiter dish as mentioned above. The strains were printed onto selective media containing X-gal and +/- 0.3mM IPTG (final concentration) metal-pronged block. The plates were incubated at 37°C and grown O/N. The level of *lacZ* expression between the strains expressing the *osmY-lacZ* and *rsi001-lacZ* fusions was compared and recorded on the following day.

2.8.2 β-galactosidase Assay

To measure the effect of the *rpoS* antisense on *osmY* and *rsi001* gene expression and its specificity for *rpoS*, IPTG-induced and noninduced cells from the experimental *E. coli* strains, carrying the *osmY-lacZ* and the *rsi001-lacZ* fusions, were collected and assayed for β-galactosidase activity.

Isolated colonies from the strains MC4100DE3 pET21 and MC4100DE3pSOPR containing the *osmY-lacZ* fusion were inoculated in 10ml of selective 1xLB media and incubated at 37°C O/N. The next day, the O/N cultures were subcultured 1:1000 in 10ml

of fresh 1xLB containing 100μg/ml ampicillin and continued to be grown at 37°C. Once the OD 600 reached 0.25-0.30, it was subcultured once more to the same dilution factor into 50ml of fresh media. When the cultures reached early exponential phase, the cultures were transferred to 200ml of the same media in larger flasks to give a starting OD 600 of 0.005–0.010. These cultures were incubated at 37°C.

At given time points, 10ml samples of each culture were transferred to 2 small flasks where one culture was induced with IPTG (to a final concentration of 0.3mM) and one was left uninduced. These small flasks were simultaneously incubated at 37°C with the large flasks. 1ml culture samples were taken in triplicate from the each of the small flasks when the cultures reached stationary phase. They were aliquoted into clean small test tubes. Protein synthesis was inhibited by the addition of 10μl of 15mg/ml chloramphenicol and storing the sample tubes on ice. OD 600 values were measured for each sample using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

β-galactosidase activity was assayed according to the previously described protocol (Miller, 1992). 50μl of cells were added to each of the reaction tube containing 950μl of Z-buffer (with β-mercaptoethanol that helped denature the proteins), and 25μl of 0.1% SDS and 50μl of 100% chloroform to lyse the cells. 200μl of ONPG, the substrate for β-galactosidase, was then added to the reaction tubes. The tubes were vortexed and the reaction was begun.

When the tubes appeared yellow-coloured, the reaction was stopped by the addition of 0.5ml of 1M Na_2CO_3. For each reaction, the absorbance was measured at 420nm using a spectrophotometer (Shimadzu). This OD 420 value was used to determine the specific β-galactosidase activity. The units of specific activity, expressed in Miller units, was calculated as:

[1000 x OD 420] / [incubation time (min) x volume of sample (ml) x OD 600]

Specific activity was plotted against time to observe the changes in expression of *osmY* with the induction of the *rpoS* antisense. This assay was repeated with the MC4100DE3 pET21 and MC4100DE3 pSOPR strains carrying the *rsi001-lacZ* fusion.

2.8.3 Determination of Relative Expression Levels of gene fusions

To clearly illustrate the effect of the induction of the *rpoS* antisense (from the pSOPR plasmid) on the expression of *osmY-lacZ* and *rsi001-lacZ*, the percent induction was determined. This was calculated in the following manner: [(β-galactosidase activity of the MC4100DE3 pSOPR strain under induced condition) / (β-galactosidase activity of the MC4100DE3 pSOPR strain under noninduced conditions)] x 100%.

It was compared to the percent induction obtained from the MC4100DE3 strain carrying the pET21 vector.

2.9 Identification of the RpoS-independent gene fusion from the 13C10 strain

To determine the identity of *rsi001*, chromosomal DNA was sequenced using a

primer (Mu c) that specifically anneals to one of the flanking ends of the prophage that is integrated in 13C10 (Roy *et al.*, 1995). Chromosomal DNA was isolated from the 13C10 strain using a small-scale protocol modified for the previously described method (Silhavy *et al.*, 1984).

This was done using 1.5ml of O/N culture that was transferred into a microcentrifuge tube and the cells were pelleted. The cells were then resuspended into 500μl of 1xTE buffer that contained SDS to help lyse the cells, proteinase K to degrade the cellular proteins, and RNase A to eliminate all the RNA. The resuspended cells were incubated at 37°C for 2 hours. Following the incubation, an equal volume of phenol/chloroform/isoamyl alcohol solution was added to extract the chromosomal DNA. The DNA was precipitated O/N by ethanol precipitation. This was achieved by adding 1/10 volume of 3M Sodium acetate, pH 5.2, to stabilize the DNA backbone, and 2 volumes of 100% ethanol to the aqueous phase and mixed. The acquired DNA pellet was resuspended into 100μl of 10mM TrisCl, pH 8. The quality of the DNA was verified by agarose gel electrophoresis and the DNA was quantified using a spectrophotometer at 260nm (Shimadzu).

5μg of purified DNA sample was sent to the Central Facility at McMaster University for sequencing with diluted Mu c end primer (Roy *et al.*, 1995). Once sequence of the fusion junction was obtained, it was aligned with all sequences annotated in GenBank using the BLAST sequence alignment program (Altschul *et al.*, 1990).

2.10 IPTG Titration

To determine the effect of IPTG concentration on the growth rate of the experimental strains, MC4100, MC4100DE3, MC4100DE3 pET21 and MC4100DE3 pSOPR, were titrated with different concentrations of IPTG. Isolated colonies of each strain were inoculated in 10ml of 1xLB media (containing the appropriate antibiotic, if required to seleect for the plasmid) in a 50ml flask and incubated at 37°C O/N. The next day, it was subcultured 1:1000 into 10ml of fresh media in a similar sized flask and grown to an OD 600 = 0.2-0.3. Meanwhile, 50ml flasks containing 10ml of 1xLB supplemented with IPTG final concentration: 0mM, 0.05mM, 0.1mM, 0.2mM, 0.3mM, 0.5mM, and 1.0mM, were prepared for each of the four strains.

Exponentially growing cells were transferred to each of these prepared flasks to give a starting OD 600 of 0.005–0.010. The cultures were all incubated at 37°C. 1ml samples were taken 15 minutes after the inoculation to serve as the initial OD, t = 0. Following this, samples were taken from each flask every hour, for a total of 5 hours. An O/N sampling was also taken. Samples were aliquoted into tubes containing 10µl of 15mg/ml chloramphenicol and stored on ice to inhibit protein synthesis. The OD 600 was measured for each sample using a spectrophotometer (Shimadzu). OD 600 values were plotted against time to observe any effects of IPTG on the growth rate of these strains.

2.11 Preparation for integration into the *E. coli* chromosome

In order to successfully integrated the desired gene/sequence (the *rpoS* sense and *rpoS* antisense) into the chromosome several preliminary steps needed to be completed. In this study the plasmid, pKD46, which encodes the λRed system, needed to be modified to allow for the selection of recombinants. The present antibiotic resistance marker, the β-lactamase gene, had to be replaced with the neomycin phosphotransferase gene, a kanamycin resistance (Kan^R) marker. This was necessary as the sequence to be integrated also carried the ampicillin resistance marker (Amp^R), β-lactamase.

2.11.1 PCR Amplification of the kanamycin resistance marker

To replace the Amp^R marker with the Kan^R marker, the Datsenko and Wanner (2000) method was utilized. The neomycin phosphotransferase ORF was PCR amplified from the pKD4 plasmid using primers that had 5' tails made of 36 base pairs (bp) that were homologous to regions upstream and downstream the β-lactamase ORF on the pKD46 plasmid. The PCR reaction tubes were set-up as the following:

PCR component	PCR reaction (µl)	PCR control (µl)
10xPCR buffer + $(NH_4)_2SO_4$	5	5
25mM $MgCl_2$	4	4
10mM dNTPs	2	2
Distilled H_2O	30.5	32.5
25pmol/µl Primer FORWARD ML1108	2	2
25pmol/µl Primer REVERSE ML1109	2	2
10ng/µl pKD4	2	0
1U/µl Taq Polymerase	2.5	2.5
Total volume	50	50

The PCR conditions were set as indicated below:

Step	Temperature (°C)	Duration	No. of cycles
Denature	95	2 min	1
Denature	95	30 sec	
Anneal	55	30 sec	30
Extend	72	1.5 min	
Extend	72	7 min	1

Once the PCR product was amplified and verified for the correct size, it was purified

using an agarose gel extraction kit (Qiagen). Following this purification step, the PCR

product was ready to be transformed into prepared electrocompetent cells, expressing the

λRed system, by electroporation.

2.11.2 Preparation of λRed system-induced electrocompetent cells

To obtain recombinants, electrocompetent cells were prepared for transformation

that already had the λRed system induced. MG1655 cells carrying the pKD46 plasmid

were grown in 500ml of 1xLB containing L-arabinose (final concentration of 20mM) to

an OD 600 value of 0.4-0.6. The cells were pelleted at 4200 rpm at 4°C for 15 minutes.

The pellet was resuspended into equal volume of ice-cold 10% glycerol and chilled on ice

for 30 minutes. Cells were pelleted as mentioned above and washed thrice in ice-cold

10% glycerol. After the final wash step, the pellet was resuspended in the same solution,

aliquoted into 1.5ml microfuge tubes and stored at –80°C.

2.11.3 Transformation of the PCR product encoding the Kan[R] marker

In order to obtain recombinants, the linear PCR product was transformed, by

electroporation, into the above prepared cells. Electroporation was achieved by pulsing

50μl of the MG1655 pKD46 electrocompetent cells and 4μl of the purified PCR product

with 2.25 kV. The pulsed cells were resuspended in 1ml of 1xLB and transferred to a

1.5ml microfuge tube. The cells were incubated at 37°C for 1 hour to allow sufficient

time for neomycin phosphorotransferase to be expressed. Following the incubation, cells

were pelleted and resuspended into 100μl of 1xLB. 50 μl of the cells were spread onto a

LB Agar + kanamycin (50μg/ml) plate and incubated at 30°C to obtain recombinants.

The putative recombined plasmid from here on would be referred to as pKAN.

2.11.4 Phenotypic Characterization of the Recombinants

For the preliminary determination of the correct integration of the Kan^R marker,

the obtained recombinants were tested for the correct phenotype on LB + Agar plates

containing different antibiotics; streptomycin, kanamycin, and ampicillin (data not

shown).

2.11.5 Confirmation of the Integration of Kan^R marker

To verify the proper integration of the neomycin phosphotransferase open reading

frame (ORF) in the pKD46 plasmid to the location of the β-lactamase ORF, PCR was

utilized. One primer was designed upstream the ORF, which would anneal to the plasmid,

and another was designed, which would anneal to the neomycin phosphotransferase ORF.

The reaction and the conditions were similar to those indicated in the two tables above

with the following exceptions: (1) isolated pKAN was used as the template; (2) The

forward and reverse primers used were ML1325 and ML1326, respectively; (3) The

extension time for the 30 cycles was reduced to1 minute since the expected product was

much smaller, about 1kb in length.

2.12 Synthesis of the PCR products to be integrated into the chromosome

2.12.1 Small-scale isolation of plasmids

To synthesize the desired PCR products, plasmid DNA, needed to be extracted

from cells to serve as template DNA, for this reaction. Plasmids, pET21, pSOPR, and

pRPOS, were isolated using a small-scale alkaline lysis preparation (Sambrook and

Russell, 2001). Cells were grown O/N and pelleted the following day. The cells were

lysed in 100μl of ice-cold Solution I (50mM glucose, 25mM Tris-Cl pH 8, 10mM EDTA

pH 8). 200μl of freshly prepared Solution II (0.2N NaOH, 1% SDS) was added and

mixed to precipitated out the protein and cell debris. 150μl ice-cold Solution III (5M

potassium acetate, glacial acetic acid) was then added. After a 5 minute incubation on ice,

the mixture was centrifuged at max speed to remove the precipitated matter. The DNA

was present in the remaining aqueous phase. The aqueous phase was transferred to a new

tube and mixed with 2 equal volumes of 100% ethanol and 0.1 volume of 3M Sodium

acetate (pH 5.2) to precipitate the DNA. Plasmid DNA was collected by centrifuging the

tubes at max speed for 30 minutes. The DNA pellet was resuspended in 20μl of 10mM

Tris-Cl (pH 8) and stored at 4°C.

2.12.2 PCR amplification of region of interest for integration

The linear double-stranded DNA fragment to be integrated into the chromosome

was generated by PCR amplifying the region of interest on the pET21 vector and its

derivatives. The PCR product would include the β-lactamase gene, the *rpoS* sense or

rpoS antisense sequence or just pET21 vector sequence, the T7 promoter and the *lacI*

gene. The primers used had a 5' tail that was made up of 36bp that were homologous to

the integration sites in the *ebg* operon (Figure 4). The reactions were set up as the

following for each plasmid:

PCR component	PCR reaction (µl)	PCR control (µl)
10x HI-FI Taq Buffer	5	5
25mM MgCl$_2$	4	4
10mM dNTPs	2	2
Distilled H$_2$O	33.5	34.5
25pmol/µl Primer FORWARD AB32933	2	2
25pmol/µl Primer REVERSE AB32934	2	2
40ng/µl plasmid template	1	0
5U/µl HI-FI Taq Polymerase	0.5	0.5
Total volume	50	50

Due to the larger size of the primers and the expected large size of the PCR product

(pET21 = 3.7kb, pSOPR = 4.7kb, pRPOS = 5.0kb), the PCR reactions were performed

using the following conditions:

Step	Temperature (°C)	Duration	No. of cycles
Denature	95	2 min	1
Denature	95	15 sec	
Anneal	55	45 sec	30
Extend	72	6 min	
Extend	72	10min	1

The correct size PCR products was verified by running it on a 0.8% agarose gel stained

with ethidium bromide and visualizing the product under an UV transilluminator. The

PCR products were purified using an agarose gel extraction kit (Qiagen) and resuspended

into 20μl of 10mM Tris-Cl (pH 8).

2.13 Integration of linear PCR products into the chromosome

To obtain the desired recombinants, purified PCR products, synthesized in the above

reactions, were transformed into λRed system expressing electrocompetent cells of the

appropriate background. The *rpoS* sense carrying PCR product (referred to as *rpoS* sense

PCR product in this study) was transformed into the RpoS⁻ background HS1600DE3 cells

and the *rpoS* antisense (referred to as *rpoS* antisense PCR product in this study) was

transformed into the RpoS⁺ background MC4100DE3 cells. As a positive control for the

transformation procedure, pRPOS was also transformed into these backgrounds.

The homologous recombination event that would allow integration of the PCR

product into the chromosome is illustrated in Figure 5.

The protocol used was the same as that followed to create pKAN; However,

recombinants were selected by plating the electroporated cell mixture onto LB Agar +

ampicillin (150μg/ml) plates and incubated at 42°C to lose the pKAN plasmid

(temperature sensitive plasmid). Ten recombinants of each were streaked out for purity.

However, if less than 10 recombinants were obtained, all colonies were streaked out.

2.13.1 Phenotype Characterization of the Putative Recombinants

Plate Assay

To test if the recombinants had the proper antibiotic resistance markers, their

phenotype was characterized by replica-plating. Six isolated colonies of each putative

recombinant were grown O/N in wells of a 96 well microtitre dish containing 200μl of

1xLB. The following day, the liquid cultures were transferred from the wells to LB Agar plates containing different antibiotics using a metal-pronged block. The antibiotics used were 100µg/ml streptomycin (S), 15µg/ml tetracycline (T), 50µg/ml kanamycin (K), and 100µg/ml ampicillin (A). These plates were incubate O/N at 37°C. Growth or no evidence of growth would determine the antibiotic resistance or sensitivity of the isolated recombinants.

Hydrogen peroxide sensitivity assay

To test for the expression of *rpoS* sense and the *rpoS* antisense, the putative recombinants were checked for their hydrogen peroxide sensitivity. They were replica-plated as indicated above on LB Agar plates containing 100µg/ml ampicillin and different IPTG concentrations (0mM to 1.0mM) to induce the expression of the gene/sequence of interest. A drop of concentrated hydrogen peroxide was deposited on top of a colony using a pipette. The degree of bubbling (oxygen released from catalase activity) was recorded.

2.13.2 Confirmation of integration into the chromosome

Confirmation using PCR

To confirm the integration of the PCR product into the *E. coli* chromosome by PCR, primers were designed upstream and downstream the chosen integration site. Each reaction was set up as follows:

PCR component	PCR reaction (µl)	PCR control (µl)
10xPCR buffer + (NH₄)₂SO₄	5	5
25mM MgCl₂	4	4
10mM dNTPs	2	2
Distilled H₂O	30	30
25pmol/µl Primer FORWARD ML1706	2	2
25pmol/µl Primer REVERSE ML1707	2	2
10% Tween 20	2.5	2.5
DNA template	Isolated colony	0
1U/µl Taq Polymerase	2.5	2.5
Total volume	50	50

The PCR conditions were the same as those used to amplify the PCR product for

integration except for the fact that the duration of the initial denaturation step was

increased to 4 mins instead of 2 mins, as this would allow the high temperature to assist

in the breaking open of cells. The PCR products were run on a 0.8% agarose gel, stained

with ethidium bromide, and visualized using an UV transilluminator.

Confirmation using Restriction Mapping

To determine whether or not the PCR products had integrated into the

chromosome, the PCR products, obtained in the above reactions from the putative

recombinants that showed the most promising phenotype from the hydrogen peroxide

sensitivity assay, were digested using different restriction endonucleases. Three enzymes,

PstI, HindIII, and EcoRI, were used to cleave the PCR products. Locations of the

cleavage sites of these restriction enzymes are shown in Figure 6. The composition of

each reaction tube is shown below:

Reaction Components	Reaction Tubes			
	—	PstI	HindIII	EcoRI
DNA sample (µl)	5	5	5	5
10x Restriction Buffer (µl)	1	1	1	1
Restriction Enzyme (µl)	0	1	1	1
Distilled H$_2$0	3	3	3	3

Isolated pRPOS plasmid was treated in a similar manner as a positive control for the activity of the restriction enzymes. The reactions were run at 37°C for 1 hour. Following the incubation, the enzymes were inhibited by the addition of 1µl of 0.5M EDTA. The digested DNA was run on a 0.8% agarose gel, stained with ethidium bromide, and visualized using an UV transilluminator. The expected restriction fragments for the integration and non-integration is shown in Table 4.

Confirmation using sequencing

To establish the identity of the site of integration, an internal primer (ML3096) was designed ~250bp downstream the 5'end of the PCR products, which was used to integrate into the chromosome. The primer would anneal to the 3'end of the β-lactamase gene and amplify outward to the junction point of the integrated PCR product and a location in the *E. coli* chromosome.

Chromosomal DNA was isolated from the most promising putative recombinant HS1600DE3 *rpoS* sense PCR product isolate no.6 (HS1600DE3 *rpoS*-6). It was RNase-treated and purified by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation following the protocol mentioned earlier.

5µg of chromosomal DNA with diluted internal primer (1pmol/µl) was sent to the

Central Facility for sequencing (Mobix). The sequence data was analyzed using the

sequence alignment program, BLAST (Altschul *et al.*, 1990).

CHAPTER 3: Results

3.1 Effects of *rpoS* antisense and *rpoS* sense expression on *osmY*-lacZ expression

To be able to quantify the effect of the *rpoS* antisense and *rpoS* sense expression on the level of RpoS activity, the strains MC4100DE3 pET21, MC4100DE3 pSOPR, HS1600DE3 pET21, and HS1600DE3 pRPOS were transduced with an *osmY-lacZ* fusion. No significant difference in *osmY-lacZ* expression was observed between the MC4100DE3 strain carrying the pET21 vector (control) and the pSOPR plasmid (*rpoS* antisense expressing plasmid) under IPTG-induced or noninduced conditions (Figure 7). The level of *osmY-lacZ* expression was similar to that observed in the MC4100DE3 *osmY-lacZ* strain which carried no plasmid.

A significant difference in *lacZ* expression was seen between the transductants of the HS1600DE3 background carrying the pET21 vector (control) and the pRPOS plasmid, the *rpoS* sense expressing plasmid under IPTG-induced and noninduced conditions (Figure 7). However, in the HS1600DE3 pRPOS strain there was a significant induction of *lacZ* even when not induced by IPTG (data not shown), most likely due to leaky expression of the plasmid encoded *rpoS* under non-induced conditions.

3.2 Determination of the specificity of the *rpoS* antisense

To qualitatively determine the specificity of the *rpoS* antisense, the level of *osmY* expression in the MC4100DE3 pET21 and MC4100DE3 pSOPR transductants carrying *osmY-lacZ* and *rsi001-lacZ* (*rpoS*-independent) fusions were compared by replica-plating on selective media. No difference in *lacZ* expression was observed between the strains carrying the *osmY-lacZ* fusion in IPTG-induced (Figure 7) or noninduced conditions.

Similar *lacZ* expression was observed in the strains carrying the *rsi001-lacZ* fusion

whether induced with IPTG (Figure 8) or not (data not shown).

3.3 Quantification of the effect of *rpoS* antisense expression

To quantify the effect of the *rpoS* antisense on *osmY-lacZ* expression, the

transductants carrying the *osmY-lacZ* and *rsi001-lacZ* fusions were grown in liquid media

and assayed for β-galactosidase activity. Inhibition of *osmY-lacZ* expression was seen in

the strain MC4100DE3 pSOPR when it was IPTG-induced. (Figure 8). However, similar

inhibition was observed in the strain MC4100DE3 pET21 when induced with IPTG

(Figure 8). Some inhibition of *rsi001-lacZ* was observed in the strain MC4100DE3

pSOPR when induced with IPTG but not when noninduced (Figure 9).

To further illustrate any effect of the *rpoS* antisense, the percent induction of

osmY-lacZ was determined. With the exception of a single outlier, there appears to be no

significant reduction in *osmY-lacZ* expression due to the expression of the *rpoS* antisense

when compared to the strain carrying the control plasmid (Figure 10). The percent

induction of *rsi001-lacZ* was also calculated. Due to errors in the spectrophometric

readings, the obtained data was not as expected since the data points increased and

decreased at random intervals (Figure 11). However, no obvious inhibition of *rsi001-lacZ*

expression was seen.

3.4 Identification of *rsi001*

The chromosomal DNA extracted from 13C10 was sent for sequencing. The

quality of the sequence obtained from the sample was good (Appendix I). By using the

sequence alignment program (BLAST), the fusion junction sequence showed exact homology to the *acpD* gene in *E. coli*.

3.5 IPTG Titration

To determine the effect of IPTG concentration on growth the strains they were grown in media containing different concentrations of IPTG (0mM-1.0mM). At any IPTG conncentration tested, no effect of the growth rate of MC4100 and MC4100DE3 was observed (Figure 12). However, the growth rate of MC4100DE3 carrying the pET21 vector or the pSOPR plasmid was affected (Figure 12). With increasing IPTG concentration (IPTG $\geq$ 0.3mM), these two strains showed a slower growth rate over time. However, the OD 600 readings of the O/N samples obtained from all four of the strains gave similar values (Figure 12).

3.6 Creation of pKAN – modified λRed expressing plasmid

To integrate the *rpoS* sense and *rpoS* antisense as a PCR product into the *E. coli* chromosome, the λRed system expressing plasmid pKD46 required modification. The β-lactamase gene (Amp[R] marker) needed to be replaced with a neomycin phosphotransferase gene (Kan[R] marker). This was because the PCR product to be integrated carried the same Amp[R] selectable marker. This exchange was achieved by homologous recombination aided by the λ Red recombinase genes encoded on the pKD46 plasmid.

Several recombinants were obtained after the transformation. The recombination efficiency was determined to be 3.5×10^4 recombinants per μg of DNA. The recombinants

were tested for their phenotype on media supplemented with different antibiotics. All recombinants were found to be kanamycin resistant and ampicillin sensitive.

The confirmation of the replacement of the β-lactamase ORF by the neomycin phosphotransferase ORF was done by PCR. The expected size of the PCR product using the confirmation primers was 278bp. The correct size fragment was amplified (Figure 13), showing that the Kan^R ORF had replaced the Amp^R ORF. This modified pKD46 plasmid was subsequently renamed pKAN and could be used for the integration of β-lactamase expressing PCR fragments.

3.7 Integration of the *rpoS* sense and *rpoS* antisense into the chromosome

The PCR fragments amplified by the 56nt primers to be integrated into the chromosome were found to be the correct size. The PCR products obtained by using pET21, pSOPR, and pRPOS as DNA template were 3.6 kb, 4.7 kb, 5 kb, respectively (data not shown).

rpoS sense and *rpoS* antisense expressing PCR products along with the empty pET21 vector fragment were integrated into the *E. coli* chromosome using the Datsenko and Wanner method (2000). This method was used previously in the creation of pKAN (see above). Since all three of the PCR fragments above express β-lactamase, pKAN was used instead of pKD46 to express the λRed system.

Putative recombinants with the *rpoS* antisense PCR product and the pET21 vector PCR product integrated into the MC4100 DE3 background were obtained. The transformation efficiency was found to be $8x10^2$ recombinants per μg of DNA and $3x10^3$

recombinants per μg of DNA, respectively. The transformation efficiency achieved by

pRPOS, as a positive control, was found to be 7.5×10^6 cells per μg DNA. Ten colonies

were streaked for purity and tested for proper phenotype (Table 5).

Putative recombinants with the *rpoS* sense PCR product and the empty pET21

PCR fragment integrated into the HS1600 DE3 background were also obtained. The

transformation efficiency in this set of reactions was much lower. 2.8×10^2 recombinants

per μg of DNA and 40 cells per μg of DNA, respectively. All the colonies were streaked

out for purity and their phenotype was tested (Table 5).

3.7.1 Expression of catalase in the putative recombinants

The putative recombinants showing the correct antibiotic resistance phenotype

were tested for catalase expression under IPTG-induced and noninduced conditions. The

recombinants, in groups of 6, were printed onto selective media plates containing varying

concentrations of IPTG (0.1mM to 1.0mM) and tested for hydrogen peroxide sensitivity.

It was noted that the putative *rpoS* antisense recombinants did not show any

inhibition of catalase expression when compared with the pET21 vector controls and the

MC4100 DE3 background at any of the tested IPTG concentrations (Table 6). However,

some of the putative *rpoS* sense recombinants did show a significant increase in catalase

expression on plates containing IPTG in comparison to those containing no IPTG (Table

6).

3.7.2 Confirmation of Integration

To confirm the integration in all of the putative recombinants obtained that

showed proper antibiotic resistance phenotype, PCR was performed using primers

upstream and downstream the integration site. The size of the PCR fragments obtained

from all the reactions was ~5 kb (Figure 14). These products obtained from the

recombinants were compared to the MC4100DE3 strain as a control for non-insertion.

Restriction mapping was utilized to establish the contents of the 5 kb PCR

product generated from the putative *rpoS* sense recombinant (*rpoS*-6). This PCR product

along with the putative pET vector fragment and the PCR product from the host cells

were treated with three different restriction endonucleases: PstI, HindIII, and EcoRI. The

pET21 vector derivative, pRPOS, was used as a positive control for the digestion. When

the digests were run on an agarose gel, all PCR products were cut with PstI, showing a

band of slightly smaller size, which is consistent with the expected 4.7 kb band (Figure

15). In the reactions using HindIII and EcoRI, no restriction patterns were observed that

would be consistent with integration of the PCR fragments.

To determine the location of the PCR product a primer was designed 250 bp

downstream of the 5'-end of the PCR product to sequence outward to the junction point

on the chromosome. The sequence obtained was of good quality (Appendix II). The

generated sequence was aligned with BLAST against all sequences in the Genbank

database. The resulting alignment indicated exact homology to expression vector

sequences. The obtained sequence was compared with the pET21 vector sequence

acquired from the manufacturer's website (Novagen), and the obtained sequence

contained only the pET21 vector and showed no recombination junction with the

chromosome. This suggests that the entire pRPOS plasmid is located within the strain and

has not recombined within the *ebg* locus.

CHAPTER 4: Discussion

4.1 Expression of the *rpoS* antisense and sense from high-copy plasmids

4.1.1 *rpoS* antisense expression does not show any effect on the RpoS-dependent *osmY-lacZ* gene fusion on solid media

To determine if expression of the *rpoS* antisense has an effect on the level of RpoS activity in the cell, an *osmY-lacZ* fusion was introduced into the MC4100DE3 strain containing either the pSOPR plasmid or the unmodified pET21 vector. The host strain MC4100 that was used carries a large deletion in the region that encodes the *lacZ* gene in its chromosome (Peters *et al.*, 2003), allowing it to be used in fusion studies. The *osmY-lacZ* gene fusion was used since it has been previously shown to be highly RpoS-dependent (Schellhorn *et al.*, 1998). *osmY* encodes a periplasmic protein that is induced in a hyperosmotic environment (Yim and Villarejo, 1992). No difference in *lacZ* expression was observed in the strains MC4100DE3 pET21 and MC4100DE3 pSOPR, whether induced or uninduced by IPTG. This result was unexpected, as previous studies have shown that the *rpoS* antisense encoded by pSOPR is effective in downregulation of RpoS regulon members including *katE* and *glgS* (Chen *et al.*, 2003). This suggested that the *rpoS* antisense was not inhibiting *rpoS* expression or that the inhibition could not be measured on plates. The reason for this discrepancy is unclear.

4.1.2 *rpoS* sense expression does show an effect on the RpoS-dependent *osmY-lacZ* gene fusion on solid media

To determine if induction of *rpoS* from a plasmid, pRPOS, results in downstream expression of members of the *rpoS* regulon, *osmY-lacZ* expression was examined in the

HS1600DE3 pET21 and HS1600DE3 pRPOS strains. There was a significant increase in

lacZ expression in the strain expressing the *rpoS* sense. However, there was also

significant basal level expression of *rpoS* under the noninduced condition. This can be

explained by the fact that even in the presence of the *lac* repressor the host RNA

polymerase can transcribe expression of the T7 RNA polymerase gene from DE3 causing

"leaky" expression of *rpoS*. This could be tested by comparing expression to the strain

carrying the vector alone. Additionally, northern analysis of T7 polymerase expression

under non-induced conditions would reveal if there was expression under non-induced

conditions (-IPTG). Nonetheless, these results suggest that *rpoS* expression could be

IPTG-induced in the host strain.

4.1.3 Comparison of the effect of *rpoS* antisense expression on a RpoS-dependent

and independent *lacZ* gene fusion on solid media

To test whether the *rpoS* antisense RNA was specific for *rpoS*, expression studies

were done using an RpoS-independent operon *lacZ* fusion, *rsi001-lacZ*, and compared to

the RpoS-dependent fusion, *osmY-lacZ*. Upon comparison of the *rpoS* antisense

expression on the *osmY-lacZ* and *rsi001-lacZ* gene fusions, it appeared that there was no

difference in expression of either gene on plates. The MC4100DE3 pET21 and

MC4100DE3 pSOPR strains expressing the *osmY-lacZ* fusion showed similar levels of β-

galactosidase expression as the same strains carrying the *rsi001-lacZ* fusion. Further

study was conducted in liquid media to test the effect of the *rpoS* antisense on these two

lacZ gene fusions.

4.1.4 *rpoS* **antisense does not inhibit expression of the RpoS-dependent** *osmY-lacZ*

gene fusion in liquid media

No inhibition in the expression of the RpoS-dependent *osmY-lacZ* gene fusion

was observed upon induction of the *rpoS* antisense from the pSOPR plasmid in the

MC4100DE3 background. It may be that there were a large number of *rpoS* transcripts

already present in the cell prior to the induction of the *rpoS* antisense, possibly

minimizing the inhibitory effect of the *rpoS* antisense.

Expression of *rpoS* is induced during early exponential phase, at an OD600 = 0.3

(Schellhorn and Stones, 1992). That means in order to achieve inhibition the *rpoS*

antisense construct needs to be induced in early log phase prior to *rpoS* expression to

allow T7 RNA polymerase some time to be expressed before it could transcribe the *rpoS*

antisense mRNA (Glick and Pasternak, 1998). If the *rpoS* antisense transcripts were

synthesized as the *rpoS* transcripts are synthesized, the antisense would stand a better

chance at inhibiting translation of *rpoS* without accumulating *rpoS* transcripts in the cell.

However, expression of the *rpoS* antisense was induced at several culture densities,

including several lower than OD600 = 0.3 (Chen *et al.,* 2003). It is not likely a problem

with the accumulation of *rpoS* transcipts. However, it may be possible that the IPTG

concentration was not high enough to allow induction of the *rpoS* antisense.

4.1.5 *rpoS* **antisense does not inhibit expression of the RpoS-independent** *rsi001-*

lacZ **gene fusion in liquid media**

No inhibition of *rsi001-lacZ* expression was evident, in liquid media, when

comparing the expression of the fusion in the MC4100DE3 pET21 and MC4100DE3 pSOPR strains (Figure 11). The data confirms that *rsi001-lacZ* is a growth phase-dependent but RpoS-independent *lacZ* fusion as its expression begins to increase in a linear fashion starting in early exponential phase. As expected the *rpoS* antisense had no effect on the expression of the *rsi001-lacZ* fusion.

4.2 *rsi001* identified as *acpD*

The growth phase-dependent and RpoS-independent *rsi001-lacZ* gene fusion was sequenced and identified as *acpD*, a gene that codes for an acyl carrier protein (ACP) phosphodiesterase in *E. coli*. ACP phosphodiesterase catalyzes the cleavage of the 4'-phosphopantetheine residue from holo-ACP generating apo-ACP (Vagelos and Larrabee, 1967). It has been determined to be a 25-kDa enzyme that is fully active at 42°C (Fischl and Kennedy, 1990). It was proposed to play a role in fatty acid synthesis (Vagelos and Larrabee, 1967). However, a more recent study indicated that this was not likely its physiological function (Jackowski and Rock, 1983). The physiological role remains unclear. More recently, *acpD* was proposed to be renamed *azoR*, an azoreductase (Nakanishi *et al.*, 2001). It was shown that the *acpD* gene product showed no ACP phosphodiesterase activity. Rather, it shared biochemical properties with azoreductase, an enzyme that catalyzed reductive cleavage of azo groups (Nakanishi *et al.*, 2001).

4.3 IPTG affects bacterial growth rate

An effect of IPTG concentration on growth rate was observed in MC4100DE3 strains carrying the pET21 vector and the pSOPR plasmid. However, no effect on growth

rate was observed for the MC4100 and MC4100DE3 strains. The strains containing the plasmids showed a significantly slower growth rate with IPTG concentration ≥0.3mM. However, the O/N samples reached similar OD600 values as the MC4100 and MC4100DE3 strains.

The concentration of IPTG used in the earlier expression studies was 0.3mM. At this concentration, there appears to be an effect on the growth rate. It is not as significant an effect as cultures grown in 1.0mM IPTG concentration but it is still showing an effect.

The observations of this titration experiment suggest that IPTG concentrations ≥ 0.3mM in the presence of a high-copy plasmid may pose a tremendous metabolic burden on the *E. coli* strains. As a result, the growth rate is reduced but the culture eventually reaches similar density as strains not carrying plasmids. This may be due to the fact as the density of the culture increases the concentration of IPTG in the cell decreases, therefore reducing the level of toxicity associated with IPTG, however this is not likely.

4.4 Overexpression using pET vector expression system

The pET vector expression system was used to express the *rpoS* sense and *rpoS* antisense from an IPTG-inducible promoter. This system was chosen for its ability to mass overproduce the target gene. The major problem faced in this study was that after IPTG induction of exponentially growing cells, the rate of cell growth was significantly lowered. A less significant effect was seen on cell growth rate when cells were induced near or during stationary phase.

An alternative expression system, pBAD, may also be utilized for expression of

the target gene under an L-arabinose inducible promoter that allows for tight regulation of gene expression (Invitrogen). Changing the concentration of arabinose can modulate levels of expression (Khlebnikov *et al.*, 2002). However, in nutrient limiting conditions *Escherichia coli* may utilize arabinose as a carbon source. In this case, transcription may be poorly induced. Another problematic area is that the native *E. coli* RNA polymerase recognizes this promoter. This may lead to leaky expression of the target gene as the case in the pET vector system. The pET vector expression system can use the pLysS plasmid to inhibit T7 RNA polymerase expression (Novagen). However, there is no way to reduce the target gene expression in the non-induced state in the pBAD system.

4.5 Integration of the *rpoS* sense and *rpoS* antisense into the host chromosome as a single copy

After analyzing the data gathered from the expression studies utilitizing the pET vector expression system, it was concluded that the *rpoS* sense and antisense should be integrated into the host chromosome. It would be less of a burden on the microorganism and be more physiologically relevant. In order to do this, the method used by Datsenko and Wanner (2000) was utilized. However, before this could be done, the plasmid carrying the λRed system, pKD46, required modification. The ampicillin resistance marker (Amp^R) was replaced with the kanamycin resistance marker (Kan^R).

4.5.1 Replacement of Amp^R marker on λRed expressing plasmid

Recombinants were successfully obtained that had replaced the Amp^R marker with the Kan^R marker. However, it was difficult to determine whether or not the

recombination efficiency was good. Datsenko and Wanner (2000) did not publish their recombination efficiencies and they performed their experiments using a different λRed expressing plasmid, pCP20. Data using the pKD46 plasmid was not shown, although they indicated that higher numbers of recombinants are obtained utilizing pKD46 relative to pCP20. In a previous study, a recombination efficiency of approximately $4x10^5$ cells per μg of DNA was obtained, utilizing a similar technique of electroporation and recombination with the λRed system (Murphy, 1998).

All recombinants were found to be kanamycin resistant and ampicillin sensitive. The neomycin phosphotransferase ORF appeared to have successfully replaced the β-lactamase ORF. The integration was verified using PCR. The PCR product was only attainable if the neomycin phosphotransferase ORF was properly integrated into the β-lactamase ORF site as one primer annealed to the neomycin phosphotransferase ORF and the other annealed to sequence on the plasmid. This proved that the Kan^R ORF had replaced the Amp^R ORF on the pKD46 plasmid.

4.5.2 Integration of the linear DNA fragment into the chromosome

Several recombinants were obtained using the Datsenko and Wanner (2000) method. The recombination efficiency was much lower than the $7.5x10^6$ cells per μg DNA transformation efficiency achieved by pRPOS as a positive control, which is an accurate representation of transformation efficiencies using plasmids (Sambrook and Russell, 2001). As previously noted, a recombination efficiency of approximately $4x10^5$ cells per μg of DNA was obtained, utilizing a similar technique of electroporation and

recombination with the λRed system (Murphy, 1998). The recombination efficiency obtained in this study was much lower.

The differences in transformation efficiency may be attributed to the preparation of the electrocompetent cells, which had the λRed system induced. The protocol used to make the cells of both MC4100DE3 pKAN and HS1600DE3 pKAN was the same but they may have been grown to different densities giving fewer cells in total that were available for transformation of the DNA. It may also be due to the nature of the preparation. This may have resulted in a decrease in the number of recombinants. Using modified media to wash the cells to remove electrolytes may have increased the transformation efficiency. Additionally, freezing and thawing of the cells or the age of the cells prior to electroporation may have influenced the transformation efficiencies.

4.5.3 Expression of catalase in the putative recombinants

Since HPII catalase (encoded by *katE*) expression is known to be highly *rpoS*-dependent (Schellhorn and Hassan, 1988), the data suggested that the *rpoS* antisense insertion is not modulating *rpoS* expression, if the insertion has properly occured. However, some of the putative *rpoS* sense recombinants did show a significant increase in catalase expression on plates containing IPTG in comparison to those containing no IPTG, suggesting that *rpoS* was being expressed. One isolate in particular, *rpoS*-6, showed the same degree of catalase activity (as determined by bubbling in the presence of H_2O_2) as the RpoS$^+$ background (MC4100 DE3). This suggested that *rpoS* was being expressed at levels similar to wild-type strains in this isolated recombinant.

We did not observe a significant difference in catalase expression between the induced and non-induced *rpoS* sense in the obtained recombinants. This is most likely due to the leaky expression of the T7 RNA polymerase gene under the control of the *lac*UV5 promoter, which allows some transcription under the non-induced condition. Transforming in a pLysS plasmid, which encodes a T7 lysozyme that inactivates T7 RNA polymerase, may control the leaky expression (Novagen), thus preventing the non-induced transcription of the gene/sequence of interest.

4.5.4 Confirmation

The product obtained by PCR indicates that the PCR product carrying the *rpoS* antisense has not inserted into the *ebg* operon in the *E. coli* chromosome. The fragment amplified should have been ~1.3kb shorter but it appeared similar in size to the strain with no integration. Due to the similarity in size of the PCR products, the reactions were not sufficient to determine if the putative *rpoS* sense fragment had replaced the *ebg* operon.

All PCR products cut with PstI showed the same restriction digestion pattern. This suggested that the PCR product had not integrated into the *ebg* operon. However, this did not eliminate the possibility that it integrated elsewhere in the genome as the putative recombinants have acquired ampicillin resistance.

Alignment of the obtained sequence and the Genbank and manufacturer's sequence suggested that pRPOS had either integrated entirely by homologous recombination with *rpoS* or randomly somewhere into the *E. coli* chromosome.

Alternatively, it was also possible that no integration into the chromosome had occurred and the plasmid was being carried in the cells.

4.6 Successful recombination is possible

The λRed system has been successfully utilized to integrate genes/sequences into a plasmid (this study) and into bacterial chromosomes (Table 7) (Murphy, 1998; Poote and Fenton, 2000; Murphy *et al.*, 2000; Yu *et al.*, 2000; Datsenko and Wanner, 2000; Stanley *et al.*, 2000; Ho and Slauch, 2001; Wilson *et al.*, 2001; Janes *et al.*, 2001; Gust *et al.*, 2003). However, the PCR products carrying the *rpoS* sense and the *rpoS* antisense were not integrated into the chosen site (*ebg* operon) in the *E. coli* chromosome. There were some differences that may have resulted in recombinantion inefficiency.

First of all, the size of the PCR fragments intended for integration exceeded the DNA fragment size that had been successfully integrated using the short regions of homology (Yu *et al.*, 2000; Datsenko and Wanner, 2000; Stanley *et al.*, 2000; Ho and Slauch, 2001; Gust *et al.*, 2003). Large fragments (≥ 3kb) have only been successfully integrate when there was ~1000bp homology to the site of integration (Murphy, 1998; Poote and Fenton, 2000; Murphy *et al.*, 2000; Wilson *et al.*, 2001; Gust *et al.*, 2003).

Secondly, the PCR products contained sequences homologous (i.e. *rpoS*) to the *E. coli* chromosome, where recombination may also occur. In the past, this technique was used to knock out or integrate genes, foreign to the microorganisms (Murphy, 1998; Poote and Fenton, 2000; Murphy *et al.*, 2000; Yu *et al.*, 2000; Datsenko and Wanner, 2000; Stanley *et al.*, 2000; Ho and Slauch, 2001; Wilson *et al.*, 2001; Janes *et al.*, 2001;

Gust *et al.*, 2003). The homologous sequences that are being integrated, in our case, could give rise to unintended recombination events.

Finally, the strains used in this study (MC4100 DE3 and its derivative) differed from those used by others, and recombination using the λRed system has not been demonstrated in this strain. Amongst other smaller mutations, MC4100 contains a large deletion in the chromosome (Peters *et al.*, 2003), which may affect the recombination ability of this strain. Perhaps there is a gene missing that is essential for the λRed system to work efficiently.

4.7 Future Work

The next component of this study that may be conducted is to modify the approach to integrating the desired sequences into the *E. coli* chromosome. Steps should be taken to eliminate any region of homology within the chromosome to the PCR product intended for integration. Primers may need to be designed that have a greater degree of base pair homology to the integration site. Also, all the non-essential sequence should be removed from the PCR product to shorten its size, as fragment size may be one of the reasons for poor recombination efficiency. To eliminate the possibility of strain variation, the DE3 should be inserted into the MG1655 wild-type strain.

It may be necessary to take a different approach to creating an inducible *rpoS* sense and *rpoS* antisense in the *Escherichia coli* chromosome. The present *rpoS* promoter region may be excised and replaced by an inducible promoter using the same λRed system. This would eliminate the problem associated with the amount of homology

required and the large PCR fragment sizes encountered in this study.

Table 1. *Escherichia coli* **strains and phage used in this study**

Strain	Genotype	Source or reference
MG1655	*E. coli* K-12, F⁻ λ⁻ *ivlG⁻ rfb*-50 *rph*-1	Yale University
MC4100	Δ(*argF-lacZ)205 ara139 flbB5301 relA1 rpsL150 thi ptsF25*	G. Weinstock
MC4100DE3	same as MC4100 but DE3 encoding T7 RNA polymerase	Chen *et al.* 2003
MC4100DE3 pET21	same as MC4100DE3 but also carrying a pET21 vector	Chen *et al.* 2003
MC4100DE3 pSOPR	same as MC4100DE3 but also carrying an *rpoS* antisense expressing vector	Chen *et al.* 2003
HS1600	same as MC4100 but *rpoS13::Tn10*	Laboratory collection
HS1600DE3	same as MC4100DE3 except *rpoS13::Tn10*	Chen and Schellhorn, 2003
HS1600DE3 pET21	same as HS1600DE3 but also carrying a pET21 vector	Chen and Schellhorn, 2003
HS1600DE3 pRPOS	same as HS1600DE3 but also carrying a *rpoS* sense expressing vector	Chen and Schellhorn, 2003
HS1091	Δ*lacU169 rpsL* but *osmY*-λplacMu53 (rsd1091)	Schellhorn *et al.* 1998
13C10	Δ*lacU169 rpsL* but carrying a growth phase dependent, RpoS-independent promoter-*lacZ* fusion	Schellhorn *et al.* 1998
P1vir	generalized transducing bacteriophage	Laboratory stock

Table 2. Plasmids used in this study

Name	Description and resistance	Source or Reference
pKD46	encodes λRed system for homologous recombination; encodes β-lactamase (Amp^R marker)	Datsenko and Wanner, 2000
pKD3	encodes CAT (Cm^R marker)	Datsenko and Wanner, 2000
pKD4	encodes neomycin phosphotransferase (Kan^R marker)	Datsenko and Wanner, 2000
pKAN	same as pKD46 except encodes Kan^R marker in place of Amp^R (Kan^R marker)	this study

Table 3. Oligonucleotides used in this study

Name	Sequence	Source
AB32933	5'-GTTACCGTTACAGCGTCAAAGAAACGCGCT TTATTTCAGCAGATTACGCGCAGAAA-3'	MOBIX
AB32934	5'-CCAGAGGCGTAAATCTGGCGGAACTGTTCT AAGTTACTCCAGCGAAAGCGGTCCT-3'	MOBIX
ML1108	5'-TGATAAATGCTTCAATAATATTGAAAAAGG AAGAGTATGATTGAACAAGATGGATTGC-3'	MOBIX
ML1109	5'-AATCTAAAGTATATATGAGTAAACTTGGTC TGACAGTCAGAAGAACTCGTCAAGAGGG-3'	MOBIX
ML1325	5'-GCGGAACCCCTATTTGTTTATT-3'	MOBIX
ML1326	5'-ATTCAGGGCACCGGACAG-3'	MOBIX
ML1706	5'-TTCACTTCCACAATCTTTCC-3'	MOBIX
ML1707	5'-AATGAGTCACCGATGGAGT-3'	MOBIX
ML3096	5'-TGGATGAACGAAATAGACAGA-3'	MOBIX

Table 4. Expected restriction fragment sizes from restriction digestion of the isolated 5kb PCR product (see Figure 6)

Restriction Enzyme	Expected Fragment sizes (bp)	
	with integration	without integration
PstI	500, 4500	300, 4700
HindIII	1800, 3200	Uncut
EcoRI	1900, 1400, 1700	Uncut

Table 5. Phenotype characterization of the putative recombinants

Putative Recombinant	Isolate	Selective media				
		LB	LB+ S	LB+ T	LB+ K	LB+ A
MC4100DE3 pET21 control PCR product	A	+	+	-	+	+
	B	+	+	-	-	+
	C	+	+	-	+	+
	D	+	+	-	+	+
	E	+	+	-	+	+
	F	+	+	-	+	+
	G	+	+	-	-	+
	H	+	+	-	+	+
	I	+	+	-	+	+
	J	+	+	-	+	+
MC4100DE3 *rpoS* antisense PCR product	A	+	+	-	+	+
	B	+	+	-	-	+
	C	+	+	-	-	+
	D	+	+	-	+	+
	E	+	+	-	+	+
	F	+	+	-	+	+
	G	+	+	-	+	+
	H	+	+	-	+	+
	I	+	+	-	+	+
	J	+	+	-	+	+
HS1600DE3 pET21 control PCR product	1	+	+	+	+	+
	2	+	+	+	-	+
HS1600DE3 *rpoS* sense PCR product	1	+	+	+	-	+
	2	+	+	+	-	+
	3	+	+	+	-	+
	4	+	+	+	-	+
	5	+	+	+	-	+
	6	+	+	+	-	+
	7	+	+	+	-	+

Abbreviations: +, growth on indicated media; -, no growth on indicated media; S, streptomycin; K, kanamycin; A, ampicillin; T, tetracycline.

Table 6. Catalase activity in putative recombinants

Strain/Recombinant	Catalase activity (degree of bubbling with H_2O_2)							
	IPTG Concentration (mM)							
	0	0.1	0.2	0.25	0.3	0.5	0.75	1.0
MC4100DE3	+++	+++	+++	+++	+++	+++	+++	+++
MC4100DE3 pET21-B	+++	+++	+++	+++	+++	+++	+++	+++
MC4100DE3 pET21-G	+++	+++	+++	+++	+++	+++	+++	+++
MC4100DE3 *rpoS* antisense-B	+++	+++	+++	+++	+++	+++	+++	+++
MC4100DE3 *rpoS* antisense-C	+++	+++	+++	+++	+++	+++	+++	+++
HS1600DE3	+	+	+	+	+	+	+	+
HS1600 DE3 pET21-2	+	+	+	+	+	+	+	+
HS1600DE3 *rpoS* sense-1	+	+	+	+	+	+	+	+
HS1600DE3 *rpoS* sense-2	+	+	+	+	+	+	+	+
HS1600DE3 *rpoS* sense-3	+	+	+	+	+	+	+	+
HS1600DE3 *rpoS* sense-4	+	+	+	+	+	+	+	+
HS1600DE3 *rpoS* sense-5	+	++	++	++	++	++	++	++
HS1600DE3 *rpoS* sense-6	++	+++	+++	+++	+++	+++	+++	+++
HS1600DE3 *rpoS* sense-7	+	++	++	++	++	++	++	++

Abbreviations: +++, high level of activity with vigorous bubbling in the presence of H_2O_2; ++, medium level of catalase activity; +, low level of catalase activity with slow bubbling in the presence of H_2O_2

Table 7. λRed system used to integrate linear DNA fragments into host chromosome

Integrated Linear Fragment size (kb)	Sequence Homology to gene of interest (bp)	Description	Reference
3.9	1000	Integration of a *lacZ::kan* fragment into the *E. coli* chromosome with the aid of a λ*red* encoding plasmid in a Δ*recBCD* strain	Murphy, 1998
4.0	1500	Integration of a *lacZ::cat* fragment into the *E. coli* chromosome of a Δ*recBCD::λred* strain	Poote and Fenton, 2000
3.0	1000	Integration of *geneX::cat* into the *E. coli* chromosome, where *geneX* was *lacZ, recR, recQ, polB, mutH,* or *mutL.*	Murphy *et al.*, 2000
1.1	30-50	Integration of a *galK:cat* fragment to introduce a single base pair change in the chromosomal *galK* of *E. coli.*	Yu *et al.*, 2000
1.0	36-50	Integration of *geneX::cat* and/or *geneX::kan* into the *E. coli* chromsome, where *geneX* was *arcB, cyaA, lacZYA, ompR-envZ, phnR, pstB, pstCA, pstS, pstscab-phoU, recA,* and *torSTRcad* genes/operons.	Datsenko and Wanner, 2000
1.0	30-50	Integration of *gipA::kan* into the *S. enterica* chromosome.	Stanley *et al.*, 2000
1.0	25-36	Integration of *grvA::kan* into the *S. enterica* chromosome	Ho and Slauch, 2001
0.1	1000	Integration of *escF* carrying a 120bp deletion into the *escF* gene in the chromosome of an enteropathogenic *E. coli* strain.	Wilson *et al.*, 2001
1.0	N/A	Integration kanamycin resistance cassette into the promoter region of *gdhA* in the *K. aerogenes* chromosome.	Janes *et al.*, 2001
1.4	39	Integration of a disruption cassette containing *acc (3)IV* (ApraR) and *oriT* into the *cyc1* and *cyc2* genes in the *S. coelicolor* chromosome.	Gust *et al.*, 2003

Figure 1. Structure of *rpoS*.

The locations of the *rpoS* promoters (P1, P2, P3) are indicated above. The primary

promoter is located within the *nlpD* ORF. The other promoters that also allow for *rpoS*

transcription are the two *nlpD* promoters. The *rpoS* self complementary region causes

secondary structure of the *rpoS* transcript; Thus, preventing its translation until proper

stimuli are present. The *rpoS* ORF is designated by the hatched region in the mRNA,

with the start and stop codons indicated at the ends.
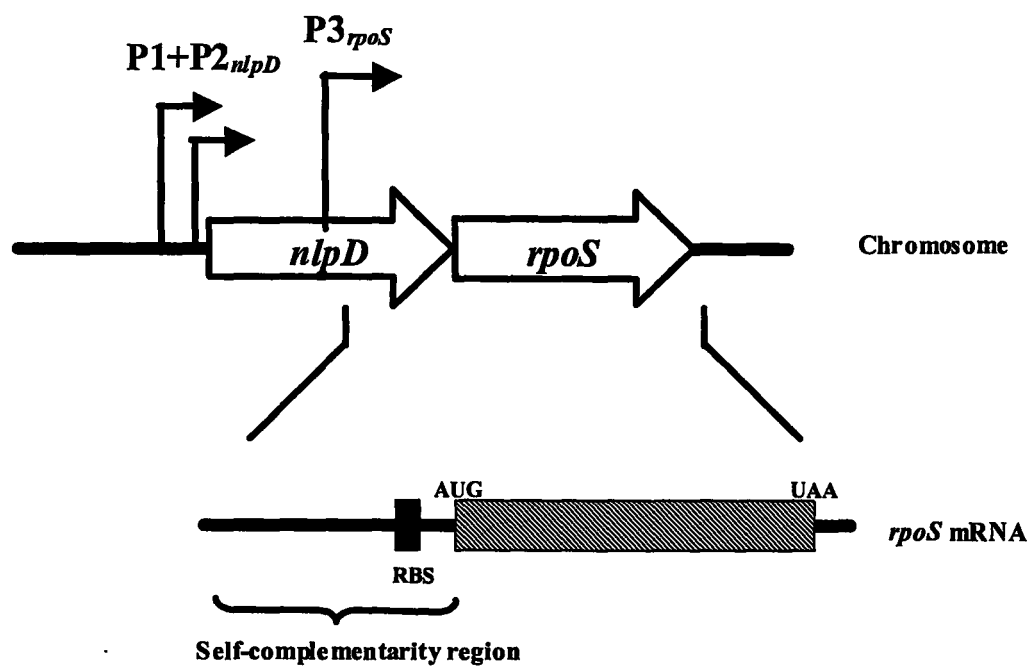
Figure 2. Graphic representation of plasmids utilized in this study.

(A) pET21, empty vector was used as a control in the expression studies; (B) pSOPR, *rpoS* was cloned downstream the P_{T7lac} promoter in the opposite orientation into a pET21 vector, creating an *rpoS* antisense; (C) pRPOS, *rpoS* was cloned downstream the P_{T7lac} promoter in the right orientation, creating a *rpoS* sense. (The pET21 derivatives were created G. Chen).
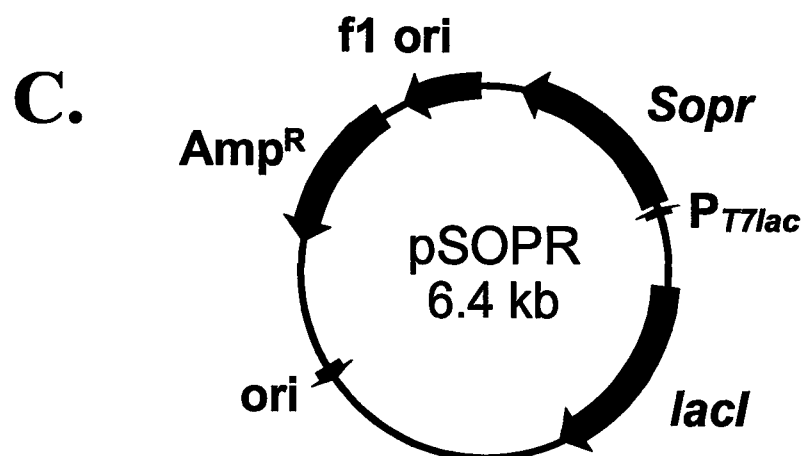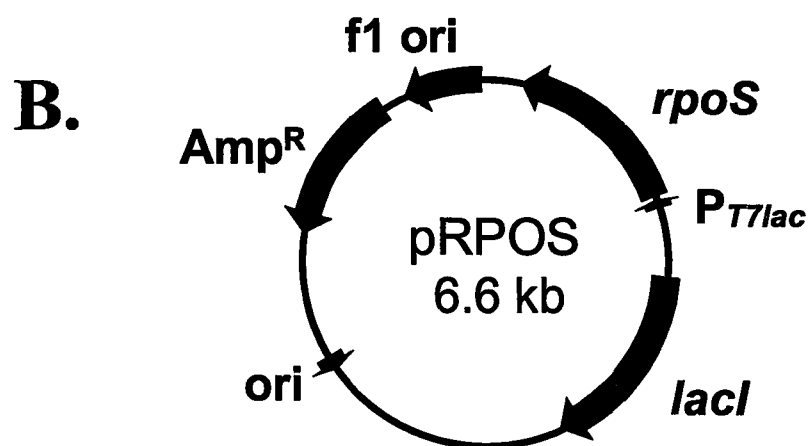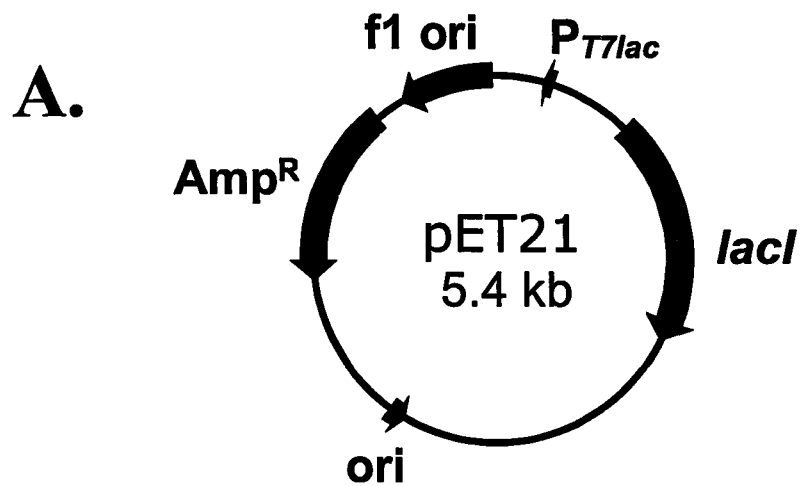
Figure 3. Structure of the *ebg* operon.

The operon is located at 69.4 min in the *E. coli* chromosome. This operon is composed of

three genes: *ebgR*, *ebgA*, and *ebgC*. The arrows represent the open reading frames

(ORFs) and the direction of transcription. The dashed line indicates the chosen site for

integration. (Note: The integration sites are not shown to scale).

1 kb

E. coli chromosome
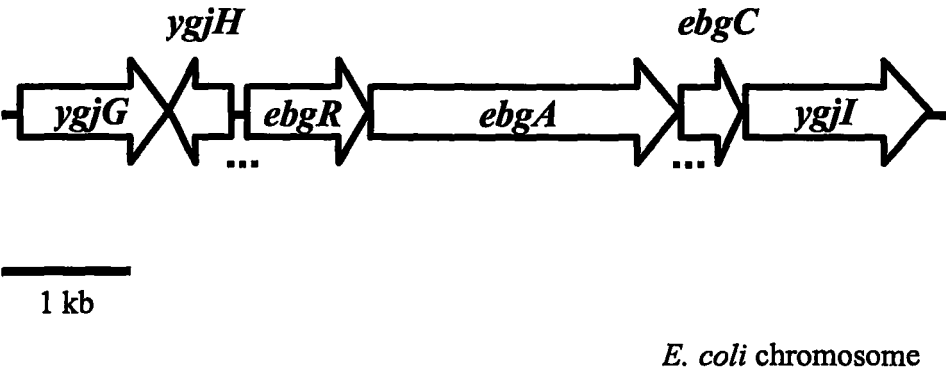
Figure 4. Annealing location of primers used to amplify linear DNA fragment for integration.

The region of interest was amplified from the pRPOS plasmid (from Chen and Schellhorn, 2003). Each primer is made of two components. The solid line represents the 20nt portion of the primer that is homologous to the plasmid. The dashed line represents the 36nt at the 5' end of the primer that is homologous to the chosen site of integration, the *ebg* operon. The same primers were used to amplify from the pSOPR plasmid (Chen *et al.*, 2003) and pET21 vector (not shown). Note: the primers are not shown to scale.

f1 ori

rpoS

Amp^R

P_{T7lac}

pRPOS
6.6 kb

lacI

ori
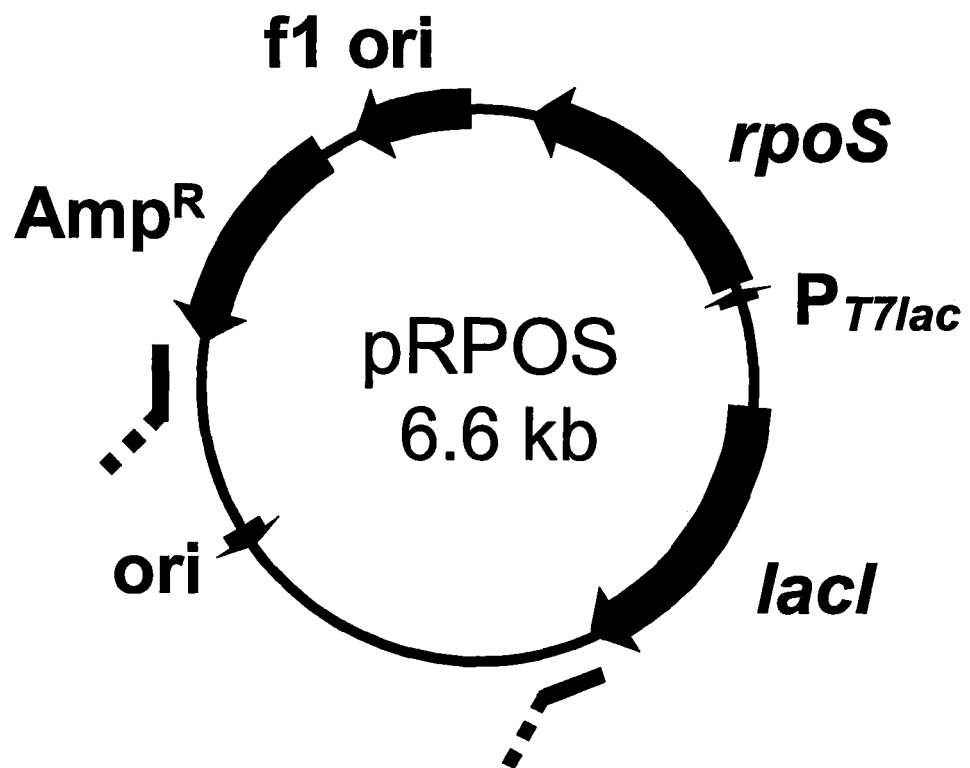
Figure 5. Representation of the homologous recombination event.

This event is made possible by the expression of λRed recombinase from a helper plasmid (i.e. pKD46). Integration of the linear fragment of DNA, which encodes the *rpoS* antisense or the *rpoS* sense, into the *E. coli* chromosome is shown. (Note: The integration of the control fragment amplified for the pET21 vector is not shown, though it would integrate in a similar manner).

(not shown to scale)
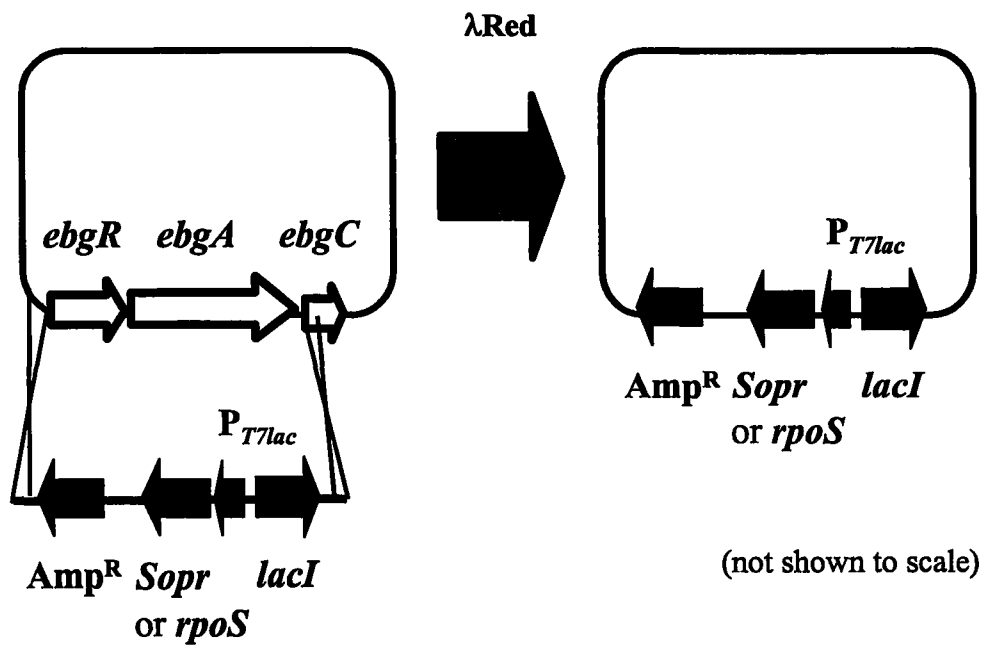
Figure 6. Restriction maps.

(A) location of PstI (P) restriction site is indicated in the *ebg* operon; (B) location

of restriction sites of PstI (P), HindIII (H), EcoRI (E) in the linear PCR fragment

that was integrated into the *E. coli* chromosome; (C) location of restriction sites in

the pRpoS plasmid that was used as a positive control for the digestion.

A.

B.

C.
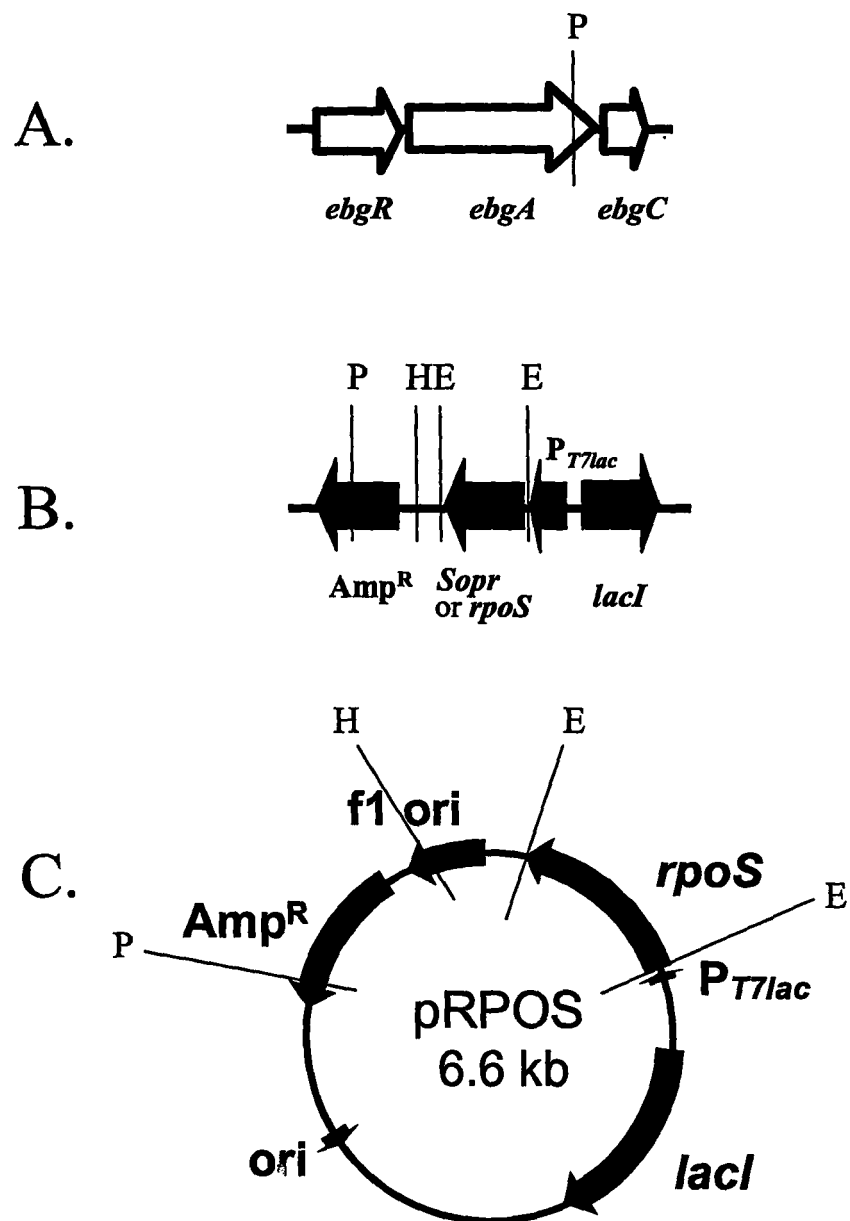
Figure 7. *osmY-lacZ* and *rsi001-lacZ* fusion expression in host strains and the

strains carrying a pET21 vector, and the pET21 vector derivative, pSOPR

and pRPOS, which encode the *rpoS* antisense and *rpoS* sense, respectively.

No reduction in *osmY-lacZ* expression was noted in the strain expressing the *rpoS*

antisense. An induction of *osmY-lacZ* expression was observed in the strain

expressing the *rpoS* sense. (A) *osmY-lacZ* fusion expression in host strains,

MC4100DE3 and HS1600DE3. The strains were printed on a LB Agar plate

containing no supplements. (B) *osmY-lacZ* fusion expression in MC4100DE3

containing the pET21 vector and the *rpoS* antisense expressing plasmid and also,

HS1600DE3 containing the pET21 vector and the *rpoS* sense expressing plasmid.

The strains were printed on a LB Agar plate containing 100µg/ml ampicillin to

select for the plasmid and 0.3mM IPTG to induce expression of the *rpoS* antisense

and *rpoS* sense from the P_{T7lac} promoter. Y in the strain name indicates that an

osmY-lacZ fusion is present in the strain. (C) No reduction in *osmY-lacZ* or

rsi001-lacZ expression was observed in the strains expressing the *rpoS* antisense.

The level of *lacZ* expression from both fusions was found to be similar to the

strains containing the pET21 vector (control plasmid).

A.

MC4100DE3Y

HS1600DE3Y
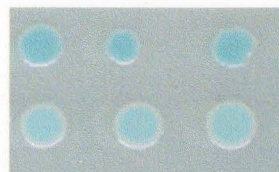
B.

MC4100DE3Y pET21

HS1600DE3Y pET21

MC4100DE3Y pSOPR

HS1600DE3Y pRPOS

C.
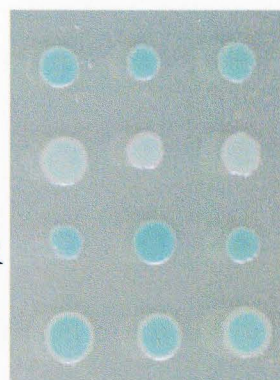
	osmY-lacZ	*rsi001-lacZ*
MC4100DE3 pET21		
MC4100DE3 pSOPR		
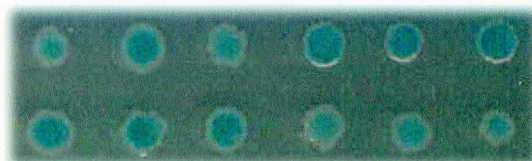
Figure 8. Growth phase dependent expression of *osmY-lacZ*, a highly RpoS-dependent fusion, in rich media.

Expression of pET21 vector and the *rpoS* antisense were induced with 0.3mM IPTG at the indicated time points and assayed for β-galactosidase once the culture reached stationary phase. Inhibition of *osmY-lacZ* expression was observed in the strain expressing the *rpoS* antisense. However, a significant difference in *osmY-lacZ* expression was seen in the strain carrying the pET21 vector under IPTG-induced and noninduced conditions.

osmY-lacZ fusion expression
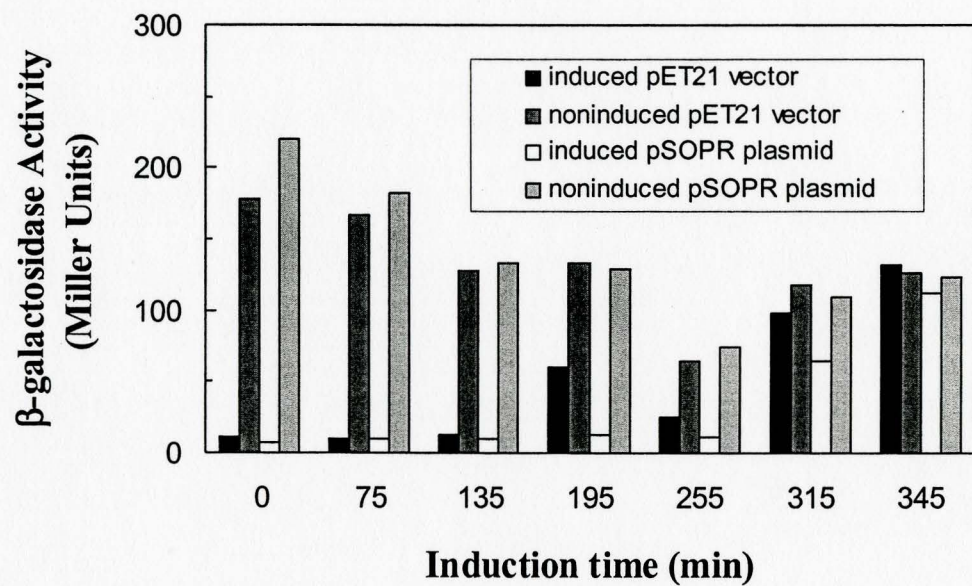
Figure 9. Growth phase dependent expression of *rsi001-lacZ*, a RpoS-

independent fusion, in rich media.

Expression of pET21 vector and the *rpoS* antisense were induced with 0.3mM

IPTG at the indicated time points and assayed for β-galactosidase once the culture

reached stationary phase. No significant inhibition of *rsi001-lacZ* expression was

observed in the strain expressing the *rpoS* antisense. However, a significant

difference in *rsi001-lacZ* expression was seen in the strain carrying the pET21

vector under IPTG-induced and noninduced conditions.

rsi001-lacZ fusion expression
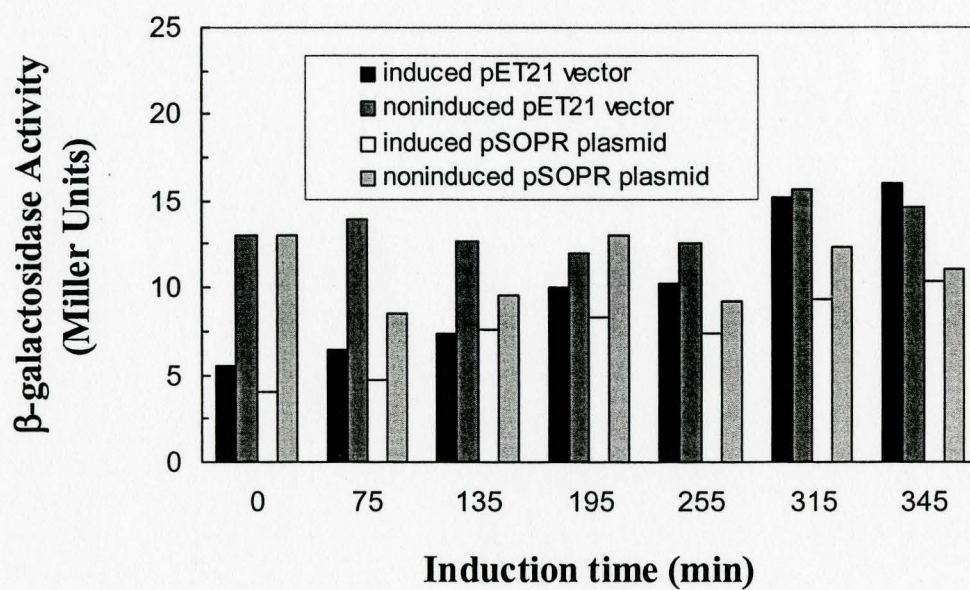
Figure 10. Relative induction of *osmY-lacZ* expression in the MC4100DE3 strains carrying the pET21 vector and pSOPR, the plasmid encoding the *rpoS* antisense.

Growth is represented by circles where open circles indicate IPTG-noninduced conditions and closed circles indicate IPTG-induced conditions. β-galactosidase activity is represented by squares where open squares indicate IPTG-noninduced conditions and closed squares indicate IPTG-induced conditions.

Induction of *osmY-lacZ*
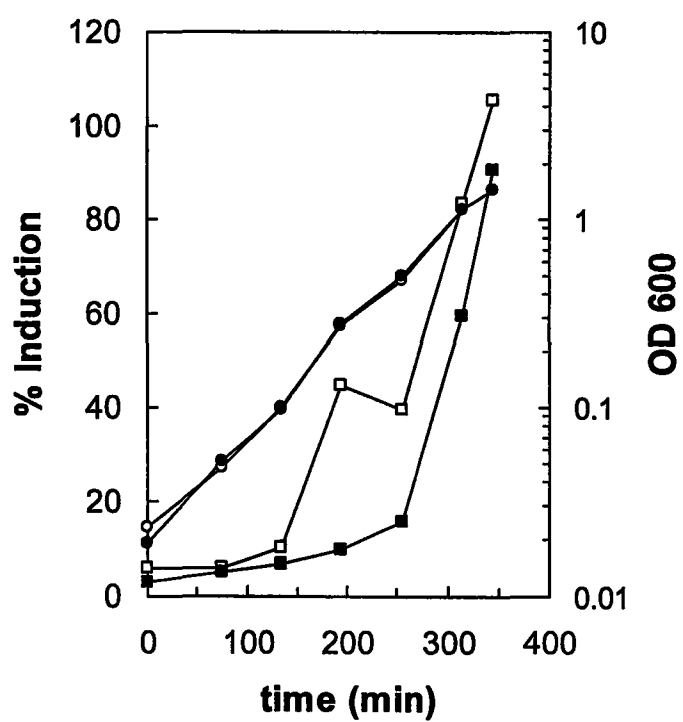
Figure 11. Relative induction of *rsi001-lacZ* expression in the MC4100DE3 strains carrying the pET21 vector and pSOPR, the plasmid encoding the *rpoS* antisense.

Growth is represented by circles where open circles indicate IPTG-noninduced conditions and closed circles indicate IPTG-induced conditions. β-galactosidase activity is represented by squares where open squares indicate IPTG-noninduced conditions and closed squares indicate IPTG-induced conditions.

Induction of *rsi001-lacZ*
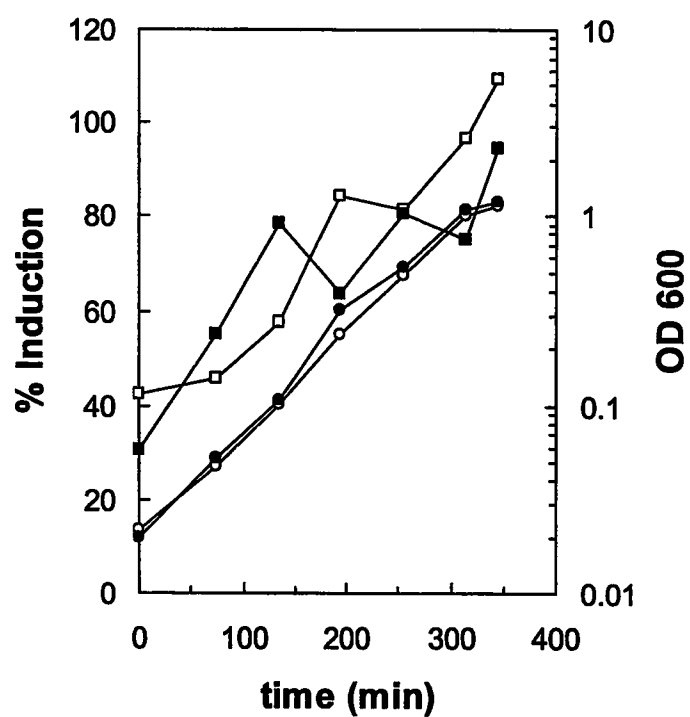
Figure 12. The Effect of IPTG concentration on selected *E. coli* strains.

MC4100 *osmY-lacZ*, MC4100DE3 *osmY-lacZ*, MC4100DE3 pET21 *osmY-lacZ*,

and MC4100DE3 pSOPR *osmY-lacZ*, were titrated with various IPTG

concentrations, ranging from 0mM to 1mM, and growth was monitored at the

indicated time points. No effect of IPTG concentration was seen in the strains,

MC4100 and MC4100DE3, that carried the *osmY-lacZ* fusion. An effect on

growth rate was observed on the strains containing plasmids, MC4100DE3 pET21

osmY-lacZ and MC4100DE3 pSOPR *osmY-lacZ*. Growth at given IPTG

concentrations is represented by the following symbols: Black diamond indicates

0mM; Black triangle indicates 0.05mM; White triangle indicates 0.1mM; Black

square indicates 0.2mM; White square indicates 0.3mM; Black circle indicates

0.5mM; White circle indicates 1.0mM.

Figure 13. Confirmation of the neomycin phosphotransferase gene integrated into the pKD46 plasmid.

Amplification product was only possible if the Kan^R marker had properly been replaced in the location of the β-lactamase gene (Amp^R marker). The lanes are: 100bp, 100bp DNA ladder (Invitrogen); C, no template control; R, confirmation PCR reaction. The numbers on the side indicate the band size in base pairs (bp). The band seen was just below 300bp, as expected.
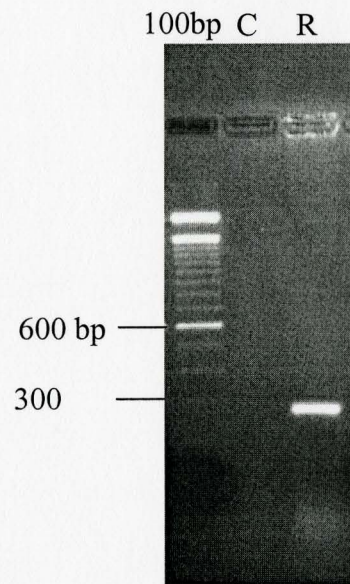
Figure 14. PCR products amplified using primers to confirm the integration of the linear fragments containing the regions of interest.

Confirmation primers were designed upstream and downstream the integration site. The PCR reactions were run on a 0.8% agarose gel along with the positive control. A 5kb band was seen in all lanes including the positive control. No bands were seen in the control lane. Lane 1,1kb DNA ladder (MBI Fermentas); lane 3, no template control; lane 4, PCR product amplified from the host cells MC4100DE3 (+); lane 5-6, putative pET21 recombinants (isolates B and G) in the MC4100DE3 background; lane 7-8, putative *rpoS* antisense expressing recombinants (isolates B and C) in the MC4100DE3 background; lane 9-10, putative pET21 recombinants (isolates 1 and 2) in the HS1600DE3 background; lane 11-17, putative *rpoS* sense expressing recombinants (isolates 1-7) in the HS1600DE3 background. The numbers on the left-hand side indicate the band size in kilobase pairs (kb). Bands at the bottom of the gel, of small size, are due to primer-dimer formation and mispriming.
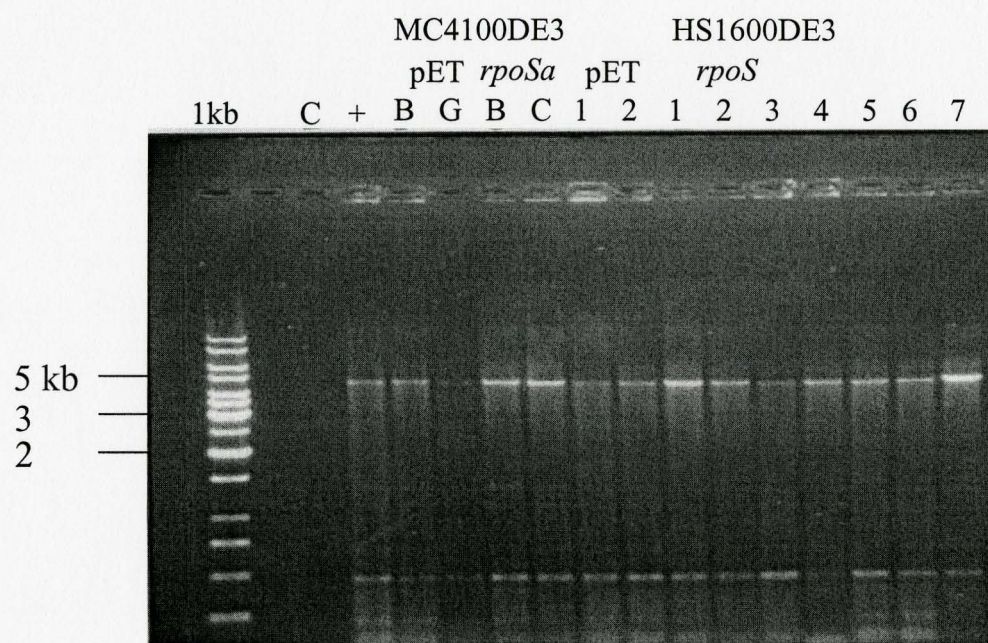
Figure 15. Fragments obtained after restriction digest of the 5kb PCR product.

The 5kb PCR products obtained from using the confirmation primers were treated with three restriction enzymes: PstI (P), HindIII (H) and EcoRI (E). These reactions including the uncut (U) sample were run on a 0.8% agarose gel along with the positive control. All the PCR products were digested using PstI. A band corresponding to a slightly smaller-sized band was seen. This was representative of the expected fragment size, 4700bp. pRpoS was cut by all three enzymes indicating that they were active. lane 1, 1kb DNA ladder (MBI Fermentas); lane 2-5; PCR product from HS1600DE3 cut with indicated enzymes; lane 6-9; PCR product from the putative pET21 recombinant isolate no.2 (pET21-2); lane 10-13, PCR product from the putative *rpoS* recombinant isolate no.6(*rpoS*-6) cut with indicated enzymes; lane 14-17, *rpoS* sense expressing plasmid (pRpoS) cut with the enzymes as a positive control. The numbers on the sides indicate the ladder band sizes in kilobase pairs (kb).
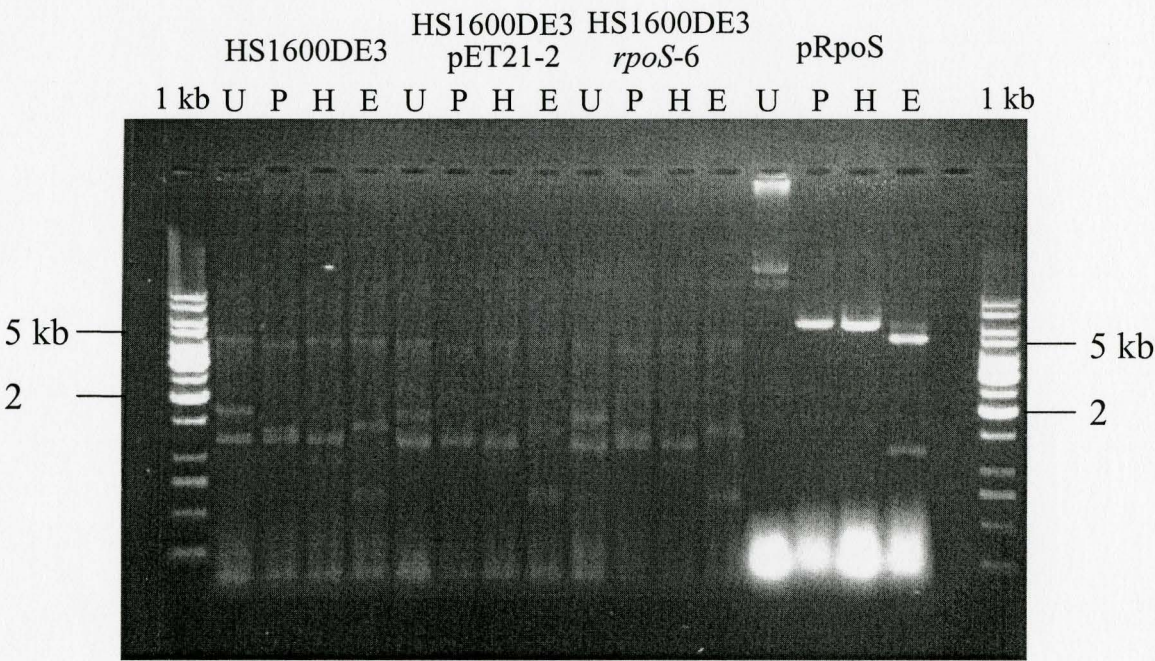
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Appendix I - 13C10 sequence

TTNTANGnNnTcAATACAATGaGCAAGGTATAnGTTCTTAAATCCAGCATCCTGGCAGGGTACTCTCAGTCTAATCAGTTGTCCGATTATTTTGTTGAACAATGGCCGCGAAAAGCACTCCGCTGATGAAATCACCGGTTCGCGACCTGCCT
10   20   30   40   50   60   70   80   90   100   110   120   130   140   15

GCAAATCCGATTCCGGTACTGGATGCCGAACTGGTTGGCGCCTCTGCCGTCCGAGCGATGCCGCCGCTGACTCCCCGTCAGCCAGGAAGCTCTGGCACTTTCCGATGAGTTGATTGCCGAGCTGAAAGCCCCGACGTTATCGTTATTCGGCC
0   160   170   180   190   200   210   220   230   240   250   260   270   280   290   3(

CCGATGTATAACTTCAACATCTCGACTCAGTTGAAAAACTATTTTGACCTGGNTGCCCGCCGCAGGCGTTACTTTCCGCTATACCGAGAACGGTCCGGAAGGTCTGGTAACNGGTAAAAAAGCCCNTNGTT
00   310   320   330   340   350   360   370   380   390   400   410   420

Appendix II - Integration junction sequence

GTG Nht AGGT GCCT CCT GaTTAAG CATTGGTAACTGT CAG ACGAAG TTTAC TCA TATATACTTTAG ATTGATTTAAAACTTCATTTTTAATTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACGAAAATCCCTTAAC
10   20   30   40   50   60   70   80   90   100   110   120   130   140   150

GTGAGTTTTCGTTCGACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTTCTGCGCGTAATCTGCTGCCTTGCAAACAAAAAAACCACCGCTACCAGCCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAAC
160   170   180   190   200   210   220   230   240   250   260   270   280   290   300

CTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC
310   320   330   340   350   360   370   380   390   400   410   420   430   440   45

AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAgGCGCAGCGGTCGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACGCGTGAGC
0   460   470   480   490   500   510   520   530   540   550   560   570   580   590

:TATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGA
600   610   620   630   640   650   660   670   680   690   700   710   720   730   740

Appendix III – Working solutions and buffers

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The following working solutions were autoclaved. If the solutions require filter-sterilization using a 0.45μm pore size filter disc, it is indicated below. They were stored at room temperature unless otherwise indicated.

General

X-gal was dissolved in dimethylformamide to make a stock concentration of 25mg/ml to be used as a working concentration of 50μg/ml. Due to its light sensitivity, the solution is store in a air-tight bottle covered in foil at –20°C.

1M IPTG, contained 0.238g per 1ml: in a 1.5ml microfuge tube, dissolve in 500μl of ddH₂O and bring up volume to 1ml. Cover tube with foil and store at - 20°C until ready for use.

10% Tween 20, made by adding 100μl of Tween 20 into 900μl of ddH₂O in a 1.5ml microfuge tube. Prepare as needed.

1M L-arabinose, contained 1.5g per 10ml.

Plasmid isolation

Solution I, contained 50mM glucose, 25mM Tris-Cl, pH 8, and 10mM EDTA, pH 8, per 100ml. 5ml of 1M glucose, 2.5ml of 1M Tris-Cl, pH 8, 2ml of 0.5M EDTA, pH 8, was mixed with 90.5 ml of ddH₂O. Stored at 4°C.

Solution II, contained 0.2N NaOH and 1% SDS, per 100ml. This was freshly prepared by mixing 2ml of 10N NaOH, 5ml of 20% SDS, and 93ml of ddH₂O.

Solution III, contained per 100ml: 60ml of 5M KAc, 11.5ml of glacial acetic acid and 28.5ml of ddH$_2$O. Stored at 4°C.

Phenol:Chloroform:Isoamyl alcohol, composed of 25ml of buffer-saturated phenol pH 8.0, 24ml of 100% chloroform, and 1ml of isoamyl alcohol to give a final volume of 50ml. Stored in an air-tight bottle covered with foil at 4°C.

3M Sodium acetate, pH 5.2, contained 408.3g per litre. pH was adjusted using glacial acetic acid.

5M Potassium acetate (KAc), contained 98.14g per litre.

1M glucose, contained 180.16g per litre.

0.5M EDTA, pH 8, 186.1g of EDTA was added to 800ml of ddH$_2$O. Approximately 20g of NaOH pellet were used to adjust the pH. The volume was then brought up to one litre.

1M Tris-Cl, pH 8 and pH 7.4, 121.1g of Tris Base was dissolved in 800ml of ddH$_2$O. The pH was adjusted using 5-10ml of highly concentrated HCl and the volume was brought up to one litre.

10xTE, pH 8, contained 100mM Tris-Cl, pH 8, and 10mM EDTA, pH 8. 50ml of 1M Tris-Cl, pH 8, 10ml of 0.5M EDTA were mixed with 440ml to give a total volume of 0.5 litre.

70% ethanol, contained 70ml of absolute ethanol and 30ml of ddH$_2$O.

Freezer stocks

20% LB glycerol, contained per 100ml: 50ml of 2xLB, 20ml of 100% glycerol and 30ml of ddH$_2$O. Best to prepare as needed.

Agarose gel electrophoresis

Ethidium Bromide solution (EtBr) was prepared by dissolving ethidium bromide in water to make a 10mg/ml stock. The working concentration was 0.5μg/ml.

50xTAE buffer, composed of 121g Tris Base, 28.55ml glacial acetic acid, and 50ml of 0.5M EDTA per litre. The working buffer concentration used for agarose gel electrophoresis was 1xTAE.

1% Agarose gel, contained 0.5g of agarose per 50ml of 1xTAE; for a large gel increased volume to 100ml.

Electrophoresis buffer, composed of 250ml of 1xTAE containing 125μl of 0.5μg/ml EtBr solution; for a large gel the volume was doubled.

Transduction

Suspension buffer, contained per 100ml: 0.5ml of 1M CaCl$_2$, 1ml of 1M MgSO$_4$, and 98.5ml of ddH$_2$O.

Dilution buffer, contained per 100ml: 1ml of 1M Tris-Cl, pH 7.4, 1ml of 1M MgSO$_4$, and 98ml of ddH$_2$O.

Top Agar, contained per 100ml: 50ml of 2xLB, 25ml of 2xAgar, and 25ml of ddH$_2$O.

1M CaCl$_2$, contained 14.7g per 100ml.

1M Sodium citrate, contained 147.05g per 500ml.

β-galactosidase assay

Chloramphenicol (15mg/ml), made by dissolving 1.5g into 80ml of ddH$_2$O and bringing up the volume to 100ml. Filter-sterilize and store at –20°C.

Z-buffer, contained per one litre: 16.1g Na$_2$HPO$_4$·7H$_2$O, 5.5g NaH$_2$PO$_4$·H$_2$O, 0.75g KCl, and 0.246g MgSO$_4$·7H$_2$O and 2.7ml β-mercaptoethanol was added when ready to use.

ONPG, to make a 4mg/ml stock solution 0.4g was added to 100ml ddH$_2$O. Due to the light-sensitive nature of this solution, the storage bottle was covered with foil and stored at 4°C.

1M Na$_2$CO$_3$, contained 105.9g per one litre.

Transformation

10% glycerol, made in a one litre glass graduated cylinder by adding 100ml of 100% glycerol into 900ml ddH$_2$O. Seal the mouth of the cylinder with a piece ofparafilm and mix together by gently inverting. Transfer the solution into four 500ml glass bottles.

Appendix IV - Point form methods

Titration of P1 lysate

To determine the number of plaque forming units (PFU) obtained from the P1 (or any lysate):

1. Grow up a wild-type strain O/N
2. Subculture 1:10 by adding 1ml of O/N culture to 10ml of 1xLB in a 50ml flask. Grow at 37°C and agitate at 200 rpm for 1 hour,
3. Centrifuge culture at max speed for 15 mins.
4. Remove the supernatant and resuspended the pellet in 2.5ml of resuspension buffer by vortexing.
5. Prepare a serial dilution of the lysate. Aliquot 990μl of dilution buffer into four 1.5ml microfuge tubes. Add 10μl of the lysate to the first tube (label 10^{-2}). Mix and transfer 10μl from this tube to the next (label 10^{-4}) and so on.
6. Combine 100μl of resuspended cells and 100μl of one of the diluted lysate solutions in a 15ml red-capped conical tube (Sarstedt).
7. Incubate at 30°C for 30 mins.
8. Mix the contents of the 15ml tube with 3ml of top agar by inverting.
9. Immediately pour the mixture on the surface of a LB+Agar plate. Gently tilt the plate to coat the entire surface. Do not move it around too long as the agar will began to harden and give a non-smooth surface.
10. Incubate the plate (lid side up) O/N
11. Count the number of plaques and calculate PFU.

Preparation of *lacZ* gene fusion lysate

Lysates containing *lacZ* gene fusions are prepared in order to transduce them into another strain background. The protocol is as follows:

1. Isolated colony from a strain carrying the desired *lacZ* gene fusion is inoculated into 5ml of 1xLB containing 5mM $CaCl_2$ and kanamycin and grown O/N.
2. Subculture 1:100 by adding 100μl of O/N culture to 10ml of 1xLB, containing no antibiotic, in a 50ml flask.
3. Grow at 37°C at 200rpm to an OD600 of 0.3.
4. Spilt culture into 2 large test tubes; one containing 6 ml and the other 4ml.
5. Add 100μl of P1 lysate to the tube with 6ml and label it "CV" for cell+virus. The other tube acts as the control.
6. Incubate until lysis is evident (usually 2-3 hours) and wait another hour before collecting the lysed culture.
7. Transfer the lysed culture (lysate) into a 15ml red-capped conical tube (Sarstedt).
8. Add 100μl of 100% chloroform and centrifuge at max speed for 15 mins.
9.
10. Transfer supernatant to new 15ml tube. Be sure not to touch the pellet/chloroform at the bottom.
11. Add 100μl of chloroform, mix together, and store at 4°C, indefinitely.

Transduction

Transductions are done to generate strains with *lacZ* gene fusions. This technique can also be used to transfer a mutation (as long as it has a selectable marker eg. *ΔrpoS::cat*). The procedure used is listed below:

1. Inoculate single colony of the recipient strain into 10ml of 1xLB containing 5mM $CaCl_2$ and grow O/N at 37°C.
2. Transfer the culture into 15ml red-capped conical tube and pellet the cells by centrifuging at max speed for 15 mins.
3. Discard the supernatant and resuspend the pellet in 250µl of 1xLB containing 5mM $CaCl_2$ by vortexing.
4. Label three 15ml tubes: "CV", "C" and "V".
5. Add 100µl of the recipient cells and 100µl of the prepared lysate into the tube labeled "CV". Add 100µl to the "C" tube and 100µl to the "V" tube as controls.
6. Mix the contents of "CV" by tapping the side of the tube (do not vortex).
7. Incubate at 37°C for 15 mins to allow phage to adsorb and inject their DNA into the cells.
8. Add 12ml of LB+100mM sodium citrate to each tube.
9. Mix and centrifuge at max speed for 10 mins at 4°C.
10. Discard the supernatant.
11. Wash the cells one more time as indicated in steps #8-10.
12. Add 12ml of LB+100mM sodium citrate to each tube again and incubate at 37°C for 30 minutes to allow for the selectable marker to be expressed.
13. Discard the supernatant and resuspended the cells in the remaining volume.
14. 100µl of the cells are plated on the surface of an appropriate selectable marker plate using a glass spreader (hockey stick).
15. Plates are incubated at 37°C O/N to obtain transductants.

This procedure may require modifications, such as increase or decrease of lysate and adsorption time, if no transductants are obtained.

Spread plating

This technique allows to evenly spread bacterial cells over the surface of solid media to obtain isolated colonies. It should be done near a lit Bunsen burner at a bench-top that has recently been disinfected or in the fumehood. A bent glass rod, commonly referred to as a hockey stick, is needed:

1. Deposit the desired volume of bacterial culture at the center of the surface of a selective media plate.
2. Dip the hockey stick in a glass dish containing 95% ethanol.
3. Immediately pass the hockey stick through the flame of the Bunsen burner to sterilize it. Do not overheat it as fracturing may occur. Wait a few seconds to allow cooling of the glass.
4. Gently touch the hockey stick to the inside edge of the solid media to ensure that it has cooled down.
5. Place the hockey stick on the drop of culture and begin to spread the culture over the surface of the plate using uniform motion. At the same time, rotate the plate to help cover the entire surface of the plate. Allow a few minutes for any excess culture to absord into the media.
6. Invert the plate and place at the desired temperature to obtain colonies.

Preparation of microtitre dish for replica-plating

Multiple strains can be grown in the same dish and transferred, simultaneously, to different selective media agar plates.

1. Add 200µl of 1xLB and necessary supplements required to select for the correct cells to each well. (The maximum strains that can be transferred are 6x8 using the metal pronged block).
2. Gently stretch a wide piece of parafilm over the surface of the dish.
3. UV-irradiate the dish and the inside of the lid for 1 minute.
4. Sterile toothpicks are used to inoculate each well by transferring an isolated colony from a plate into the well through the parafilm.
5. After inoculation is complete, the parafilm is carefully removed (to prevent cross-contamination).
6. The plate is incubated at the appropriate temperature O/N.

Sampling of Cell Culture

To construct a growth curve and determine the growth rate, cells are collect a regular intervals and their absorbance is read at OD600. Cells are also collected in a similar manner to test for β-galactosidase activity in *lacZ* gene fusion strains. The below is the protocol for measuring every 30 mins (or another desired interval). This protocol can also be used to obtain only exponential phase and stationary phase cell samples.

1. Inoculate an isolated colony in 10ml of 1xLB in a 50 ml flask and grow at 37°C O/N and agitating at 200rpm.
2. Subculture 1:1000 by adding 50μl of the O/N culture into 50ml of 1xLB in a 250ml flask. Grow to OD600 = 0.3 at 37°C while agitiating at previous rpm.
3. Subculture into the "sampling flask" by adding log phase culture to 10ml of 1xLB in a 50ml flask to give a starting OD600 of 0.01-0.05. Continue growing at 37°C and shaking at 200rpm.
4. Sample every 30 mins. Remove the flask from the shaker. Flame the foil cap, flame the lip of the flask, and take out 1ml using a pipet.
5. Add the 1ml of sample culture to a 13x100mm disposable borosilicate tube that contains 10μl of 15mg/ml chloramphenicol. Vortex to mix together.
6. Label the tube with the time point, place in a small test tube rack and then place on ice. If O/N storage is necessary, cover the tubes in the rack with a piece of parafilm followed by a piece of foil and then place the rack on fresh ice and store at 4°C.

IPTG induction

To induce expression from an IPTG-inducible promoter on an expression vector, cells carrying the plasmid are induced with IPTG in early-log phase cultures. In the case of this study, 3μl of 1M IPTG was added to 50ml of culture for a final concentration of 0.3mM to induce expression of the *rpoS* antisense from the plasmid encoding it, pSOPR.

1. Inoculate an isolated colony in 10ml of 1xLB in a 50 ml flask and grow at 37°C O/N and agitating at 200rpm.
2. Subculture 1:1000 by adding 50μl of the O/N culture into 50ml of 1xLB in a 250ml flask. Grow to OD600 = 0.3 at 37°C while agitiating at previous rpm.
3. Subculture into the "induction flask" by adding early-log phase culture to 50ml of 1xLB containing IPTG to the desired final concentration in a 250ml flask to give a starting OD600 of 0.01-0.05. Continue growing at 37°C and shaking at 200rpm.
4. Sampling may be done as indicated in the above protocol or cultures may be grown to stationary phase where cells can be collected to recover gene products of the expression vector.

Measuring OD600 values using a spectrophotometer (spec)

1. Zero the spec at 600nm using a 1.5ml semi-micro cuvette containing 1ml of ddH$_2$O.
2. Decant the ddH$_2$O and using a pipet add 1ml of cell culture to the cuvette. If the culture in dense (OD600 ≥ 0.5), dilute the culture by adding 100μl to 900μl of ddH$_2$O in the cuvette.
3. Read the absorbance at 600nm and record the value.
4. Dispose of the culture, unless it is required for further expression studies, then return to the borosilicate tube.
5. Rinse the cuvette with ddH$_2$O to ensure no trace of the cell sample is left and repeat, if necessary.

β-galactosidase Assay

β-galactosidase activity can be quantified by a simple reaction: lysed cells in a buffer solution are mixed with an analog enzyme substrate, ONPG. In the presence of β-galactosidase, ONPG is cleaved into galactose and o-nitrophenol, which gives the reaction a yellow colour. This reaction is done in triplicate. The procedure to conduct this assay is indicated below:

1. To each reaction tube, add:
      950µl of Z-buffer that contains β-mercaptoethanol
      50µl of 100% chloroform
      25µl of 0.1% SDS
2. Add 50µl of cells to each tube. If a large volume of cell culture is desired, the adjust the volume of the Z-buffer accordingly. For example, you want to add 100µl of cells then the reaction tube should contain 900µl of Z-buffer.
3. As controls for the reaction, leave a few reaction tubes without cells.
4. Vortex to mix the solutions and lyse open the cells.
5. Add 200µl of 4mg/ml ONPG and mix quickly using a vortex.
6. START the timer. This is the start of the reaction (t = 0h).
7. When the tubes appear yellow-coloured, STOP the reaction by adding 0.5ml of 1M Na_2CO_3 to the tube and vortexing.
8. Zero the spec at 420nm using 1ml of ddH_2O in a 1.5ml semi-micro cuvette.
9. Once the chloroform has settled at the bottom of the tube, pipet 1ml of the reaction solution in the cuvette.
10. Measure the OD420 and record the value. (For values to be in the linear range OD420 values should be higher than 0.2 and less than 1.5).
11. Measure the OD420 of the control tube in a similar manner. Subtract this value from the OD420 value obtained from the reaction tubes.
12. Determine the Specific Activity (Miller's units) using the following equation:

$$[1000 \times OD_{420}]/[\text{incubation time (min)} \times \text{volume of sample (ml)} \times OD_{600}]$$

IPTG titration

IPTG is a toxic compound that can not be metabolized by *E. coli*. To determine the effect it may have on growth rate the experimental strain(s) can be grown in the presence of varying concentrations of IPTG and the growth can be monitored.

1. Inoculated an isolated colony into 10ml of 1xLB and any appropriate antibiotics in a 50ml flask. Grow O/N at 37°C and shaking at 200rpm.
2. Subculture 1:1000 by adding 10µl of O/N culture in 10ml of the same media in a 50ml and grown at 37°C to $OD_{600} = 0.3$.
3. Add IPTG to final concentrations ranging from 0mM to 1.0mM to the same media in new 50ml flasks.
4. Add log phase culture to each of these flasks containing a known concentration of IPTG to give a starting value OD_{600} of 0.010.
5. The culture is grown at 37°C while shaking at 200rpm for 15 minutes. A 1ml sample is taken from each flask and placed in a disposable borosilicate tube containing 10µl of 15mg/ml chloramphenicol to stop protein synthesis. This serves as time zero sample (t = 0).
6. Every hour, take 1ml samples and place in tubes with chloramphenicol.
7. Measure the absorbance at 600nm. Record the OD_{600} value.
8. Plot OD_{600} values versus time and observe the trends.

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Small-scale plasmid isolation by Alkaline lysis

Plasmids can be transformed into other strain background by transormation or be used as a DNA template for PCR. To isolate a plasmid from a cell culture, a small scale protocol (modified from Sambrook and Russell, 2001) can be used:

1. Inoculate a single isolated colony from the strain carrying the plasmid in 10ml of 1xLB plus the appropriate antibiotic to select for the plasmid. Grow at 37°C, if temperature sensitive plasmid grow at 30°C, and shake at 200rpm O/N.
2. Place 1.5ml of O/N culture into a 1.5ml microfuge tube.
3. Centrifuge for 30 seconds at max speed to pellet cells.
4. Decant supernatant and resuspend the pellet in 100μl of ice-cold of SolutionI. Mix by vortexing.
5. Add 200μl of freshly made SolutionII. Mix the contents by inverting the tube five times. Store the tube on ice for a few minutes.
6. Add 150μl of ice-cold SolutionIII. Mix by inverting a few times. Store the tube on ice for 5 mins.
7. Centrifuge the lysed cells at max speed for 5 mins. Transfer the supernatant to a new tube.
8. Add equal volume of phenol:chloroform:isoamyl alcohol solution. Mix by inverting and centrifuge at max speed for 2 mins.
9. Transfer the aqueous phase (top layer) into a new tube.
10. Repeat step#8-9 until no white interface is seen.
11. Add 2 volumes of absolute ethanol to the aqueous phase. Mix by vortexing and allow to precipitate O/N. Store at –20°C.
12. Centrifuge tube for 30 mins at max speed at 4°C.
13. Remove the ethanol and wash the pellet with 1ml of 70% ethanol.
14. Centrifuge at max speed for 2 mins and remove all supernatant.
15. Allow pellet to air dry until no traces of ethanol seen.
16. Resuspend the plasmid DNA is 15-20μl of 10mM Tris-Cl, pH 8.
17. To remove the RNA, the isolated plasmid can be treated with Rnase A and re-extracted by phenol:chloroform:isoamyl alcohol and re-precipitated with ethanol.

Agarose Gel Electrophoresis

This technique is used to separate DNA based on its size and charge using an electric current.

Preparation of agarose gel:

1. To make a small gel, add 0.4-0.5g of agarose powder to 50ml of 1xTAE buffer in a 250ml flask. To make a large gel, add 0.8-1.0g pf agarose to 100ml of 1xTAE buffer in a 250ml flask
2. Loosely plug the mouth of the flask with 2 sheets of KimWipes.
3. Melt the agarose in a standard household microwave (Watts??) by heating the flash for 30 second interval. Swirl between each interval until no agarose particles are visible.
4. Add ethidium bromide (final concentration) and swirl. Instead of adding the ethiduim bromide now, the gel may also be stained and destained afterwards.
5. Pour the molten agarose into the gel tray (whose sides have been sealed by creating a barrier using 1 inch autoclave tape).
6. Place the comb into the molten agarose. Make sure it is parallel to the edge of the gel.
7. Allow the gel to set for at least 30 minutes.
8. Carefully remove the comb and the tape from the edges.
9. Place gel tray into the gel box.
10. Fill the box with 1XTAE buffer. The gel must be fully submerged.
11. Add ethidium bromide (final concentration) to the buffer.

Loading of agarose gel:

1. Add 2µl of loading dye to each sample.
2. Deposit the nucleic acid sample into the well using a pipet. Do not pierce the bottom of the well.
3. Load a ladder (of known volume and concentration) on one end of the gel to serve as a marker.

Running of the agarose gel:

1. Set the voltage to 100V.
2. Place the lid on the gel box.
3. Plug in the black wire to (+) and the red wire (-) of the power supply box.
4. Run the agarose gel for 45-60 mins.

Agarose Gel Extraction

To purify large volumes of PCR products the QIAEX II Agarose Gel Extraction Kit (Qiagen) can be used.

1. Load 20-25µl of PCR product into a wide mouth well of a large 1% agarose gel.
2. Run the gel as indicated above.
3. Weigh an empty 1.5ml microfuge tube. Record the weight.
4. Once gel is done running, place on an UV transilluminator and cut out the band corresponding to the PCR product using a scalpel blade (BLAH). Wear a full-face shield, lab coat and gloves when doing this step.
5. Transfer the piece of agarose containing the PCR product into the pre-weighed microfuge tube using the blade.
6. Weigh the tube again, now containing the piece of agarose. Record the weight.
7. Calculate the weight of the piece of agarose.
8. Use the protocol and the reagents provided by the manufacturer to extract the PCR product from the agarose.

PCR

PCR is used to amplify a large quantity of DNA. This technique can be used to amplify sequences for cloning or to verify the integration or deletion of sequence or the mere presence of specific DNA sequence (as done in forensics).

PCR using DNA as template:

1. The PCR components are added into a 0.5ml PCR tube (thinner wall than regular microfuge tube).
2. Add the components listed below in the following order:

PCR component	PCR reaction (µl)	PCR control (µl)
10xPCR buffer + $(NH_4)_2SO_4$	5	5
25mM $MgCl_2$	4	4
10mM dNTPs	2	2
Distilled H_2O	30.5	32.5
25pmol/µl Forward Primer	2	2
25pmol/µl Reverse Primer	2	2
10ng/µl DNA template	2	0
1U/µl Taq Polymerase	2.5	2.5
Total volume	50	50

3. Gently tap the side of the tube to mix the contents. Centrifuge the tube for 10 secs to bring all liquid from the sides to the bottom
4. Warm up the PCR machine for 30 mins. Turn on the heated lid.
5. Set the PCR conditions in the thermal cycler, Techne GeneE, to the settings indicated below:

Step	Temperature (°C)	Duration	No. of cycles
Denature	95	2 min	1
Denature	95	30 sec	
Anneal	55	30sec	30
Extend	72	1 min/kb	
Extend	72	7 min	1

6. When the PCR reaction is complete, carefully lift the lid, and remove the tubes.
7. Run the PCR product on a 1% agarose gel stained with ethidium bromide.
8. Visualize the size of the band by an UV transilluminator.

PCR using a colony as template:

Whole cell PCR is used to quickly obtain a PCR product using an isolated colony as the source of DNA template. The isolated colony is transferred to the reaction tube using a sterile toothpick. The PCR reaction is set up the same way except an additional component, 10% Tween 20, is required to help lyse the cells. The initial denaturation cycle time is increased from 2 mins to 4 mins to help with the cell lysis. The rest of the PCR condition remains the same.

1. The PCR components are added into a 0.5ml PCR tube (thinner wall than regular microfuge tube).
2. Add the components listed below in the following order:

PCR component	PCR reaction (µl)	PCR control (µl)
10xPCR buffer + $(NH_4)_2SO_4$	5	5
25mM $MgCl_2$	4	4
10mM dNTPs	2	2
Distilled H_2O	30	30
25pmol/µl Forward Primer	2	2
25pmol/µl Reverse Primer	2	2
10% Tween 20	2.5	2.5
DNA template	Isolated colony	0
1U/µl Taq Polymerase	2.5	2.5
Total volume	50	50

3. Gently tap the side of the tube to mix the contents. Centrifuge the tube for 10 secs to bring all liquid from the sides to the bottom
4. Warm up the PCR machine for 30 mins. Turn on the heated lid.
5. Set the PCR conditions in the thermal cycler, Techne GeneE, to the settings indicated below:

Step	Temperature (°C)	Duration	No. of cycles
Denature	95	2 min	1
Denature	95	30 sec	
Anneal	55	30sec	30
Extend	72	1 min/kb	
Extend	72	7 min	1

6. When the PCR reaction is complete, carefully lift the lid, and remove the tubes.
7. Run the PCR product on a 1% agarose gel stained with ethidium bromide.
8. Visualize the size of the band by an UV transilluminator.

High Fidelity PCR:

If the PCR product amplified is to be used for the expression of a gene, the regular Taq polymerase is not appropriate for obtaining a product. High fidelity (HI-FI) Taq polymerase (5U/µl) allows for minimal or no errors to occur during the extension step of the PCR. For each reaction, 2.5U of HiFi Taq polymerase is added and the volume of ddH$_2$O is adjusted to have the total volume remain 50µl.

1. The PCR components are added into a 0.5ml PCR tube (thinner wall than regular microfuge tube).
2. Add the components listed below in the following order:

PCR component	PCR reaction (µl)	PCR control (µl)
10x HI-FI Taq Buffer	5	5
25mM MgCl$_2$	4	4
10mM dNTPs	2	2
Distilled H$_2$O	33.5	34.5
25pmol/µl Forward Primer	2	2
25pmol/µl Reverse Primer	2	2
40ng/µl DNA template	1	0
5U/µl HI-FI Taq Polymerase	0.5	0.5
Total volume	50	50

3. Gently tap the side of the tube to mix the contents. Centrifuge the tube for 10 secs to bring all liquid from the sides to the bottom
4. Warm up the PCR machine for 30 mins. Turn on the heated lid.
5. Set the PCR conditions in the thermal cycler, Techne GeneE, to the settings indicated below:

Step	Temperature (°C)	Duration	No. of cycles
Denature	95	2 min	1
Denature	95	30 sec	
Anneal	55	30sec	30
Extend	72	1 min/kb	
Extend	72	7 min	1

6. When the PCR reaction is complete, carefully lift the lid, and remove the tubes.
7. Run the PCR product on a 1% agarose gel stained with ethidium bromide.
8. Visualize the size of the band by an UV transilluminator.

Preparation of Electrocompetent cells

Electrocompetent cells are required for transforming DNA into a host strain. The cells are prepared as follows:

1. Inoculate isolated colony into 10ml of 1xLB in a 50ml flask. Grow at 37°C and shake at 200rpm O/N.
2. Subculture 1:100 by adding 5ml of O/N culture into 500ml of 1xLB with any appropriate anitbiotics in a 2L flask. Grow at 37°C and shake at 200rpm to OD600 0.4-0.6.
3. Equally split the culture into two centrifuge bottles and incubate on ice for 30 mins. Swirl frequently to help cool down the culture.
4. Centrifuge the bottles in the bucket centrifuge (Beckman) at 4200rpm at 4°C for 15mins.
5. Aspirate off the supernatant and add 250ml of ice-cold 10% glycerol to each bottle. Mix by swirling.
6. Repeat step #4-5.
7. Aspirate off the supernatant and add 50ml of ice-cold 10% glycerol to each bottle. Mix by swirling.
8. Transfer the mixture into 50ml blue-capped conical tubes (Falcon).
9. Centrifuge at max speed in the table-top centrifuge at 4°C for 15 mins.
10. Aspirate off the supernatant. Resuspend the pellet into 1ml of sterile ice-cold 10% glycerol. Mix by vortexing.
11. Aliquot 100µl into sterile pre-chilled 1.5ml microfuge tubes.
12. Flash freeze using liquid nitrogen and store at -80°C.

Induction of λ Red system

The λ Red recombinase encoded on the pKD46 plasmid (or the modified pKAN plamid) can be induced during the preparation of electrocompetent cells by the addition of 1M L-arabinose to a final concentration of 20mM. Since these plasmids are temperature-sensitive, the cultures must be grown at 30°C.

1. Inoculate isolated colony into 10ml of 1xLB in a 50ml flask. Grow at 30°C and shake at 200rpm O/N.
2. Subculture 1:100 by adding 5ml of O/N culture into 500ml of 1xLB with any appropriate anitbiotics and 10ml of 1M L-arabinose in a 2L flask. Grow at 30°C and shake at 200rpm to OD600 0.4-0.6.
3. The culture is treated in the same manner as in the protocol for the preparation of electrocompetent cells.

Transformation of DNA

DNA can be transformed into electrocompetent cells by electroporation. The cells must stay at -80°C until immediately ready to use. The apparatus to conduct the electroporation is called GenePulser® (BioRad). The procedure to transform the DNA is listed below:

1. Add 50µl of electrocompetent cells to a pre-chilled electroporation cuvette (BioRad).
2. Add 2µl of plasmid DNA or 4µl of linear DNA into the cells. Tap the cuvette on the bench to mix. Store on ice for 1 minute.
3. Pulse the contents of the cuvette at 2.25kV (25µF capacitance).
4. Add 1ml of 1xLB into the cuvette and mix together by pipetting up and down.
5. Transfer the electroporated mixture from the cuvette into a new sterile 1.5ml microfuge tube.
6. Incubate the tube at the appropriate temperature, 37°C or 30°C, if a temperature sensitive plasmid, for 1-2 hours.
7. Perform a serial dilution for the transformation with plasmid DNA. Aliquot 90 µl of 1xLB into five 1.5ml microfuge tubes. Add 10µl of the lysate to the first tube (label 10^{-1}). Mix and transfer 10µl from this tube to the next (label 10^{-2}) and so on. For transformation with linear DNA, no dilution series is required.
8. Plate 50µl of each dilution on the appropriate selective media and incubate them at the appropriate temperature O/N to obtain transformants. For transformation with linear DNA, plate the entire mixture.

Restriction Digestion

To identify a piece of DNA by restriction mapping or to cut a fragment of DNA from a plasmid for cloning purpose, the DNA can be treated with restriction endonucleases (enzyme) that cut a specific sequences. The restriction digest for a 20µl reaction volume can be set up as follows:

1. Add 2µl of the DNA sample (~10ng/µl) to a 1.5ml microfuge tube.
2. To the tube, add:
   15µl of ddH$_2$O
   1µl of 10x Restriction Buffer (specific for the enzyme)
   2µl of Restriction Enzyme
3. Mix the contents of the tube by gently tapping the side. Centrifuge for 10 secs if there is liquid on the sides.
4. Incubate the tube in a 37°C waterbath for 1 hour.
5. Inactivate the restriction enzyme by incubating the tube at 80°C for 30 secs or by adding 1µl of 0.5M EDTA.
6. Run half of the reaction on 0.8% agarose gel stained with ethidium bromide.
7. Visualize the restriction pattern on an UV transilluminator.

If the amount of plasmid DNA is limiting, the reaction can be done in a 10µl volume by reducing the volumes of all reagents by half.

Small-scale isolation of bacterial chromosomal DNA

This procedure is modified from Silhavy *et al.*, 1984. It is utilized to isolate chromosomal DNA from bacteria to use as template for PCR and sequencing.

1. Inoculate isolated colony into 10ml of 1xLB in a 50ml flask. Grow O/N at 37°C and agitate at 200rpm.
2. Transfer 1.5ml of O/N culture into a 1.5ml microfuge tube and centrifuge at max speed for 2 mins.
3. Discard the supernatant and add 462μl of TE buffer, 30μl of 10% SDS, 3μl of 20mg/ml Proteinase K, and 5μl of 20mg/ml RNase A to the pellet and mix well.
4. Incubate the mixture for 2 hours at 37°C (attach the tube to the rotating wheel and allow it to turn).
5. Add 500μl of phenol/chloroform/isoamyl alcohol and mix by inverting
6. Centrifuge the tube for 2 mins at max speed.
7. Carefully transferred the aqueous phase to a new tube. Do not transfer white precipitated material between the two phases or any organic phase. Cutting the end of the tip makes it easier to pipet. If it is too viscous add a known volume TE buffer to slightly dilute the DNA.
8. Repeat steps #5-7 until no white precipitate is seen between the two phases.
9. Transfer the aqueous phase to a new tube.
10. Add 1/10 volume 3M NaAc and 2 volumes of absolute ethanol. Mix by inverting. (a cottonball-like precipitate should begin to be visible). Allow the DNA to precipitate O/N at -20°C.
11. Centrifuge tube at max speed for 30 mins.
12. Remove ethanol by pipetting. Wash the DNA pellet with 70% ethanol twice.
13. Dry the pellet until no evidence of ethanol remains. Do not over-dry.
14. Resuspend the pellet in 20-50μl of 10mM Tris-Cl, pH 8 or ddH₂O, pH 8.

Quantification of DNA

By Absorbance reading:

1. Set the wavelength of the spectrophotometer (spec) to 260nm.
2. Add 1000μl of ddH$_2$O to a quartz cuvette.
3. Zero the spec using the ddH$_2$O.
4. Add 2μl of isolated DNA to the same cuvette.
5. Measure the absorbance (Abs.) at 260nm.
6. Calculate the concentration by this formula:

$$\text{Concentration (μg/ml)} = \text{Abs.} \times 50\text{μg/ml} \times \text{Dilution Factor (1000/2)}$$
$$= \text{Concentration (ng/μl)}$$

By Gel analysis:

1. Run the 1μl of the isolated DNA on a 0.8% agarose gel with a 1kb ladder of known volume and concentration.
2. Compare the band from the isolated DNA to one in the ladder. Match it to one with the same intensity and thickness.
3. Use this equation to estimate the concentration;

Concentration (ng/μl)

$$= \frac{\text{kb of the band that matches the DNA}}{\text{Total kb in ladder}} \times \text{ng of ladder loaded on gel}$$

Preparation for Sequencing

To sequence specific sites in chromosomal DNA or *lacZ* gene fusion junction points, the following must be sent to the Central Facility (Mobix):

1. 5 μg of purified DNA resuspended in 10mM Tris-Cl, pH 8, or ddH$_2$O, pH 8.
2. One primer diluted to 1pmol/μl. For sequencing of fusion junctions, diluted Mu c end primer is used (Roy *et al.*, 1995).

Appendix V – Catalogue of Strains Used

.

Box	Row	Strain Name	Genotype	Antibiotic Resistance	Source/Reference
Saima-1	B-1	MC4100	del(argF-lac)205 araD139 flbB5301relA1 rpsL150 thi ptsF25	StrR	Schellhorn and Stones, 1992
Saima-1	B-2	MC4100	del(argF-lac)205 araD139 flbB5301relA1 rpsL150 thi ptsF25	StrR	Schellhorn and Stones, 1992
Saima-1	B-3	MC4100 DE3	as MC4100 but carrying DE3	StrR	Chen et al., 2003
Saima-1	B-4	MC4100 DE3	as MC4100 but carrying DE3	StrR	Chen et al., 2003
Saima-1	B-5	MC4100 DE3 pET21	as MC4100 but carrying DE3 and transformed with pET21b vector	StrR, AmpR	Chen et al., 2003
Saima-1	B-6	MC4100 DE3 pET21	as MC4100 but carrying DE3 and transformed with pET21b vector	StrR, AmpR	Chen et al., 2003
Saima-1	B-7	MC4100 DE3 pGC217a	as MC4100 but carrying DE3 and transformed with pGC217a	StrR, AmpR	Chen et al., 2003
Saima-1	B-8	MC4100 DE3 pGC217a	as MC4100 but carrying DE3 and transformed with pGC217a	StrR, AmpR	Chen et al., 2003
Saima-1	B-9	MC4100 DE3 pGC218	as MC4100 but carrying DE3 and transformed with pGC218	StrR, AmpR	Chen et al., 2003
Saima-1	B-10	MC4100 DE3 pGC218	as MC4100 but carrying DE3 and transformed with pGC218	StrR, AmpR	Chen et al., 2003
Saima-1	C-1	HS1600	as MC4100 but rpoS::Tn10	StrR, TetR	Mukhopadhyay et al., 2000
Saima-1	C-2	HS1600	as MC4100 but rpoS::Tn10	StrR, TetR	Mukhopadhyay et al., 2000
Saima-1	C-3	HS1600 DE3	as HS1600 but carrying DE3	StrR, TetR	Chen and Schellhorn, 2003
Saima-1	C-4	HS1600 DE3	as HS1600 but carrying DE3	StrR, TetR	Chen and Schellhorn, 2003
Saima-1	C-5	HS1600 DE3 pET21	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Saima-1	C-6	HS1600 DE3 pET21	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Saima-1	C-7	HS1600 DE3 pGC217	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Saima-1	C-8	HS1600 DE3 pGC217	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Saima-1	D-3	MC4100 DE3 osmY	as MC4100 but carrying DE3 and osmY-lacZ	StrR, KanR	Chen et al., 2003; G. Chen
Saima-1	D-4	MC4100 DE3 osmY	as MC4100 but carrying DE3 and osmY-lacZ	StrR, KanR	Chen et al., 2003; G. Chen
Saima-1	D-5	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and osmY-lacZ and transformed with pET21b vector	StrR, AmpR, KanR	Chen et al., 2003; G. Chen
Saima-1	D-6	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and osmY-lacZ and transformed with pET21b vector	StrR, AmpR, KanR	Chen et al., 2003; G. Chen
Saima-1	D-7	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and osmY-lacZ and transformed with pGC217a vector	StrR, AmpR, KanR	Chen et al., 2003; G. Chen
Saima-1	D-8	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and osmY-lacZ and transformed with pGC217a vector	StrR, AmpR, KanR	Chen et al., 2003; G. Chen
Saima-1	E-3	HS1600 DE3 osmY	as HS1600 but carrying DE3 and osmY-lacZ	StrR, TetR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	E-4	HS1600 DE3 osmY	as HS1600 but carrying DE3 and osmY-lacZ	StrR, TetR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	E-5	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and osmY-lacZ and transformed with pET21b vector	StrR, TetR, AmpR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	E-6	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and osmY-lacZ and transformed with pET21b vector	StrR, TetR, AmpR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	E-7	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and osmY-lacZ and transformed with pGC217 vector	StrR, TetR, AmpR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	E-8	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and osmY-lacZ and transformed with pGC217 vector	StrR, TetR, AmpR, KanR	Chen and Schellhorn, 2003; G. Chen
Saima-1	F-1	HS1014	as GC4468 but katE-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-2	HS1014	as GC4468 but katE-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-3	HS1091	as GC4468 but osmY-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-4	HS1091	as GC4468 but osmY-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-5	HS1094	as GC4468 but otsA-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-6	HS1094	as GC4468 but otsA-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	F-7	HS1098	as GC4468 but otsA-lacZ	StrR, KanR	Schellhorn et al. 1998
Saima-1	G-1	MC4100 DE3 pKD46	as MC4100 but transformed with pKD46	StrR, AmpR	Datsenko and Wanner, 2000; S.Tariq
Saima-1	G-2	MC4100 DE3 pKD46	as MC4100 but transformed with pKD46	StrR, AmpR	Datsenko and Wanner, 2000; S.Tariq
Saima-1	G-3	MC4100 DE3 pKD46 osmY	as MC4100 but transformed with pKD46 and osmY-lacZ	StrR, AmpR, KanR	Datsenko and Wanner, 2000; S.Tariq
Saima-1	G-4	MC4100 DE3 pKD46 osmY	as MC4100 but transformed with pKD46 and osmY-lacZ	StrR, AmpR, KanR	Datsenko and Wanner, 2000; S.Tariq
Saima-1	G-5	MC4100 DE3 pKAN	as MC4100 but transformed with pKAN	StrR, KanR	S.Tariq
Saima-1	G-6	MC4100 DE3 pKAN	as MC4100 but transformed with pKAN	StrR, KanR	S.Tariq

NOTE: pGC217a has been renamed as pSOPR, pGC217 is pRPOS and pGC218 is pSOPRL

Box	Row	Strain Name	Genotype	Antibiotic Resistance	Source/Reference
Salma-2	B-1	MC4100 DE3 osmY	as MC4100 but carrying DE3 and *osmY-lacZ*	StrR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	B-2	MC4100 DE3 osmY	as MC4100 but carrying DE3 and *osmY-lacZ*	StrR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	B-3	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	B-4	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	B-5	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pETGC217a vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	B-6	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pETGC217a vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	C-1	HS1600 DE3 osmY	as HS1600 but carrying DE3 and *osmY-lacZ*	StrR, TetR, KanR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	C-2	HS1600 DE3 osmY	as HS1600 but carrying DE3 and *osmY-lacZ*	StrR, TetR, KanR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	C-3	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, TetR, KanR, AmpR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	C-4	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, TetR, KanR, AmpR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	C-5	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pGC217 vector	StrR, TetR, KanR, AmpR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	C-6	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pGC217 vector	StrR, TetR, KanR, AmpR	Chen and Schellhorn, 2003; S.Tariq
Salma-2	E-1	MC4100 osmY	as MC4100 but carrying osmY-lacZ	StrR, KanR	Schellhorn and Stones, 1992; S.Tariq
Salma-2	E-2	MC4100 osmY	as MC4100 but carrying osmY-lacZ	StrR, KanR	Schellhorn and Stones, 1992; S.Tariq
Salma-2	E-3	MC4100 DE3 osmY	as MC4100 but carrying DE3 and *osmY-lacZ*	StrR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-4	MC4100 DE3 osmY	as MC4100 but carrying DE3 and *osmY-lacZ*	StrR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-5	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-6	MC4100 DE3 pET21 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-7	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pGC217a vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-8	MC4100 DE3 pGC217a osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pGC217a vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-9	MC4100 DE3 pGC218 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pGC218 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	E-10	MC4100 DE3 pGC218 osmY	as MC4100 but carrying DE3 and *osmY-lacZ* and transformed with pGC218 vector	StrR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-1	HS1600 osmY	as HS1600 but carrying DE3	StrR, TetR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-2	HS1600 osmY	as HS1600 but carrying DE3	StrR, TetR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-3	HS1600 DE3 osmY	as HS1600 but carrying DE3 and *osmY-lacZ*	StrR, TetR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-4	HS1600 DE3 osmY	as HS1600 but carrying DE3 and *osmY-lacZ*	StrR, TetR, KanR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-5	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, TetR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-6	HS1600 DE3 pET21 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pET21 vector	StrR, TetR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-7	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pGC217 vector	StrR, TetR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	F-8	HS1600 DE3 pGC217 osmY	as HS1600 but carrying DE3 and *osmY-lacZ* and transformed with pGC217 vector	StrR, TetR, KanR, AmpR	Chen *et al.*, 2003; S.Tariq
Salma-2	H-1	13C10	as GC4468 *acpD-lacZ*	StrR, KanR	Schellhorn *et al.*, 1998
Salma-2	H-2	13C10	as GC4468 *acpD-lacZ*	StrR, KanR	Schellhorn *et al.*, 1998
Salma-2	G-1	MC4100 13C10	as MC4100 but carrying *acpD-lacZ*	StrR, KanR	S.Tariq
Salma-2	G-2	MC4100 13C10	as MC4100 but carrying *acpD-lacZ*	StrR, KanR	S.Tariq
Salma-2	G-3	MC4100 DE3 13C10	as MC4100 but carrying DE3 and *acpD-lacZ*	StrR, KanR	S.Tariq
Salma-2	G-4	MC4100 DE3 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pET21 vector	StrR, KanR	S.Tariq
Salma-2	G-5	MC4100 DE3 pET21 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	S.Tariq
Salma-2	G-6	MC4100 DE3 pET21 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pET21 vector	StrR, KanR, AmpR	S.Tariq
Salma-2	G-7	MC4100 DE3 pGC217a 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pGC217a vector	StrR, KanR, AmpR	S.Tariq
Salma-2	G-8	MC4100 DE3 pGC217a 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pGC217a vector	StrR, KanR, AmpR	S.Tariq
Salma-2	G-9	MC4100 DE3 pGC218 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pGC218 vector	StrR, KanR, AmpR	S.Tariq
Salma-2	G-10	MC4100 DE3 pGC218 13C10	as MC4100 but carrying DE3 and *acpD-lacZ* and transformed with pGC218 vector	StrR, KanR, AmpR	S.Tariq
Salma-2	I-1	HS1600 13C10	as MC4100 but carrying *acpD-lacZ*	StrR, TetR, KanR	S.Tariq
Salma-2	I-2	HS1600 13C10	as MC4100 but carrying *acpD-lacZ*	StrR, TetR, KanR	S.Tariq

NOTE: pGC217a has been renamed as pSOPR, pGC217 is pRPOS and pGC218 is pSOPRL

Box	Row	Strain Name	Genotype	Antibiotic Resistance	Source/Reference
Salma-3	B-1	MC4100 DE3 pET21 PCR - b	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-2	MC4100 DE3 pET21 PCR -g	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-4	MC4100 DE3 pET21 PCR -c	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-5	MC4100 DE3 pET21 PCR -f	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-6	MC4100 DE3 pET21 PCR -h	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-7	MC4100 DE3 pET21 PCR -2	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	B-8	MC4100 DE3 pET21 PCR -4	as MC4100 but carrying DE3 and pET21b PCR product	StrR, AmpR	S.Tariq
Salma-3	C-1	MC4100 DE3 pGC217a PCR - b	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-2	MC4100 DE3 pGC217a PCR - c	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-4	MC4100 DE3 pGC217a PCR - d	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-5	MC4100 DE3 pGC217a PCR - e	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-6	MC4100 DE3 pGC217a PCR - g	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-7	MC4100 DE3 pGC217a PCR - h	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-8	MC4100 DE3 pGC217a PCR - l	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-9	MC4100 DE3 pGC217a PCR - j	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	C-10	MC4100 DE3 pGC217a PCR - 7	as MC4100 but carrying DE3 and pGC217a PCR product	StrR, AmpR	S.Tariq
Salma-3	E-1	HS1600 DE3 pET21 PCR -1	as HS1600 but carrying DE3 and pET21b PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-2	HS1600 DE3 pET21 PCR -2	as HS1600 but carrying DE3 and pET21b PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-4	HS1600 DE3 pGC217 PCR -1	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-5	HS1600 DE3 pGC217 PCR -2	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-6	HS1600 DE3 pGC217 PCR -3	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-7	HS1600 DE3 pGC217 PCR -4	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-8	HS1600 DE3 pGC217 PCR -5	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-9	HS1600 DE3 pGC217 PCR -6	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	E-10	HS1600 DE3 pGC217 PCR -7	as HS1600 but *carrying* DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-1	HS1600 DE3 pET21 PCR -1	as HS1600 but carrying DE3 and pET21b PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-2	HS1600 DE3 pET21 PCR -2	as HS1600 but carrying DE3 and pET21b PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-4	HS1600 DE3 pGC217 PCR -1	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-5	HS1600 DE3 pGC217 PCR -2	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-6	HS1600 DE3 pGC217 PCR -3	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-7	HS1600 DE3 pGC217 PCR -4	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-8	HS1600 DE3 pGC217 PCR -5	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-9	HS1600 DE3 pGC217 PCR -6	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	F-10	HS1600 DE3 pGC217 PCR -7	as HS1600 but carrying DE3 and pGC217 PCR product	StrR, TetR, AmpR	S.Tariq
Salma-3	H-1	HS1094	as GC4468 but *otsA-lacZ*	StrR, KanR	Schellhorn *et al.*, 1998
Salma-3	I-1	MC4100	del(argF-lac)205 araD139 flbB5301relA1 *rpsL*150 thi ptsF25	StrR	Schellhorn and Stones, 1992
Salma-3	I-2	MC4100	del(argF-lac)205 araD139 flbB5301relA1 *rpsL*150 thi ptsF26	StrR	Schellhorn and Stones, 1992
Salma-3	I-3	MC4100 DE3	as MC4100 but carrying DE3	StrR	Chen *et al.*, 2003
Salma-3	I-4	MC4100 DE3	as MC4100 but carrying DE3	StrR	Chen *et al.*, 2003
Salma-3	I-5	MC4100 DE3 pET21	as MC4100 but carrying DE3 and transformed with pET21b vector	StrR, AmpR	Chen *et al.*, 2003
Salma-3	I-6	MC4100 DE3 pET21	as MC4100 but carrying DE3 and transformed with pET21b vector	StrR, AmpR	Chen *et al.*, 2003
Salma-3	I-7	MC4100 DE3 pGC217a	as MC4100 but carrying DE3 and transformed with pGC217a	StrR, AmpR	Chen *et al.*, 2003
Salma-3	I-8	MC4100 DE3 pGC217a	as MC4100 but carrying DE3 and transformed with pGC217a	StrR, AmpR	Chen *et al.*, 2003
Salma-3	I-9	MC4100 DE3 pGC218	as MC4100 but carrying DE3 and transformed with pGC218	StrR, AmpR	Chen *et al.*, 2003
Salma-3	I-10	MC4100 DE3 pGC218	as MC4100 but carrying DE3 and transformed with pGC218	StrR, AmpR	Chen *et al.*, 2003
Salma-3	J-1	HS1600	as MC4100 but *rpoS::Tn*10	StrR, TetR	Mukhopadhyay *et al.*, 2000
Salma-3	J-2	HS1600	as MC4100 but *rpoS::Tn*10	StrR, TetR	Mukhopadhyay *et al.*, 2000
Salma-3	J-3	HS1600 DE3	as HS1600 but carrying DE3	StrR, TetR	Chen and Schellhorn, 2003
Salma-3	J-4	HS1600 DE3	as HS1600 but carrying DE3	StrR, TetR	Chen and Schellhorn, 2003
Salma-3	J-5	HS1600 DE3 pET21	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Salma-3	J-6	HS1600 DE3 pET21	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Salma-3	J-7	HS1600 DE3 pGC217	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003
Salma-3	J-8	HS1600 DE3 pGC217	as HS1600 but carrying DE3 and transformed with pET21b vector	StrR, TetR, AmpR	Chen and Schellhorn, 2003

NOTE: pGC217a has been renamed as pSOPR, pGC217 is pRPOS and pGC218 is pSOPRL