REGULATION OF THE GABDTP OPERON AND GABA UTILIZATION IN E. COLI

REGULATION OF EXPRESSION

OF THE *GABDTP* OPERON

AND

UTILIZATION OF GABA AS A SOLE NITROGEN SOURCE

IN

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Abstract

The *gabDTP* operon is regulated by a number of different signals and stress conditions including general stress via σ^S, carbon limitation, cell-to-cell signaling, alkaline pH and nitrogen limitation. The mechanism of regulation by many of these conditions is unclear. Operon *lacZ*-fusions to the genes of the *gabDTP* operon, isolated in a previous search for *rpoS*-dependent genes in *E. coli*, showed a 10-fold *rpoS*-dependence in rich LB media. Here we provide evidence, utilizing Northern analysis of wildtype *E. coli* strains without any insertions in the *gabDTP* operon, that expression of the *gabDTP* operon was highly RpoS- and stationary phase-dependent. The corresponding enzymes GabD (SSDH) and GabT (GSST) showed a 20 fold higher activity in stationary phase cultures of wildtype cells compared to isogenic *rpoS*-mutant strains. However, contrary to expected results, growth on GABA as a sole nitrogen source is better in *rpoS*$^-$ mutants relative to wildtype strains. Similar growth patterns are observed when the same strains are grown on L-glutamate as the sole nitrogen source, suggesting that the limitation for growth with GABA as the nitrogen source may be due to an inability to utilize glutamate efficiently as a sole nitrogen source. These results confirm that the *gabDTP* operon is part of the RpoS regulon, and suggest the metabolism of GABA plays a role in stationary phase stress resistance in *E. coli*.

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List of Abbreviations

bp	base pair
ddH_2O	deionized distilled water
°C	Degrees Celsius
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
g	gram
h	hour(s)
kb	kilobase pair
kg	kilogram
kV	kilovolt
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
s	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

1.0 Nitrogen Source Utilization in *Escherichia coli*

The synthesis of 1 g of *E. coli* requires 11 mmoles of nitrogen (reviewed in Reitzer, 2003). In the biosynthesis of all nitrogen-containing organic molecules in the cell, nitrogen is donated from two key intermediates, glutamine and glutamate (Reitzer, 1996b). Glutamate contributes about 75% of the nitrogen for biosynthesis and glutamine contributes the remaining 25% (Reitzer, 1996a). Glutamate is used directly, or indirectly as the nitrogen donor for the α-amino groups in the synthesis of all amino acids, half the nitrogen for synthesis of nitrogenous bases (in nucleic acids) and the amino group for adenine (Reitzer, 1996a). Glutamine provides nitrogen for amino sugars, NAD, *p*-aminobenzoate and the remaining nitrogen for the nitrogenous bases, histidine, tryptophan and asparagines (Reitzer, 1996a). *E. coli* can use a number of different nitrogen sources for growth (Tyler, 1978; Reitzer, 1996b), including ammonia, nitrates and nitrites (if used as an electron acceptor under anaerobic conditions), and a limited number of organic molecules, mostly amino acids (Tyler, 1978; Reitzer, 1996b).

1.1 Utilization of Ammonia as a Nitrogen Source

1.11 Transport and Assimilation of Ammonia as a Nitrogen Source

In *Escherichia coli*, the optimum nitrogen source is ammonia (NH_3), because it supports the fastest rate of growth. When sufficient ammonia is present in the media, assimilation of ammonia results in repression of enzymes for the catabolism of alternative

nitrogen sources (reviewed in Reitzer, 1996b). At physiological pH in *E. coli* both NH_3 and NH_4^+ are present in the media and NH_3 can readily permeate the plasma membrane because it is small and uncharged. This means that transport is likely unnecessary at high ammonia concentrations. Conversely, this also means that NH_3 can leak out of the cells at low external ammonia concentrations (Reitzer, 1996b).

E. coli has a specific ammonia/methylammonia (Amt) carrier, the gene for which has not been identified (Jayakumar *et al.,* 1989). Expression of the carrier is positively regulated by the Ntr response (the regulation of genes in response to nitrogen limitation, discussed below) and is negatively regulated by glutamine, allosterically (Jayakumar *et al.,* 1987). Transport involves antiport of ammonia with a potassium ion, driven by the electrochemical potassium gradient (Jayakumar *et al.,* 1985). Additionally, *E. coli* has a second transport protein, Ammonia transport B (AmtB) (Soupene *et al.,* 1998), encoded by the *amtB* gene. AmtB is induced by nitrogen limitation (via the Ntr response) (Soupene *et al.,* 1998), and catalyzes the facilitated diffusion of NH_3 into and out of the cell, but not NH_4^+ (Soupene *et al.,* 1998). The AmtB transporter is required for ammonia transport under acidic conditions when ammonia concentrations are low (50 nM or less) (Soupene *et al.,* 1998).

Assimilation at High Ammonia Concentrations

At high concentrations of ammonia, the NH_3 is assimilated directly into glutamate using the Glutamate Dehydrogenase enzyme (product of the *gdhA* gene) in the following

reaction:

NH_3 + α-ketoglutarate + NADH ⟷ glutamate + NAD^+

The reaction is very rapid and efficient, however, the K_m for ammonia of the enzyme is very high (1 mM) (Reitzer, 1996a) explaining the requirement for relatively high ammonia concentrations.

Assimilation at Low Ammonia Concentrations

At low ammonia concentrations, the NH_3 can only be assimilated by an alternative pathway, which has a higher affinity for ammonia (Reitzer, 1996a). The first step is the assimilation of ammonia into glutamine by the enzyme Glutamine Synthetase, GS, (encoded by the *glnA* gene) in the following reaction:

NH_3 + glutamate + ATP ⟶ glutamine + ADP + P_i

The second step is the transfer of the amide on the glutamine side chain to α-ketoglutarate to generate two glutamate molecules. The reaction is catalyzed by the enzyme Glutamate Synthase, GOGAT, which has two subunits (encoded by the *gltBD* genes) as follows:

glutamine + α-ketoglutarate + NADH ⟷ 2 glutamate + NAD^+

In total, these two reactions assimilate one NH_3 into glutamate with the use of energy from NADH and ATP (Reitzer, 1996a). The reactions require more energy than that used by GDH because of the ATP requirement of Glutamine Synthase. In high ammonia-concentration conditions, both GDH and the GS-GOGAT pathways may contribute to ammonia assimilation (Reitzer, 1996a). However, under energy limiting

conditions GDH is likely more important as it does not utilize as much energy in the synthesis of glutamate (Reitzer, 1996a).

1.12 Regulation of Ammonia and Alternative Nitrogen Source Assimilation

Although ammonia is the preferred nitrogen source for *E. coli*, cultures can grow on a variety of other organic compounds as sole nitrogen sources (reviewed in Reitzer, 1996b). Usually growth on these alternative nitrogen sources induces the Ntr response when they are supplied as the sole source of nitrogen (Reitzer, 1996b). The Ntr response induces expression of genes that are important for the transport and catabolism of a number of potential organic nitrogen sources.

Interestingly, it is not ammonia that acts as a signal of nitrogen adequacy in the regulation of the two ammonia assimilation pathways, and the Ntr response, but rather it is the intracellular level of glutamine (and partly α-ketoglutarate) that is an indicator of nitrogen sufficiency (Reitzer and Schneider, 2001). Glutamate plays no role as a signal of sufficient nitrogen levels (Reitzer and Schneider, 2001), even though it is the primary source of nitrogen for the synthesis of most nitrogen containing organic molecules in the cell. This may be due, in part, to the other functions of glutamate in the cell, including its role in osmoregulation as a counter-ion for potassium (McLaggan *et al.*, 1994).

The Regulators of the Ntr Operons

The key regulators of transcription of Ntr genes are RpoN, NR_I (NtrC) and Nac. RpoN is an alternative sigma factor, the only one in *E. coli* that does not have homology

to the main housekeeping sigma factor RpoD (σ^{70}) (Merrick, 1993). RpoN and NR_I together promote transcription from approximately 30 promoters, corresponding to approximately 100 genes (Zimmer *et al.,* 2000; Reitzer, 2003), many of which are directly responsible for transport or utilization of a nitrogen source (Zimmer *et al.*, 2000; Reitzer and Schneider, 2001). A few of the RpoN–dependent operons are not directly involved in nitrogen source utilization, but could potentially contribute to cellular homeostasis supporting optimal nitrogen assimilation (Reitzer and Schneider, 2001). For example the FhlA regulator is RpoN-dependent and regulates genes important for the production of the FHL (formate hydrogenlyase) complex (Reitzer and Schneider, 2001). FHL breaks down formate into CO_2 and H_2, reducing acidity during fermentation. Increasing the pH of the external environment may help to prevent NH_3 loss from the cell (Reitzer and Schneider, 2001).

Expression of the RpoN sigma factor is not regulated by nitrogen availability and is thought to be constitutive (Castano and Bastarrachea, 1984). RpoN is the third most abundant sigma factor during exponential phase, behind σ^{70} and σ^{F} (Jishage *et al.*, 1996), and has the highest affinity for core RNA polymerase of all the alternative sigma factors (Maeda *et al.*, 2000). RpoN is unable to initiate the transcription of genes (Open Complex Formation) without the assistance of a transcriptional enhancer, NR_I~P (Nitrogen Regulator I, phosphorylated) (reviewed in Reitzer and Schneider, 2001). NR_I sites are often found an unusually large distance away from the RpoN-binding site, and can promote transcription with RpoN regardless of their orientation (Buck *et al.*, 2000).

Usually activation is facilitated by DNA looping and supercoiling (Liu *et al.*, 2001). In at least one case, at the *glnH*p2 promoter, transcription is also IHF-dependent (Claverie-Martin and Magasanik, 1991), which facilitates looping of the DNA between the NR_I~P enhancer and the RpoN-containing RNA Polymerase (Claverie-Martin and Magasanik, 1991).

A number of additional operons that are also involved in nitrogen utilization are not directly controlled by RpoN and NR_I~P, but are controlled by the positive transcriptional regulator Nac (Muse and Bender, 1998). Nac is the product of the *nac* gene (which is monocistronic) (Muse and Bender, 1998) and requires RpoN and NR_I~P for expression (Muse and Bender, 1998). The regulation and properties of Nac have been most extensively studied in *Klebsiella aerogenes*. Nac is responsible for regulation of σ^D-dependent promoters, involved in nitrogen utilization (Muse and Bender, 1998). There is no known ligand that binds to Nac, and it appears to be constitutively active (Muse and Bender, 1998). Interestingly, most of the operons regulated by Nac are involved in transport and scavenging of potential nitrogen sources (Zimmer *et al.*, 2000).

Control of the Ntr Response

The Ntr response is controlled by a complex cascade of regulators and signal proteins starting with the bifunctional UTase/UR enzyme (reviewed in Reitzer and Schneider, 2001). Depending on the intracellular concentration of glutamine, this enzyme works to either add UMP to the proteins P_{II} and GlnK at low glutamine concentrations (UTase activity), or it removes the UMP group from P_{II} and GlnK at high levels of

glutamine (UR activity) (Jiang *et al.*, 1998).

At high glutamine concentrations the P_{II} protein is unmodified and interacts with the sensor kinase of the system, NR_{II} (Jiang and Ninfa, 1999). Unmodified P_{II} stimulates NR_{II} to dephosphorylate the response regulator NR_I (Jiang and Ninfa, 1999). As discussed above, NR_I~P is a positive transcriptional factor for Ntr operons, and along with RpoN, it up-regulates the transcription from Ntr promoters (Zimmer *et al.*, 2000). When NR_I is dephosphorylated, it is unable to induce expression of the Ntr genes and their expression is low (Jiang and Ninfa, 1999).

When nitrogen becomes limiting, the P_{II} protein is covalently modified with a UMP group and is unable to interact with NR_{II} (Jiang and Ninfa, 1999). NR_{II} subsequently autophosphorylates itself and transfers the phosphate group is then to NR_I, and NR_I~P induces expression of the Ntr regulon.

The key operon involved in nitrogen assimilation and regulation is the *glnALG* operon (reviewed in Magasanik, 1996). The *glnA* gene encodes the Glutamine Synthase enzyme and the *glnLG* genes encode the NR_{II} (NtrB) and the NR_I (NtrC) regulators respectively (Magasanik, 1996). The operon has three promoters, two are upstream of the *glnA* gene, and one is located between *glnA* and *glnL* (Magasanik, 1996). Under conditions of nitrogen excess the first and third promoters are transcribed at a low level. All of the promoters are repressed by NR_I (Magasanik, 1996) and a low level of the each of the corresponding proteins is found in the cell (Magasanik, 1996). The low level of GS enzyme is sufficient for synthesis of glutamine under conditions of nitrogen excess

(Reitzer, 1996a). The NR_I regulator is found at about 5 copies per cell (Reitzer, 1996a) and is sufficient for control of the *glnALG* operon in response to nitrogen availability.

Nitrogen limitation (due to low levels of ammonia) leads to an increase in the levels of NR_I~P as described previously. The resulting phosphorylated NR_I~P activates transcription from the second promoter (*glnA*p2) greatly increasing the transcription of all three genes (Magasanik, 1996). In this manner, NR_I autoregulates its own expression. Once the NR_I~P activator accumulates in the cell (to about 70 copies per cell) (Reitzer, 1996a), it allows transcription at other Ntr promoters for genes/operons involved in the transport and utilization of alternative nitrogen sources.

Regulation of GOGAT, GS and GDH

Glutamate Dehydrogenase is the product of the *gdhA* gene (Sakamoto *et al.*, 1975; Helling, 1990), located at 38 minutes on the *E. coli* chromosome. The gene is monocistronic and contains a single known promoter (Riba *et al.*, 1988). Expression of GDH is repressed by glutamate or aspartate at the transcriptional level (Riba *et al.*, 1988). The mechanism of glutamate-dependent repression is not currently known. The single known promoter is also repressed by carbon limitation through binding of CRP to the promoter (Riba *et al.*, 1988). This may be important in preventing the removal of α-ketoglutarate from the citric acid cycle during carbon limitation. Expression of GDH is also repressed by the Nac regulator in response to nitrogen limitation (Riba *et al.*, 1988).

Glutamine Synthetase is the product of the *glnA* gene and is a member of the *glnALG* operon (discussed above) (Magasanik, 1996). The operon has 3 known

promoters, *glnAp*1 and *glnAp*2 upstream of the *glnA* gene, and *glnLp* in between the *glnA* and *glnL* genes (Magasanik, 1996). All three promoters are repressed by NR_I (unphosphorylated) (Magasanik, 1996) and transcription from *glnAp*2 is highly upregulated by NR_I~P (phosphorylated) in response to nitrogen limitation (Magasanik, 1996). The *glnAp*1 and *glnLp* promoters are both σ^{70}-dependent and are responsible for basal level transcription of the operon during nitrogen excess (Magasanik, 1996). The *glnAp*1 promoter is positively regulated by cAMP-CRP during carbon limitation (Magasanik, 1996). The *glnLp* promoter does not require an activator (Magasanik, 1996). During nitrogen limited grown, most transcription occurs from the *glnAp*2 promoter, which is σ^{54}-dependent and requires NR_I~P (Magasanik, 1996).

The catalytic activity of the GS enzyme is also regulated by nitrogen availability. The P_{II} protein (discussed above) is in an unmodified state during nitrogen excess (Jiang and Ninfa, 1999), and stimulates the activity of adenylyltransferase (ATase) (Reitzer, 1996a). The function of ATase is to add AMP to one of the subunits of GS, thereby inactivating it (Reitzer, 1996a), in the following reaction:

$$GS + ATP \longrightarrow GS\text{-}AMP + PP_i$$

GS is a dodecamer of 12 identical subunits (Reitzer, 1996a) and adenylylation inactivates only the modified subunit (Reitzer, 1996a). Each subunit may be modified by adenylylation independently (Reitzer, 1996a). Conversely, during nitrogen limitation, P_{II}-UMP stimulates adenylyltransferase to remove AMP from the subunits of GS (Reitzer, 1996a). Thus, when nitrogen becomes limiting the GS enzyme becomes more active.

Additionally, GS is sensitive to feedback inhibition by alanine, glycine, serine, histidine, tryptophan, CTP, AMP, carbamyl-phosphate and glucosamine 6-phosphate (Reitzer, 1996a). The sensitivity to feedback inhibition is directly related to the adenylylation state of GS. When none of the subunits of GS are adenylylated (nitrogen limitation) the enzyme is not feedback inhibited (Reitzer, 1996a) and its primary role is nitrogen assimilation (Reitzer, 1996a). When just one of the subunits becomes adenylylated (nitrogen excess) the remaining subunits become sensitive to feedback inhibition (Reitzer, 1996a) and the primary role of GS is to synthesize glutamine for cellular biosynthesis (Reitzer, 1996a).

Glutamate synthase (GOGAT) is comprised of two non-identical subunits, a larger subunit encoded by the *gltB* and a smaller subunit encoded by *gltD* (reviewed in Reitzer, 1996a). Both genes are part of the *gltBDF* operon at 69 min on the chromosome (Reitzer, 1996a). The third gene of the operon, *gltF*, encodes a hypothetical transcriptional regulator (Reitzer, 1996a). The major promoter for the operon precedes the first gene, and minor promoters exist between *gltBD* and between *gltDF* (Reitzer, 1996a). Levels of GOGAT are high in ammonia containing media (Reitzer, 1996a), and are repressed by glutamate, or growth on nitrogen sources that generate glutamate in nitrogen limited conditions (Reitzer, 1996a). The third gene in the operon, *gltF*, has been proposed to participate in the glutamate-dependent repression of the operon (Reitzer, 1996a). Growth in rich media fails to induce expression of the operon, likely due to low levels of the transcriptional activator LRP (Reitzer, 1996a). Additionally, the operon is

repressed by carbon limitation by cAMP-CRP, which binds the major promoter upstream of *gltB*, and overlaps the –35 RNA polymerase binding site (Reitzer, 1996a).

1.2 Transport and Utilization of Amino Acids as Nitrogen Sources

E. coli is capable of utilizing a number of different amino acids as a sole nitrogen source (reviewed in Tyler, 1978; Reitzer, 1996b), including L- and D-Alanine, Arginine, Asparagine, Tryptophan, Aspartate, Glutamate, Glutamine, Threonine, Glycine and Serine.

Generally the amino acid is metabolized by oxidative deamination to its corresponding keto acid. There are four general mechanisms for this:

1) The amino group is removed by a non-specific flavoprotein oxidase, which feeds the electrons directly into the electron transport chain (White, 2000). There are a number of D-amino oxidases as well, which are important for metabolizing D-amino acids such as those found in peptidoglycan (White, 2000).
2) Similarly the amino group may be removed by $NAD(P)^+$-linked dehydrogenases, which are usually more specific, and the electrons are stored as NAD(P)H (White, 2000).
3) The amino group may be transferred to another organic molecule such as pyruvate or α-ketoglutarate to yield alanine or glutamate, respectively. The alanine can then be split back into pyruvate and ammonia by L-alanine dehydrogenase (White, 2000).
4) Lastly the amino group may be removed by specific amino acid deaminases

(White, 2000).

For growth on a nitrogen source, *E. coli* has an absolute requirement for ammonia to be produced, because it is required for glutamine synthesis by the enzyme GS (Reitzer, 1996a). In the degradation of some amino acids (including aspartate, proline and GABA) the amino group is transferred directly to glutamate (Reitzer, 1996a). For these amino acids, the reaction that generates ammonia is not currently known, even though *E. coli* can grow on these as sole nitrogen sources.

Discussed below are the degradation pathways for utilization of Glutamine, Glutamate, Aspartate, Arginine, Putrescine, Agmatine and Ornithine because of their relation to this thesis. Degradation of other amino acids as nitrogen sources is reviewed in (Reitzer, 1996a).

Transport and Utilization of Glutamine

E. coli can utilize glutamine efficiently as a nitrogen source. There are two separate transport systems for glutamine. The first is a high affinity system, utilizing a periplasmic binding protein specific for L-glutamine (Km = 0.1 μM) (Willis *et al.*, 1975). The components are encoded by the *glnHPQ* operon (Nohno and Saito, 1987). Expression of the operon is stimulated by nitrogen limitation through the Ntr response. NR_I and Integration Host factor are required for expression from the Ntr regulated promoter (Claverie-Martin and Magasanik, 1991). The *glnHPQ* operon has a second promoter that is σ^{70}-dependent and is unregulated (Nohno and Saito, 1987).

The second transport system for glutamate is lower affinity and is detectable only

in mutants of the high affinity system (Km = 10 μM) (Willis *et al.*, 1975). However if glutamine levels are high, the low affinity system is sufficient for growth on glutamine as a sole nitrogen source (Reitzer, 1996a). The low affinity system is most likely a L-glutamate transport system (Nohno and Saito, 1987).

Glutamate synthase, GS, is likely the main enzyme responsible for glutamine catabolism, as mutants of this enzyme grow much more slowly on glutamine as the sole nitrogen source (McFall and Newman, 1996). In addition, there are two glutaminases in *E. coli*, A and B, which can convert glutamine to L-glutamate and NH_3 (McFall and Newman, 1996). No structural genes have been identified for either glutaminase and their role in glutamine utilization is unclear (McFall and Newman, 1996).

Transport and Degradation of Aspartate and Glutamate

Five transport systems have been identified for aspartate and glutamate, however little is known about their expression and regulation (Schellenberg and Furlong, 1977; reviewed in McFall and Newman, 1996). System 1 is L-glutamate specific, sodium-dependent and has a Km of 1.5 μM. The permease is encoded by *gltS* (Schellenberg and Furlong, 1977). System 2 and 3 transport both L-aspartate and glutamate. System 2 is higher affinity for glutamate (Km = 0.5 μM) and utilizes a binding protein. System 3 has the lowest affinity for glutamate (Km = 5 μM). The majority of glutamate uptake is through the third system (Schellenberg and Furlong, 1977). The permease for system 3 is encoded by *gltP* (McFall and Newman, 1996), and utilizes the proton gradient across the membrane to drive transport (McFall and Newman, 1996). In addition there are two

transport systems that can take up aspartate but not glutamate. The first system is a L-aspartate specific (McFall and Newman, 1996); the second is a system for uptake of dicarboxylic acids, including succinate, malate, fumarate and L-aspartate (McFall and Newman, 1996).

The primary pathway for utilization of L-glutamate (reviewed in McFall and Newman, 1996) was shown to be transamination by the enzyme Aspartate aminotransferase in the following reaction:

Glutamate + oxaloacetate $\longrightarrow$ aspartate + α-ketoglutarate

Aspartate is then degraded to fumarate and ammonia by the enzyme Aspartate ammonia-lyase (Aspartase) in the following reaction:

Aspartate $\longrightarrow$ fumarate + NH_4^+

The Aspartate Aminotransferase is encoded by *aspC* located at 21 minutes on the *E. coli* chromosome, directly downstream of *ompF* (Koga-Ban *et al.*, 1983). The regulation of its expression is poorly understood. The structural gene for Aspartase, *aspA*, is located at 94 minutes on the *E. coli* chromosome and is regulated by CRP and FNR (Golby *et al.*, 1998). Under aerobic conditions the level of Aspartase is low, but it is up-regulated 5-fold in the presence of glutamate as the sole carbon source (Golby *et al.*, 1998). It is unclear whether this regulation is due in part to direct regulation by glutamate and/or aspartate, or solely by indirect regulation through CRP.

Transport and Degradation of Arginine, Agmatine, Ornithine and Putrescine

Transport of arginine occurs by at least three binding-protein-dependent systems

(reviewed in Reitzer and Schneider, 2001). The genes involved in the individual transport systems have not been extensively studied. The first is high-affinity, with a Km of 5 $x10^{-3}$ μM for arginine (Reitzer and Schneider, 2001). The binding protein is encoded by the *argT* gene (Reitzer and Schneider, 2001), and it transports lysine, arginine and ornithine (Reitzer and Schneider, 2001). The expression of *argT* is repressed by lysine and arginine (Reitzer and Schneider, 2001) and is dependent on σ^{54} for expression (Zimmer *et al.*, 2000; Reitzer and Schneider, 2001). The second transport system is lower-affinity, with a Km of 0.125 μM for arginine (Reitzer and Schneider, 2001), and it transports arginine and ornithine (Reitzer and Schneider, 2001). The binding protein for the lower-affinity system is encoded by the *abpS* gene (Reitzer and Schneider, 2001). Synthesis of AbpS is repressed by arginine and ornithine (Reitzer and Schneider, 2001). The gene was mapped to 63.5 minutes on the *E. coli* chromosome, however it is missing from the published MG1655 sequence (Reitzer and Schneider, 2001). The third transport system is the most recently discovered and is encoded by the *artPIQM-artJ* operons (Wissenbach *et al.*, 1995). The periplasmic binding protein for this system is the product of the *artJ* gene (Wissenbach *et al.*, 1995), which is specific for arginine but not ornithine (Wissenbach *et al.*, 1995). ArtI is also a periplasmic protein (Wissenbach *et al.*, 1995), which is likely involved in transport, but its substrate is currently unknown. The expression of the third transport system is also repressed by arginine (Wissenbach *et al.*, 1995). Interestingly, the regulator of arginine biosynthetic gene, ArgR (Maas, 1994), does not regulate the expression of the arginine transport genes, but they are regulated by ArgP (Celis, 1999).

There are at least 4 potential transport systems for putrescine uptake. The *potFGHI* (Igarashi and Kashiwagi, 1999; Reitzer and Schneider, 2001) and b1440-1444 (Reitzer and Schneider, 2001) operons each control an Ntr-regulated putrescine transport system (Zimmer *et al.*, 2000). The *potABCD* (transports spermidine primarily, but also transports putrescine) (Igarashi and Kashiwagi, 1999; Reitzer and Schneider, 2001) operon and *potE* gene encode two Ntr-independent operons respectively (Igarashi and Kashiwagi, 1999; Reitzer and Schneider, 2001). The relative contribution and physiological relevance of the transport systems have yet to be resolved.

Arginine, agmatine, ornithine and putrescine are all degraded by a common pathway (reviewed in Reitzer, 1996b; Reitzer and Schneider, 2001). Additionally, a second pathway exists for the catabolism of arginine for use as a nitrogen source (Schneider *et al.*, 1998). Both pathways are shown in Figure 4. The first pathway is called the Arginine Decarboxylase (ADC) pathway, after the first enzyme in the pathway. Arginine Decaboxylase (*speA* gene product) (Reitzer, 1996b) produces agmatine and CO_2 from arginine. The second step in the pathway is the conversion of agmatine to putrescine and urea by the enzyme Agmatine ureohydrolase (*speB* gene product) (Reitzer, 1996b). Additionally, ornithine may be converted to putrescine and CO_2 by Ornithine decarboxylase (*speC* gene product) (Reitzer, 1996b). Putrescine is then degraded to γ-aminobutyrate (GABA) by Putrescine aminotransferase (encoded by *pat*) (Reitzer, 1996b) followed by Pyrroline dehydrogenase (encoded by *prr*) (Reitzer, 1996b), with γ-aminobutyraldehyde as an intermediate (GABA degradation is discussed below). In the

process of degradation by the ADC pathway, nitrogen is released as glutamate and urea. However, *E. coli* cannot use urea as a nitrogen source (Reitzer, 1996b). For growth on arginine as a sole nitrogen source, arginine must be degraded by an alternative pathway, termed the Arginine Succinyltransferase (AST) pathway (Schneider *et al.*, 1998). The AST pathway degrades arginine through a series of succinylated intermediates, resulting in the production of ammonia and glutamate as the nitrogen containing products (Schneider *et al.*, 1998). The enzymes of this pathway are encoded by the genes of the *astCADBE* operon (Schneider *et al.*, 1998). Expression of the operon is Ntr-dependent (Schneider *et al.*, 1998), and is up-regulated upon entry into stationary phase by *RpoS* and cell-to-cell signaling (Baca-DeLancey *et al.*, 1999).

1.3 GABA as a Nitrogen and Carbon Source

1.31 Sources of GABA

GABA (γ-aminobutyric acid) is one of the alternative nitrogen sources that can be utilized by *E. coli* (Schneider *et al.*, 2002). During growth, *E. coli* produces GABA as a by-product of the enzyme Glutamate Decarboxylase (GAD) (Smith *et al.*, 1992) and as an intermediate in putrescine and arginine metabolism (Reitzer, 1996b).

E. coli has 2 Glutamate Decarboxylase enzymes, encoded by the *gadA* and *gadB* genes (Smith *et al.*, 1992). Expression of *gadA and gadBC* is regulated by acidic pH (Castanie-Cornet *et al.*, 1999; De Baise *et al.*, 1999), the specific regulators GadX (positive regulator) and GadW (repressor) (Ma *et al.*, 2002), HN-S, cAMP and RpoS (Ma

et al., 2002). The *gadX* and *gadW* genes are located downstream of *gadA* (Ma *et al.*, 2002). The enzymatic activity of Glutamate Decarboxylase is also controlled by pH (Capitani *et al.*, 2003). The Glutamate Decarboxylase reaction occurs as follows:

Glutamate $\longrightarrow$ GABA + CO_2

The reaction consumes protons and is important in aiding the cell during low pH stress (Castanie-Cornet *et al.*, 1999). A third gene, *gadC*, is transcribed with *gadB* and encodes a hypothetical glutamate-GABA antiporter (De Baise *et al.*, 1999), which takes up glutamate into the cell from the extracellular media and exports GABA.

During the catabolism of arginine and polyamines by the ADC pathway (discussed in more detail above) (Reitzer and Schneider, 2001), the end product is GABA, which can then be broken down into succinate and glutamate by the products of the *gabDTP* operon.

1.32 Metabolism of GABA: The *gabDTP* Operon and Adjacent *csiD* and *ygaF* Genes

It was originally reported that *E. coli* K-12 could not use GABA as a carbon or nitrogen source (Dover and Halpern, 1972). However $GABA^+$ utilization mutants could be created, which remained uncharacterized (Dover and Halpern, 1972). An operon was identified at 60.1 min. on the *E. coli* chromosome, which was responsible for the metabolism of GABA. The identified *gabDTP* operon in *E. coli* consists of 3 genes in the order *gabD*, *gabT* and *gabP* (Niegemann *et al.*, 1993). The structural gene *gabT* encodes the enzyme Glutamate-Succinic Semialdehyde Transaminase (GSST) and *gabD* encodes

the NADP-dependant Succinic Semialdehyde Dehydrogenase (SSDH). Together, these genes catalyze the conversion of GABA and α-ketoglutarate into glutamate and succinate (Dover and Halpern, 1972). The third structural gene, *gabP* encodes the integral membrane GABA permease, which selectively binds and transfers GABA into the cell using membrane potential to drive transport (Niegemann *et al.*, 1993).

The *csiD* gene is a strongly *rpoS*-dependent gene, which is induced by carbon starvation conditions (Marschall *et al.*, 1998). *csiD* expression is positively regulated by CRP-cAMP and LRP in the presence of the poor carbon source glycerol (Marschall *et al.*, 1998). *csiD* is closely linked to the *gabDTPC* operon, at a position coinciding with the b2659 open reading frame (Metzner *et al.*, 2004), however the length of the *csiD* coding sequence is shorter than the annotated b2659 ORF (Marschall *et al.*, 1998). The function of *csiD* is unknown.

Between *csiD* and the *gabDTP* operon is a relatively uncharacterized gene, *ygaF*. The *ygaF* open reading frame has clear homology to dehydrogenase enzymes in *E. coli* (Schneider *et al.*, 2002); however, its exact size and function are still unclear.

1.4 Regulation of the *gabDTP* Operon

1.41 Regulation by "GabC"

During the initial studies on the metabolism of GABA and the *gabDTP* operon, a control locus was identified upstream of the *gab* operon, termed *gabC* (Metzer *et al.*, 1979). Mutations at this locus result in an increase in expression of the *gab* operon and

thus it was thought *gabC* encoded a repressor protein (Metzer *et al.*, 1979). The locus remained uncharacterized beyond this initial observation. After sequencing of the *E. coli* genome was completed the original *gabC* locus was located to the *ygaT* open reading frame (Genbank Accession number U68243, unpublished). Interestingly, the location of the *csiD* gene, a highly *rpoS*-dependent and carbon-starvation inducible gene (Metzner *et al.*, 2004), coincides with the original "*gabC*" locus so I will refer to the original *gabC* locus as *csiD*.

A fourth gene follows the *gab* operon, *ygaE*, which may be a GntR-like transcriptional repressor that represses expression of the entire *gabDTP* operon (Schneider *et al.*, 2002). Because the originally identified *gabC* locus remained uncharacterized, this new downstream regulator was subsequently renamed *gabC* (Schneider *et al.*, 2002). Mutations in the downstream *ygaE* (*gabC)* gene show a ten-fold increase in SSDH and GSST enzyme activities (Schneider *et al.*, 2002). GABA itself does not specifically induce expression of the *gab* operon, suggesting that GABA does not bind to YgaE. During initial studies on the function of the repressor by Schneider *et al.* (2002), they were unable to identify a small molecule that acts as a co-repressor with YgaE, to either facilitate repression or to allow de-repression upon binding to YgaE (Schneider *et al.*, 2002). Based on this evidence, Schneider *et al.* (2002) proposed that the YgaE (GabC) repressor may not have a specific inducer and its role may be to reduce the nitrogen source-dependent variation in *gabDTP* expression that is observed when *ygaE* mutants are grown on a variety of different nitrogen sources.

1.42 Regulation by Stationary Phase and the RpoS Sigma Factor

The *gab* operon is upregulated upon entry into stationary phase through the RpoS sigma factor (Schellhorn *et al.*, 1998; Baca-DeLancey *et al.*, 1999; Stancik *et al.*, 2002). RpoS is strongly induced by several stress conditions (reviewed in Hengge-Aronis, 2002) including weak acids (Schellhorn and Stones, 1992), hyperosmotic shock (Hengge-Aronis, 1996) and carbon starvation (Lange and Hengge-Aronis, 1994). There are over 100 known genes that are regulated by RpoS (reviewed in Ishihama, 2000), many of which are redundant (meaning that they have a paralog that is differentially regulated) or have no known function. Most of the genes that have known functions are involved in resistance to a variety of stresses (Ishihama, 2000). The *gabDTP* operon has two RpoS-dependent promoters, one preceding the *csiD* gene upstream of the operon (Metzner *et al.*, 2004), which transcribes the entire *csiD-ygaF-gabDTP* operon, and a second RpoS-dependent promoter within the *ygaF* ORF that transcribes only the *gabDTP* genes (Metzner *et al.*, 2004).

1.43 Regulation by Cell Density Signals

Growth in conditioned rich media up-regulates expression of a *gabT-lacZ* fusion (Baca-DeLancey *et al.*, 1999), suggesting that cell-to-cell signaling (the accumulation of a signalling molecule in the extracellular media at high culture density) regulates the *gab* operon. The *gabT-lacZ* fusion is partially responsive to indole (Wang *et al.*, 2001), suggesting that the indole may play a role as a messenger molecule, but the component that is mostly responsible for up-regulation of the *gabT* gene by conditioned media has

yet to be determined and has been termed "signal-2" (Wang *et al.*, 2001). A clue to the identity of the signal-2 molecule was found in a recent search for mutants impaired in *gabT* expression (Joloba and Rather, 2003). Mutations in the genes *deoB* and *deoC* had reduced signal-2 secretion into the extracellular media, suggesting that the signal molecule may be a nucleoside derivative (Joloba and Rather, 2003). The mechanism of control of the *gab* operon by cell-to-cell signaling has yet to be elucidated.

1.44 Regulation by pH

Alkaline pH in the media, similar conditions to a growth-saturated culture in LB, induces the expression of a *gabT-lacZ* fusion (Stancik *et al.*, 2002). This effect depends on stationary phase, and an alkaline pH of at least 9. The mechanism of this regulation is not currently known. The *gabDTP* operon is also inducible by acidic pH (Metzner *et al.*, 2004), a condition that is known to strongly induce RpoS expression (Lee *et al.*, 1995). Expression due to acidic pH is dependent on RpoS (Metzner *et al.*, 2004).

1.45 Regulation by Carbon and Nitrogen Source Limitation

The *gabDTP* operon is sensitive to a special type of catabolite repression where expression is repressed on glucose minimal media and this repression is relieved by growth on poor nitrogen sources including GABA itself (Zaboura and Halpern, 1978). However, GABA does not specifically induce expression of the *gab* operon in *E. coli* (Schneider *et al.*, 2002). The *gab* operon is also regulated by nitrogen limitation by the Ntr response via the Nac transcriptional activator (Schneider *et al.*, 2002). Nac promotes

transcription from a σ^{70}-dependent promoter within the *ygaF* gene, upstream of the *gabDTP* operon (Schneider *et al.*, 2002). Catabolite repression of the operon may be explained in part by regulation at the *csiD* promoter, which also transcribes the *gabDTP* operon, and is regulated by cAMP-CRP (acting as an activator) and LRP (Marschall *et al.*, 1998; Metzner *et al.*, 2004).

1.46 Regulation by CsiD and CsiR

Recently, it was shown by Metzner *et al.* (2004) that the *ygaE* gene downstream of the *gab* operon encodes a negative regulator of the *csiD* gene, located upstream of the *gab* operon. Because of this, it was proposed that the *ygaE* gene be renamed as *csiR* (Metzner *et al.*, 2004). The *csiD* gene has previously been shown to be regulated by both RpoS and global catabolite repression through the CRP regulator (Marschall *et al.*, 1998). The function of *csiD* is unknown. Additionally, it has been proposed that the promoter driving expression of the *csiD* gene, also transcribes *ygaF* and the *gab* operon, such that *csiD-ygaF-gabDTP* comprise an entire operon (Metzner *et al.*, 2004). Interestingly, Metzner *et al.* (2004) reported that a mutation in the *csiD* gene up regulates expression of the entire *csiD-ygaF-gabDTP* operon, suggesting that CsiD and/or YgaF are involved in the metabolism of a compound, which is involved in the regulation of the operon. This compound may bind to YgaE (CsiR) to allow derepression of the *csiD* promoter (Metzner *et al.*, 2004), however, the mechanism of regulation is still unclear and requires further study.

5.7 Goals of This Study

The first work showing that expression of the *gabDTP* operon is *rpoS*-dependent was reported by Schellhorn *et al.*, (1998). They isolated random operon *lacZ*-fusions to the *gabD* and *gabP* genes, which showed a 10-fold *rpoS*-dependence in rich media. However, Schneider *et al.* (2002) suggested that the *rpoS*-dependent expression reported by Schellhorn *et al.* (1998) was due to an artifact resulting from polar *lacZ*-fusions within the operon used in the report. The primary goal of this study was to systematically examine the expression of all three members of the *gabDTP* operon utilizing strains that were unaltered in the *gabDTP* operon. We also wished to determine if the differences in expression in the *gabD* and *gabT* genes observed between wildtype and *rpoS*$^-$ cultures corresponded to differences in enzymatic activity of the *gabD* and *gabT* gene products, SSDH and GSST, respectively. We also wanted to examine the ability of wildtype and *rpoS*$^-$ cultures to utilize GABA as a sole nitrogen source. Lastly, we wished to determine if two upstream, highly RpoS-dependent genes, *csiD* and *ygaF* were part of the *gabDTP* operon.

Figure 1: Pathway of Uptake and Utilization of GABA by the Products of the *gabDTP* Genes.
The enzyme which catalyses each reaction is shown above the arrow and the gene that encodes each enzyme is shown in brackets. Abbreviations: GSST, Glutamate-succinic semialdehyde transaminase; SSDH, succinic semialdehyde dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; TCA, tri-carboxylic acid cycle.

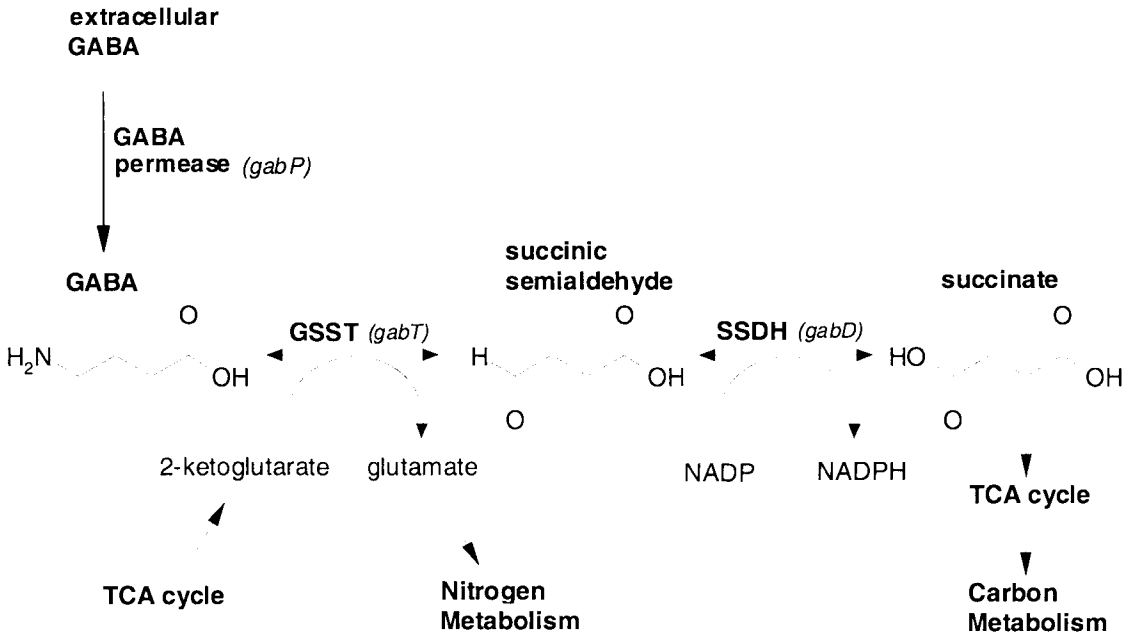
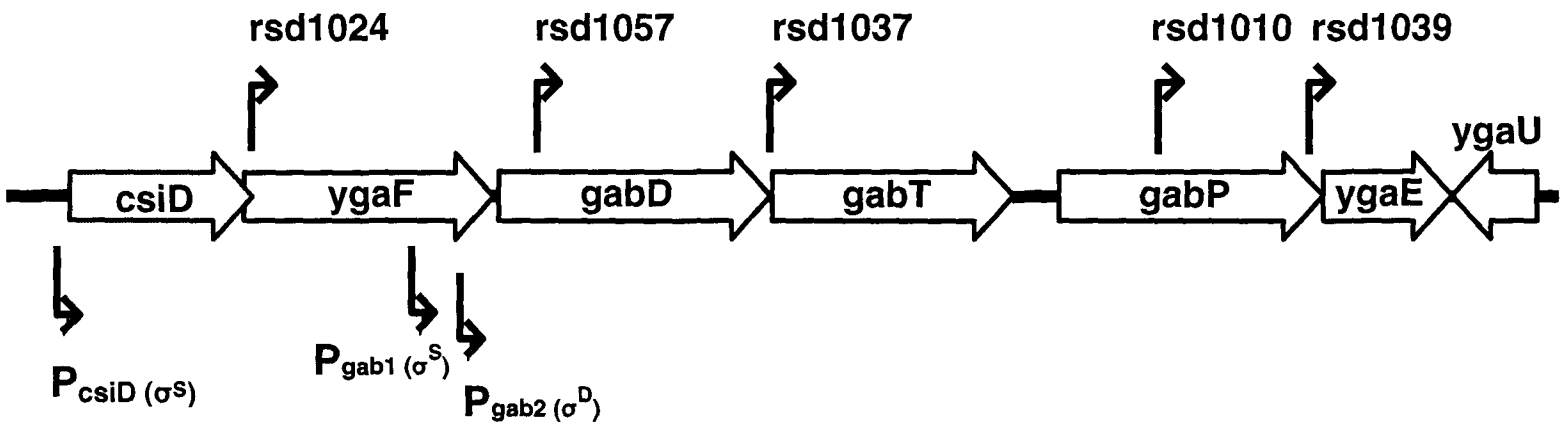

extracellular
GABA
GABA
permease (gabP)
GABA
succinic
semialdehyde
succinate
GSST (gabT)
SSDH (gabD)
2-ketoglutarate
glutamate
NADP
NADPH
TCA cycle
TCA cycle
Nitrogen
Metabolism
Carbon
Metabolism

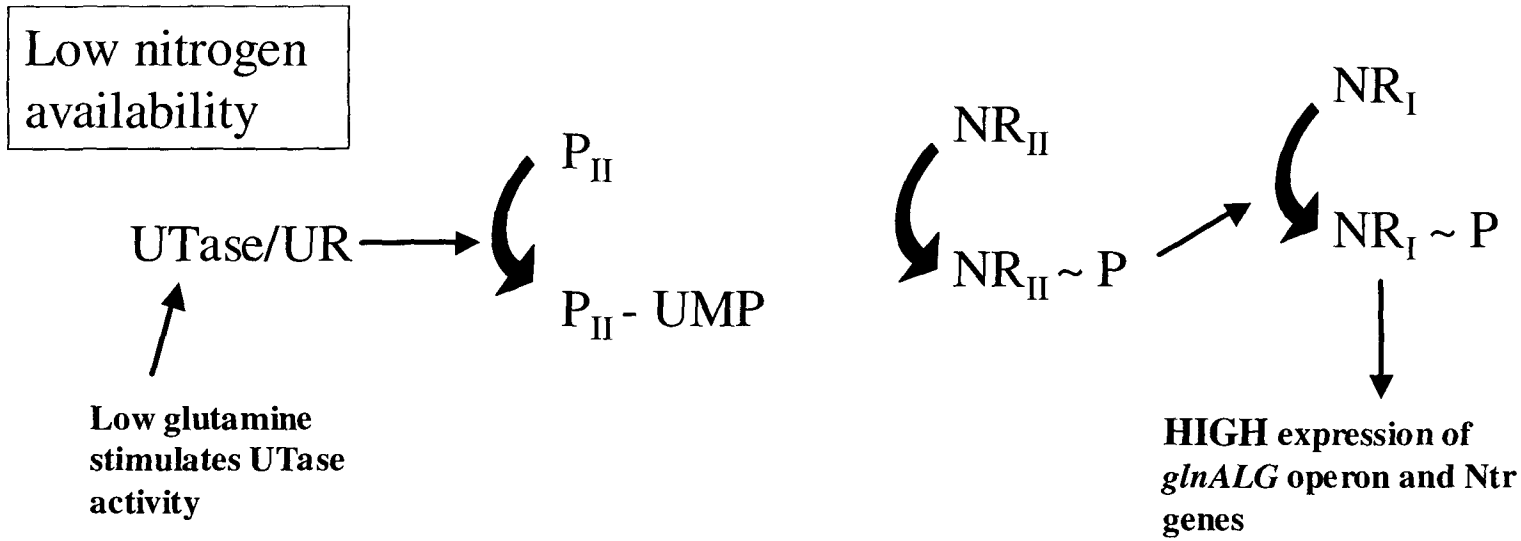
Figure 2: Genetic Map of the *gabDTPC* Operon in *E. coli* and Surrounding Genes. The identified and annotated promoters for the genes are shown as arrows below the diagram and locations of identified fusions are shown as arrows above the diagram. There are three repetitive extragenic palindromic (REP) elements located between the *gabT* and *gabP* ORFs. The map is derived from the Genbank sequence (Genbank Accession number AE000351) and the *csiD* gene is annotated from (Marschall *et al.*, 1996). The start codon for the *ygaF* gene is unclear, but is based on the annotated Genbank sequence. The P_{csiD} promoter is *rpoS*-dependent and regulated by catabolite repression via CRP and YgaE (CsiR) (Marschall *et al.*, 1996; Metzner *et al.*, 2004). The P_{gabD1} promoter is RpoS-dependent and was identified by Metzner *et al.* (2004). The P_{gabD2} promoter is σ^D-dependent and is regulated by nitrogen availability via Nac and the YgaE (GabC) repressor (Schneider *et al.*, 2002)



Alternate gene designations:

csiD: b2659, *ygaT*
ygaF: b2660
gabD: b2661
gabT: b2662
gabP: b2663
ygaE: b2664, *gabC, csiR*

Figure 3: Regulation of Nitrogen Source Utilization by the Ntr Response.
The signaling pathway is shown for conditions of nitrogen sufficiency on the bottom (high glutamine) and nitrogen limitation on top (low glutamine) (reviewed in Reitzer and Schneider, 2001). Arrows (→) indicate the activation pathway, and block arrows (⊣) indicate inhibition. Thick block arrows indicate the modification of a protein by addition/removal of UMP or a phosphate. Abbreviations: UMP, uracil monophosphate; ~P, phosphate; UTase/UR, Uridylyl transferase/uridylyl-removing enzyme; NR_I, Nitrogen regulator I (NtrC); NR_{II}, Nitrogen Regulator II (NtrB).


Low nitrogen availability
UTase/UR
P_{II}
P_{II} - UMP
Low glutamine stimulates UTase activity
NR_{II}
NR_{II} ~ P
NR_I
NR_I ~ P
HIGH expression of *glnALG* operon and Ntr genes

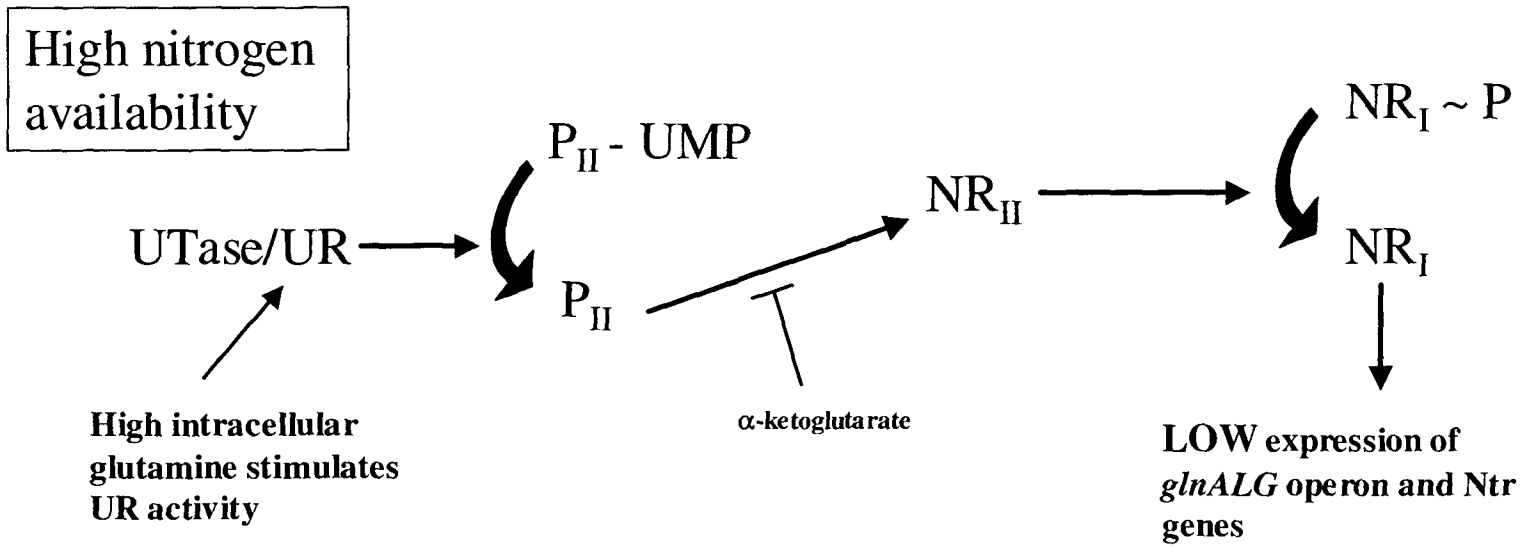
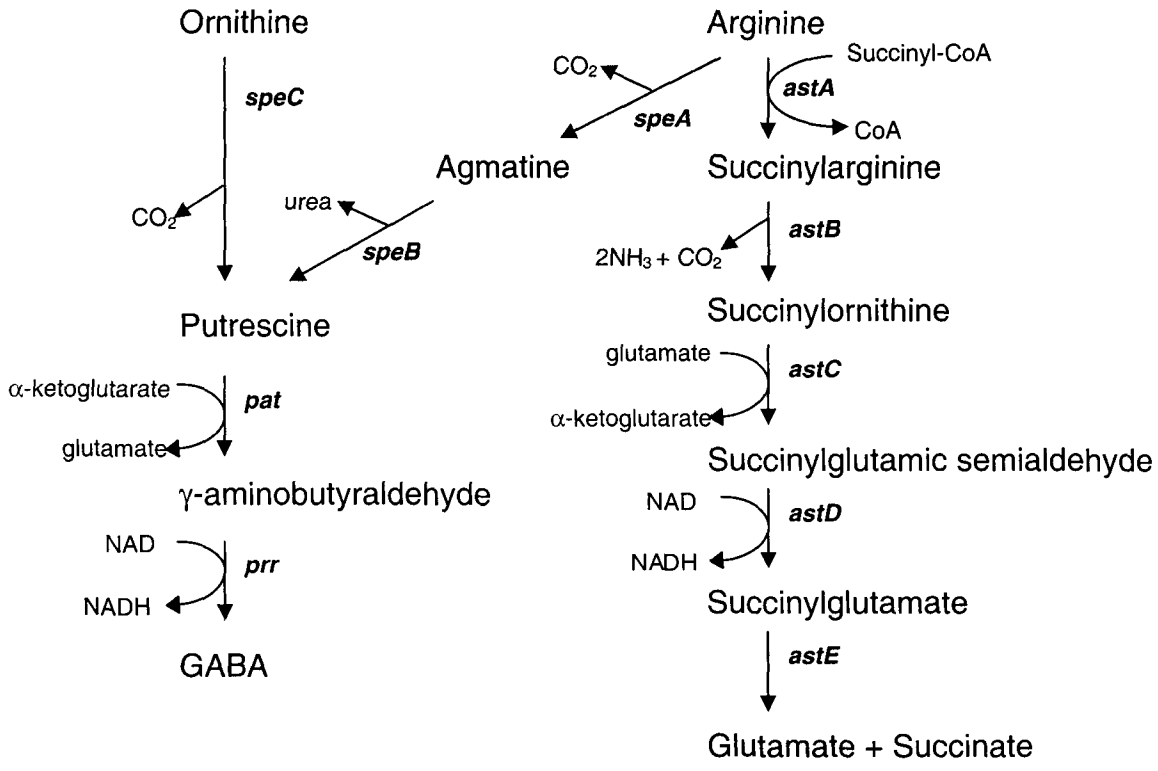

High nitrogen availability
UTase/UR
P_{II} - UMP
P_{II}
High intracellular glutamine stimulates UR activity
α-ketoglutarate
NR_{II}
NR_I ~ P
NR_I
LOW expression of *glnALG* operon and Ntr genes

Figure 4: Catabolic Pathways for Arginine, Agmatine, Ornithine and Putrescine Degradation

The arginine decarboxylase (ADC) pathway is shown on the left and the arginine succinyltransferase (AST) pathway is shown on the right (reviewed in Reitzer, 1996b; Reitzer and Schneider, 2001). The gene encoding the enzyme that is responsible for each step is shown on the right of the arrow for each reaction catalyzed, respectively. Abbreviations: GABA, γ-aminobutyrate; CoA, co-enzyme A.


Ornithine
speC
CO2
urea
speB
Agmatine
Putrescine
α-ketoglutarate
pat
glutamate
γ-aminobutyraldehyde
NAD
prr
NADH
GABA
Arginine
CO2
speA
Succinyl-CoA
astA
CoA
Succinylarginine
astB
2NH3 + CO2
Succinylornithine
glutamate
astC
α-ketoglutarate
Succinylglutamic semialdehyde
NAD
astD
NADH
Succinylglutamate
astE
Glutamate + Succinate

Chapter 2 Materials and Methods

2.1 Media and Growth Conditions

All bacterial strains were grown in Luria-Bertani (LB) broth or M9 minimal salts (Miller, 1992) as indicated. Strains were grown overnight in media supplemented with the appropriate antibiotics, subcultured into fresh media and maintained in the early exponential-phase (OD_{600} of <0.2) for at least 8 generations prior to the start of the experiment. Cultures were grown in flasks at 37°C with aeration at 200 rpm. For plate gridding experiments single colonies were inoculated into LB broth overnight in a microtitre dish, washed once with M9 salts and gridded onto plates containing different supplements: glucose (0.5%), succinate (1%), glycerol (1%) and GABA (1%) as carbon sources and NH_4^+Cl (0.05%), glutamate (1%), glutamine (1%) and GABA (1%) as nitrogen sources as indicated.

2.2 RNA Isolation Methods for Northern Analysis and RT-PCR

2.21 Isolation of Total RNA from Strains Harbouring the *gab* Operon *lacZ*-Fusions for Northern Analysis of *gabD* and *gabP* Expression

Cultures were grown as described above, and aliquots were removed from exponential- ($O.D._{600} = 0.3$) and stationary-phase ($O.D._{600} = 1.6$) cultures. Total RNA was extracted from wild type GC4468 and *rpoS*⁻ GC122 strains and the strains harbouring the identified *rpoS*-dependent, *gab* operon, *lacZ*-fusions using a modification of the hot-phenol method (Kohrer and Domdey, 1991). Samples were suspended in 250 µl

Resuspension buffer (0.3 M sucrose, 10 mM sodium acetate, pH 4.2) and 37.5 µl of 0.5 M EDTA and the samples were incubated on ice for 5 min. To each sample, 375 µl of Lysis buffer (2% SDS, 10 mM sodium acetate, pH 4.2, 1% β-Mercaptoethanol) was added; the tubes were vortexed and incubated at 65°C for 3 min. The samples were extracted three times with 700 µl acidic phenol (Sigma Aldrich) heated to 65°C. The samples were extracted with 700 µl of acidic phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature followed by a final extraction with 700 µl of chloroform:isoamyl alcohol (24:1). The RNA samples were precipitated by adding 0.1 volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol at -20°C. The RNA was pelleted by centrifugation at ~12, 000 rpm for 30 min. at 4°C in a microfuge. The RNA was rinsed with 70% ethanol, allowed to air dry for 15 min. and resuspended in 50 µl of sterile DEPC-treated water. The RNA was quantified spectrophotometrically by measuring OD_{260}. RNA integrity was examined by running 1 µg aliquots on a 1% non-denaturing agarose gel. RNA samples were stored at -80°C until used.

2.22 Isolation of RNA from MG1655 and GC4468 Cultures for Northern Analysis of *ygaF* and *gabDTP* Expression and RT-PCR Analysis of the *gab* Operon

Total RNA was isolated as indicated above from stationary phase cultures ($O.D._{600}$ = 1.6) in wildtype and *rpoS*⁻ strains except the cultures were first preserved by adding 1 volume of culture to 2 volumes of RNAlater (Ambion) on ice to preserve the RNA and incubating for 1 hour. The cells were then collected by centrifugation. Cell pellets were

stored at –80°C until RNA was isolated by the hot acidic-phenol method described above.

2.23 Purification of mRNA for Northern Analysis of *gabD* Expression in Wildtype, *rpoS*⁻ and *gabC*⁻ Mutants

Cell pellets from cultures in exponential phase and stationary phase that were preserved in RNAlater (Ambion) were lysed and RNA isolated using the hot acidic phenol method described above. To further purify and prepare the RNA, the RNA samples were reprecipitated to remove any residual salts and resuspended in 0.1 mM EDTA. 7.5 µg of total RNA in a 15 µL volume was purified using a Microbe Express kit (Ambion) to remove the rRNA. The remaining mRNA was ethanol precipitated and resuspended in a 20 µL volume of RNase-free ddH_2O and stored at –80°C until ready for use.

2.3 Probe Preparation and Northern Analysis

Oligonucleotide primers (listed in Table 2) were synthesized to generate PCR-amplified probes to detect the specific mRNA transcripts of *ygaF*, *gabD*, *gabT* or *gabP*. Each PCR tube contained 1X PCR buffer, 50 pmol of each of the forward and reverse primers, 0.2 mM each of the four dNTPs, 2 mM $MgSO_4$, ~10 ng *E. coli* chromosomal DNA and 2.5 U of *Taq* polymerase in a final volume of 50 µl. Reactions were run for 30 cycles under the following conditions: (i) 95°C for 15s; (ii) 60°C for 30s; and (iii) 72°C for 45s. The PCR product was separated on a 2% agarose gel to verify the size of the

probes and the fidelity of the reaction. The PCR product was purified from a 1% agarose gel using a QIAex II Gel Extraction Kit (Qiagen) and was radiolabelled with [α-^{32}P] – dCTP (NEN Life Science Products) by PCR labeling (Sambrook and Russell, 2001) using identical reaction conditions as above. Radiolabelled probes were purified from unincorporated nucleotides prior to use using a Sephadex G-50 microspin column (Amersham Pharmacia).

Northern analysis was performed on the total RNA samples. The samples were prepared, run on a formaldehyde gel and transferred, overnight, onto a Hybond N+ Nylon Membrane (Amersham Pharmacia) according to the instructions provided by the manufacturer. RNA was fixed to the membrane by UV crosslinking in a Stratalinker 2400 (Stratagene). Prehybridization and hybridization was performed at 68°C with gentle agitation in hybridization bottles (Amersham Pharmacia).

2.4 RT-PCR of the *gab* Operon and Upstream Genes

RNA isolated from GC4468 in stationary Phase ($O.D._{600}$ = 1.6) was used to conduct RT-PCR analysis of the *csiD-ygaF-gabD* region of the chromosome. Primers (listed in table 2) were designed to span the intergenic regions between the genes. The isolated total RNA was treated with DNase I and purified using the DNA*free* kit (Ambion) according to the manufacturers instructions. Purified RNA was run on a 1% agarose gel to check for the absence of contaminating genomic DNA. To generate cDNA, reverse transcription of the RNA was performed using Superscript II (Invitrogen) Reverse Transcriptase according to the accompanying instructions. Briefly, 1 μg of total RNA

was incubated with 2 pmol of the *gabDRev* or *gabTRev* primer (Table 2) and 10 nmol of dNTPs in a 12 μL volume at 65°C for 5 min. The reactions were chilled on ice and the additional reagents 1X First-Strand Buffer (50 mM Tris-HCl, 75 mM KCl, and 3mM $MgCl_2$), 10 mM DTT, and 40 U of RNase OUT Ribonuclease Inhibitor (Invitrogen) were added to the reaction and incubated at 42°C for 2 min. 200 U of Reverse Transcriptase was added and the reaction was incubated for 50 min at 42°C. The reaction was terminated by heating to 70°C for 15 min. To disrupt the synthesized RNA-cDNA duplexes, 2 U of RNase H (Invitrogen) was added to the reaction and incubated for 20 min at 37°C. RT^- controls were prepared identically except an equal volume of ddH_2O was added in place of the Reverse Transcriptase and RNase H. The product of the cDNA reaction was used as template for PCR. Each PCR tube contained 1X PCR buffer, 50 pmol of each of the forward and reverse primers, 0.2 mM each of the four dNTPs, 2 mM $MgSO_4$, 2 μL of the synthesized cDNA and 2.5 U of *Taq* polymerase in a final volume of 50 μl. Reactions were run for 25 cycles under the following conditions: (i) 95°C for 15s; (ii) 60°C for 30s; and (iii) 72°C for 2:00 min. The PCR products were visualized on a 1% agarose gel.

2.5 Enzyme Assays

2.51 β-Galactosidase Assays

β-Galactosidase activity was assayed using o-nitrophenol-β-D-galactoside (ONPG) as previously described (Schellhorn *et al.*, 1998). Units of activity were calculated as

[1,000 X $O.D._{420}$]/[time of incubation (min) X volume (ml) X $O.D._{600}$] and were expressed as Miller Units (Miller, 1972).

2.52 Isolation of Crude Protein Extracts

Cultures were grown in LB media to exponential phase ($O.D._{600}$ = 0.3) and stationary phase ($O.D._{600}$ = 1.8). At exponential phase 50 ml of culture was isolated and at stationary phase 10 ml of culture was removed. 150 μg/ml chloramphenicol was added to stop protein synthesis and the cultures were chilled on ice to halt growth. Cells were collected by centrifugation and washed with 0.1M KPO_4 buffer (pH 7.5) to remove residual media and cells pellets were frozen at –80°C. To obtain crude protein extracts, the cell pellets were resuspended in storage buffer (0.1M KPO_4 [pH 7.5], 1mM DTT, 1mM PMSF, 9% glycerol) and lysed by sonication for 3 pulses of 5s each. Extracts were centrifuged at 120,000 x *g* for 90 min to remove the cell debris and protein extracts were stored at 4°C. Enzyme activities were measured within 2 days. Protein concentrations were determined using the Bradford assay (Bradford, 1976) (BioRad Laboratories, Hercules CA).

2.53 Determination of Succinic Semialdehyde Dehydrogenase (SSDH) and GABA Transaminase (GSST) Activity

Assays for SSDH and GSST activity were performed as described previously (Schneider *et al.*, 2002). Reaction rates for SSDH were determined by measuring the reduction of NADP spectrophometrically at 340 nm in a UVIKON 930 spectrophotometer (Kontron Instruments) and the oxidation of NADH was measured to

determine GSST activity. For SSDH activity, the reaction mix contained: 0.1M KPO_4 (pH 7.8), 0.28 mM NADP, and 0.6 mM succinic semialdehyde at 30°C. For GSST, the reaction mix contained: 0.1M HEPES (pH 7.0), 40 mM L-glutamate, 40 mM NH_4Cl, 0.15 mM NADH, 1.6 U L-Glutamic Dehydrogenase, 0.4 mM succinic semialdehyde at 30°C. For each reaction a control was run, containing no succinic semialdehyde, to measure background NADP reduction or NADH oxidation respectively. This background was subtracted from the total enzyme activity to obtain the specific activity. All strains and growth conditions were assayed from triplicate independent cultures.

2.6 Deletion of the *ygaE*, *nac*, and *cyaA* Genes

To create *ygaE, nac* and *cyaA* deletion mutants, the method of (Datsenko and Wanner, 2000) was followed. Briefly, the target strain MG1655 was transformed with the pKD46 plasmid expressing the λ Red recombinase system under the control of an AraC-P_{araBAD} inducible promoter. To express the λ Red recombinase system the cells were grown in LB broth containing 20mM of the inducer L-arabinose. The cells were grown to an $O.D._{600}$ = 0.4 and made electro-competent by 4 washes with 10% glycerol in ddH_2O. Cells were flash frozen in liquid nitrogen and stored at –80°C until used. PCR products for mutagenesis were prepared using the disruption primers listed in Table 2. The resulting PCR products were transformed into the prepared MG1655 pKD46 strain by electroporation using a Gene Pulser II (BioRad) and grown on plates containing 25μg/ml chloramphenicol at 37°C to select for recombinants. The resulting deletions were

confirmed by PCR using the confirmation primers listed in Table 2. The deletions were transduced into the GC4468 background and strains harbouring the operon *lacZ*-fusions to the members of the *gabDTP* operon to examine their effect on expression.

2.7 Isolation of GABA$^+$ Utilization Mutants

Spontaneous mutants of *E. coli* K-12 that are capable of increased growth on GABA as a nitrogen or carbon source can be obtained by plating high numbers of cells onto minimal media containing GABA as the sole source of Nitrogen or Carbon for growth. Cultures of GC4468 and GC122 (*rpoS*$^-$) were grown overnight in M9 minimal media supplemented with 0.5% Glucose and 0.05% NH_4Cl. The strains were diluted 1:1000 into fresh media and grown to an $O.D._{600}$ = 0.8. The cultures were then chilled on ice for 30 min to stop growth and cells were isolated by centrifugation. The cell pellets were washed once in 1X M9 salts containing no carbon or nitrogen source and resuspended in the same buffer. 1 $X10^8$ cells were added to the surface of M9 plates containing either 0.5% Glucose, 1% Glycerol or 1% GABA as the carbon source and 0.05% NH_4Cl or 1% GABA as the source of nitrogen. Plates were incubated for 3 days at 37°C and GABA$^+$ mutant colonies were counted.

2.8 Determination of Growth Rate of *E. coli* Cultures on Various Nitrogen Sources

Overnight cultures of GC4468 (wildtype), GC122 (*rpoS::Tn*10), HS2529 (Δ*gabC*) and HS2530 (Δ*gabC rpoS::Tn*10) were prepared in M9 minimal media supplemented

with 0.5% Glucose and 0.05% NH_4Cl. The cultures were diluted 1:1000 into 10 ml of M9 media supplemented with 0.5% Glucose or 1% Glycerol as the carbon source and 0.05% NH_4Cl or 1% GABA as the nitrogen source. Cultures were allowed to grow for 2 hours at 37°C before sampling. 1 ml aliquots were removed from the culture at regular intervals and the $O.D._{600}$ was measured. A growth curve was created using at least 3 time points and the slope of the curve was used to determine the growth rate constant (k). The generation time (G) was determined using the equation $G = (\ln 2)/k$.

Table 1: Strains, Phage and Plasmids Used in this Study

Strain	Genotype	Source/Reference
A) Strains		
MG1655	Prototrophic *E. coli* K-12, F⁻ *λ⁻ ivlG⁻ rfld-50 rph-1*	Yale University
GC4468	*ΔlacU169 rpsL*	(Schellhorn and Stones, 1992)
GC122	as GC4468 but *rpoS13*::Tn*10*	(Schellhorn and Stones, 1992)
HS1024	as GC4468 but *csiD/ygaF*-λ*plac*Mu53(rsd1024)	(Schellhorn *et al.*, 1998)
HS1024p	as HS1024 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS1057	as GC4468 but *gabD*-λ*plac*Mu53(rsd1057)	(Schellhorn *et al.*, 1998)
HS1057p	as HS1057 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS1037	as GC4468 but *gabDT* intergenic-λ*plac*Mu53(rsd1037)	(Schellhorn *et al.*, 1998)
HS1037p	as HS1037 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS1010	as GC4468 but *gabP*-λ*plac*Mu53(rsd1010)	(Schellhorn *et al.*, 1998)
HS1010p	as HS1010 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS1039	as GC4468 but *gabP*-λ*plac*Mu53(rsd1039)	(Schellhorn *et al.*, 1998)
HS1039p	as HS1039 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS2507	as GC4468 but Δ*rpoN::cat*	This study
HS2508	as HS1024 but Δ*rpoN::cat*	This study
HS2509	as HS1057 but Δ*rpoN::cat*	This study
HS2511	as HS1010 but Δ*rpoN::cat*	This study
HS2513	as GC4468 but Δ*cyaA::cat*	This study
HS2514	as HS1024 but Δ*cyaA::cat*	This study
HS2515	as HS1057 but Δ*cyaA::cat*	This study
HS2517	as HS1010 but Δ*cyaA::cat*	This study
HS2521	as GC4468 but Δ*nac::cat*	This study
HS2522	as GC4468 but Δ*nac::cat rpoS13*::Tn*10*	This study
HS2523	as HS1024 but Δ*nac::cat*	This study
HS2524	as HS1024p but Δ*nac::cat*	This study
HS2525	as HS1057 but Δ*nac::cat*	This study
HS2526	as HS1057p but Δ*nac::cat*	This study
HS2527	as HS1010 but Δ*nac::cat*	This study
HS2528	as HS1010p but Δ*nac::cat*	This study
HS2529	as GC4468 but Δ*gabC::cat*	This study
HS2530	as GC4468 but Δ*gabC::cat rpoS13*::Tn*10*	This study
HS2531	as HS1024 but Δ*gabC::cat*	This study
HS2532	as HS1024p but Δ*gabC::cat*	This study
HS2533	as HS1057 but Δ*gabC::cat*	This study
HS2534	as HS1057p but Δ*gabC::cat*	This study

Table 1 continued: Strains, Phage and Plasmids Used in this Study

Strain	Genotype	Source/Reference
A) Strains		
HS1033	as HS1010 but Δ*gabC::cat*	This study
HS2534	as HS1010p but Δ*gabC::cat*	This study
B) Phage		
$P1_{vir}$	generalized transducing phage	Laboratory stock
C) Plasmids		
pKD3	template plasmid for gene disruption *cat* is flanked by FRT sites. 2804 basepairs	(Datsenko *et al.*, 2000)
pKD46	λ Red recombinase expression plasmid under control of an araC-P_{araBAD} inducible promoter. 6329 basepairs	(Datsenko *et al.*, 2000)

Table 2: Oligonucleotide Primers Used in this Study

Primer name	Sequence	Synthesis #
Deletion primers		
*rpoN*For1	TTTGCGACGTTTTAGCAGGAGAGTACGATTCTGAA CGTGTAGGCTGGAGCTGCTTC	AB30920
*rpoN*Rev1	ATGTTGAGCTGCATAGTGTCTTCCTTATCGGTTGGG CATATGAATATCCTCCTTAG	AB30921
*cyaA*For1	CACAGTCATGACGGGTAGCAAATCAGGCGATACGT CGTGTAGGCTGGAGCTGCTTC	AB30922
*cyaA*Rev1	TGCATGCCGGATAAGCCTCGCTTTCCGGCACGTTC ACATATGAATATCCTCCTTAG	AB30933
*nac*For1	CGGCACTTGGGCCGATTCTTAAAAACCGGAGGCAA CGTGTAGGCTGGAGCTGCTTC	ML280
*nac*Rev1	TAAAAACCCGCATCTTATTCCATCCCGATATAACAC CATATGAATATCCTCCTTAG	ML281
*ygaE(gabC)*For1	TCATAATACGCGCTGATCATGATCAGGAGTCACAC CGTGTAGGCTGGAGCTGCTTC	ML282
*ygaE(gabC)*Rev1	TATCCGGGGCAAGTGTTGCGTATTCCGGAAGAGTA GCATATGAATATCCTCCTTAG	ML283
Confirmation primers		
*rpoN*For2	CGCGCTTATATCGTCAGTCA	AB31048
*rpoN*Rev2	CCTGGTTGATTCGGTCAAAA	AB31049
*cyaA*For2	ACGGTCAATCAGCAAGGTGT	AB31046
*cyaA*Rev2	AAAGGCGATGAGTGGATTTG	AB31047
*nac*For2	ATCGTGGTGCATACCCTCCT	ML1110
*nac*Rev2	TTTCGATGCCAAGTTCATCC	ML1111
*ygaE(gabC)*For2	CAATTATGGCTCGCTGGAAA	ML1112
*ygaE(gabC)*Rev2	GAGTCAGGAGGCGAAGGAGA	ML1113
Northern Probe primers		
*ygaF*For1	GTATCGGGTGGCGTCTATGT	ML646
*ygaF*Rev1	CCGTATGAAACGCCTGATG	ML647
*gabD*For1	GGTAGCGTGCCGAAAATGG	AB30519
*gabD*Rev1	TCAATAAAGGAGGCGGCGTAG	AB30520
*gabT*For1	TTCACCCGATTTTCGCTGAC	AB30521
*gabT*Rev1	GCCCGGCACCTTCTGATT	AB30522
*gabP*For1	GGCAATCATCGCAACCACA	AB30523
*gabP*Rev1	CGGCATAGGTGGAAAACGAA	AB30524
RT-PCR primers		
*csiD*For2	AAGCGGATGAGATGGTGAAG	ML2725
*ygaF*Rev2	GGAACGGGCAGCTGATAAAG	ML2726
*ygaF*For2	CAGTCCAGAGGCGGTGAA	ML2727
*gabD*For2	TTGAAAACAGGATGTAGCGATG	ML2952
*gabD*Rev2	CCATTTTCGGCACGCTAC	ML2728
*gabT*Rev1	GCCCGGCACCTTCTGATT	AB30524

Chapter 3 Results

3.1 Fusions to Members of the *gabDTP* Operon Show *rpoS*-Dependent Expression

In a systematic search for *rpoS* dependent genes in *E. coli* utilizing random operon *lacZ* fusions, six fusions mapping to the *gab* operon region have been identified suggesting that the metabolism of GABA plays an important role in stationary phase (Schellhorn *et al.*, 1998). With the findings that *gabT* is upregulated in response to cell-to-cell signaling (Baca-DeLancey *et al.*, 1999), alkaline pH (Stancik *et al.*, 2002), and nitrogen limitation (Schneider *et al.*, 2002) the regulation of the *gab* operon and the role of GABA in stress resistance seems important and is being reexamined.

The strains harbouring *lacZ*-fusions to each of the *gab* operon members, when grown on LB plates, clearly showed that each of the genes *csiD, gabD, gabT* and *gabP*, are RpoS-dependent (Figure 5). To confirm that the members of the operon were both RpoS-dependent and stationary phase dependent, β-Galactosidase activity was assayed at 30 min intervals over the growth curve of *E. coli* in rich liquid LB broth (Figure 6). Each of the members of the operon was induced upon entry into stationary phase, and was maximally expressed in early stationary phase at an OD_{600} of approximately 1.8. In an *rpoS*⁻ deletion background, each of the fusions showed significantly lower activity. The *rpoS* dependence of the *gabD-lacZ* and *gabP-lacZ* fusions was approximately 10-fold while the $gabDT_{intergenic}$-*lacZ* fusion showed only a 7-fold *rpoS* dependence. Even in the absence of RpoS, the fusions were stationary phase dependent suggesting that the genes

were upregulated in stationary phase in an *rpoS*-independent manner as well, although this control was less significant than the regulation by RpoS. Expression of the rsd1037 (*gabDT*-intergenic *lacZ*-fusion) was less than either the *gabD-lacZ* or the *gabP-lacZ* fusions, which was unexpected. The data from northern analyses (see below) and from other reports (Metzner *et al.*, 2004), suggest that the levels of *gabD* and *gabT* transcription should be about equal, so the cause of this difference is unclear. However, despite the reduced β-Galactosidase activity, the expression of the rsd1037 fusion was still clearly *rpoS*-dependent (Figure 5, Figure 6B). One possibility is that there was a mutation in the *lacZ* gene, causing decreased β-galactosidase activity of the enzyme, but not interfering with the regulation of expression of the fusion.

3.2 Northern Analysis of the *gab* Operon in Wildtype Strains

To confirm that the expression of the *gabDTP* operon is maximal in stationary phase and dependent on RpoS, northern analysis was performed on each of the genes in wildtype strains that do not carry any insertions or deletions in the *gab* operon (Figure 7). The results showed the presence of *gab* operon mRNA in stationary phase, wild type *E. coli* cultures, but not in exponential phase or an *rpoS*$^-$ mutant. This is in agreement with the β-Galactosidase assay results and confirms the *rpoS*-dependence of the *gab* operon. To ensure that these results were not strain specific, RNA was isolated from both GC4468 and MG1655 strains as well as their isogenic *rpoS*$^-$ derivatives, GC122 and HS2210, respectively. The same pattern of expression is seen in both strain backgrounds (Figure 7), indicating that the *rpoS*-dependence of the *gab* operon was general to *E. coli*

K-12. The Northern analysis did not reveal any expression of the *gab* operon in the *rpoS*⁻ mutant strain, although a low level of β-Galactosidase activity is seen in *rpoS*⁻ mutants (Figure 6). The level of expression may be below the limit of detection by the Northern analysis.

In the original screen an operon *lacZ*-fusion mutant was also identified that mapped to the overlapping segments of the annotated *csiD* and *ygaF* genes (see Figure 2). The *csiD* gene is relatively well characterized and is known to be highly *rpoS*-dependent (Marschall *et al.*, 1998) but the regulation of *ygaF* is unclear. Northern analysis was performed to determine if *ygaF* is also *rpoS*-dependent (Figure 7). The regulation of the *ygaF* gene is similar to that of the *gabDTP* genes, where *ygaF* expression can only be detected in *rpoS* ⁺, stationary phase cultures, indicating that *ygaF* is also highly *rpoS*-dependent.

3.3 GSST (GabD) and SSDH (GabT) Enzyme Activities are Highest in *rpoS*⁺, Stationary Phase Cultures

Previous work by other groups (Dover and Halpern, 1972; Schneider *et al.*, 2002) examined the expression of the *gabD* and *gabT* genes by assaying the enzymatic activity of their gene products. To determine if the products of the *gabD* and *gabT* genes, SSDH and GSST respectively, also show RpoS-dependent regulation, the levels of enzymatic activity for each were examined in wildtype and *rpoS*⁻ strains in both exponential and stationary phase. We expect that the elevated transcription of the *gabDTP* operon in stationary phase will result in higher GSST (GabT) and SSDH (GabD) enzyme activities

in stationary phase relative to exponential phase. Figure 8 shows that the maximal levels of activity of both enzymes were observed in stationary phase cultures of the wildtype strain GC4468. The level of SSDH enzyme activity rose from 1.78 (μmol product/min mg protein) to 78.1 (μmol product/min mg protein) upon entry into stationary phase (Figure 8, Table 7). No GABA Transaminase (GSST) activity could be detected in exponential phase cultures. As expected, in the *rpoS* mutants, the levels of SSDH activity were 19-fold lower and the levels of GSST activity were 23-fold lower, which is consistent with the observed relative differences in transcription of the *gab* operon between wildtype and *rpoS*⁻ cultures (Figure 6).

3.4 Northern Analysis of *gab* Operon Expression in Strains Harbouring the Identified Operon *lacZ*-fusions

Since the identified operon *lacZ*-fusions have a polar effect on downstream genes, we can use them to probe the structure of the operon using northern analysis. Probes for the *gabD* gene and the *gabP* gene were used to identify the sizes, number and presence of any *gab* operon transcripts. The northern analysis (Figure 9) revealed some interesting features: 1) There are two transcripts visible that contain the *gabD* gene (Figure 9). The size of the smaller transcript is approximately 3.2 kb, which corresponds to the size of the *gabD* and *gabT* genes combined. The larger transcript is approximately 5 kb in length, and may be a transcript corresponding to the entire *gabDTP* operon, or may be a transcript encompassing *csiD-ygaF-gabDT*. The presence of a transcript the size of the *gabDT* genes suggests there is either a specific RNA cleavage site between *gabDT* and

gabP or alternatively there may be a weak terminator located between *gabT* and *gabP*. 2) The two fusions in *gabD* and the *gabDT* intergenic region (rsd1057 and rsd1037 respectively) both had a polar effect on *gabP* expression (Figure 9), which conclusively shows that *gabP* doesn't have a separate promoter and is dependent on the promoter preceding *gabD*.

3.5 Relationship Between *csiD*, *ygaF* and *gabDTP* Expression

The operon *lacZ*-fusion (rsd1024) located in the overlapping segment of the *csiD* and *ygaF* open reading frames (Figure 2) significantly reduced the expression of both *gabD* and *gabP* observed in northern analysis (Figure 9). This locus was first identified as a negative regulator of the *gabDTP* operon (Metzer *et al.*, 1979), and subsequently as the highly *rpoS*-dependent, carbon starvation inducible gene, *csiD* (Marschall *et al.*, 1998). The reduction in *gabDTP* expression as a result of the *lacZ*-fusion mutation may have been either a polar effect, by preventing the *csiDp* promoter from transcribing the *gabDTP* operon, or alternatively may have been due to disruption of the *csiD* gene, preventing production of the CsiD protein, which may have a role in regulating expression of the *gabDTP* operon. The genes *csiD*, *ygaF* and the *gabDTP* operon are all transcribed in the same orientation, and are all *rpoS*-dependent, so it is possible that they are co-transcribed. To test this, RT-PCR analysis was performed on RNA isolated from early stationary phase cultures of GC4468 (wildtype) to determine if a transcript is produced that includes the *csiD, ygaF* and *gabD* genes (Figure 11, Figure 12). PCR primers were designed to amplify between the 5' end of *gabD* and the known *gabDp*

promoter, *gabD* and the mid point of *ygaF*, and between *gabD* and the *csiD* ORF. Positive controls using genomic DNA isolated from *E. coli* resulted in high fidelity amplification of the expected PCR fragments. Negative controls, without the addition of Reverse Transcriptase, were performed for each reaction to ensure any observed PCR amplification was due to generated cDNA, and not residual contaminating genomic DNA in the RNA preparations. All negative controls failed to produce an amplification product. An RT-PCR amplification product was observed between the *gabDp* promoter and the 5' end of *gabD* (Figure 11, lane 6), however the reactions failed to produce an amplification product upstream of the known *gabDp* promoter, either between *gabD* and the midpoint of *ygaF* (Figure 11, lane 7) or between *gabD* and *csiD* (Figure 11, lane 9). To ensure the reverse transcription reaction was able to produce a sufficiently long cDNA template, a reverse primer homologous to the 5' end of *gabT* was used to generate cDNA as well. A PCR amplification product was observed between the *gabDp* promoter and the 5' end of *gabT* (Figure 11, lane 10), however no amplification was observed upstream of the *gabDp* promoter, using either the *ygaF* or *csiD* primers. Together, these results fail to show a transcript between the *csiD* promoter and the *gabDTP* operon, and suggest that transcripts initiated from the *csiDp* promoter do not include the *gabDTP* operon. Closer inspection of *gabD* expression in the strain harbouring the *csiD lacZ*-fusion mutation reveals reduced expression of both bands containing *gabD* transcript (Figure 9). We would expect to observe abolished expression from a transcript originating from the *csiDp* promoter and equal expression of the *gabDT* transcript originating from the

gabDp1 promoter if transcription if the *gabDTP* operon were transcribed from the *csiDp* promoter. These observations suggest that *csiD-ygaF-gabDTP* do not comprise a single transcript and that *csiD-ygaF* and the *gabDTP* operon are likely transcribed separately under the conditions tested.

3.6 Regulation of the *gab* Operon by *ygaE (gabC)* and *nac*

Both operon *lacZ*-fusions located in the *gabP* gene, rsd1010 and rsd1039, show similar expression levels of *gabD* compared to the wildtype GC4468 strain (Figure 9). These fusions would be expected to have a polar effect on the downstream gene, *ygaE (gabC)*, if this gene was part of the operon (as reported in Schneider *et al.*, 2002), and would be expected to show increased transcription of *gabD* as a result. The finding that the *gabP* mutations have no effect on observed *gabD* expression levels suggests two possibilities: 1) *ygaE* may not regulate the *rpoS*-dependent transcription of the operon seen in stationary phase, or 2) the *ygaE* gene may not be part of the *gabDTP* operon. To test whether *rpoS* and *ygaE* (*gabC)* regulate the *gabDTP* operon independently, northern analysis was performed on GC4468 (wildtype), GC122 (*rpoS*$^-$), HS2529 (Δ*ygaE*) and HS2530 (*rpoS*$^-$, Δ*ygaE*) cultures. In Figure 10, no significant difference in expression of *gabD* was observed between wildtype and Δ*ygaE* cultures in stationary phase. This suggests that YgaE does not modulate expression of the *gabDTP* operon significantly in stationary phase, and that there must be an additional *rpoS*-dependent promoter that transcribes the *gab* operon, which is independent of the previously identified promoter (Schneider *et al.*, 2002) that is regulated by nitrogen limitation and YgaE (GabC).

To study the effects of Ntr regulation on the *gab* operon and negative regulation by *ygaE*, deletion mutants were constructed in the *nac* and *ygaE* genes and the knockouts were transduced into strains harboring the fusions to *csiD/ygaF* and the *gabDTP* operon. Similar levels of *gab* operon expression were observed in Δ*nac* and Δ*ygaE* mutants relative to wildtype strains in media containing 0.5% glucose and 0.05% ammonia (Figure 13B). Conversely, in this media, the expression of the *gab* operon showed a high level of *rpoS*-dependence (Figure 13B). Similar results were observed with 1% glycerol (a poorer carbon source than glucose) as the sole carbon source. Together these results show that the *gabDTP* operon is most highly regulated by RpoS under conditions of nutrient sufficiency and carbon limitation. To test regulation of the operon by nitrogen limitation, expression was examined on 0.5% glucose 1% GABA plates. Under these conditions the *rpoS*$^-$ Δ*nac* double mutants have less expression of all three fusions assayed (*csiD/ygaF-lacZ, gabD-lacZ* and *gabP-lacZ*) relative to the *rpoS*$^-$ mutants. This suggests that the entire region is under control by nitrogen limitation via the Nac transcriptional regulator. Regulation of the *gabDTP* operon by Nac has been reported previously (Schneider *et al.*, 2002), but regulation of *csiD* by nitrogen limitation has not been reported. The decrease in expression may be due in part to decreased levels of growth, as Nac may be necessary for growth on GABA as a nitrogen source (Schneider *et al.*, 2002). Regulation of the *gabDTP* operon by nitrogen limitation is not as clear in RpoS-positive strains due to deduced growth relative to RpoS mutants (discussed below). Interestingly, the *rpoS*$^-$ *ygaE* double mutant has increased levels of expression of the

csiD/ygaF-lacZ fusion compared to the *rpoS*⁻ mutant. This is unexpected, as the *csiD* gene should not be expressed in *rpoS*⁻ cultures (Marschall *et al.*, 1998).

A Δ*cyaA* mutation was also created, to further elucidate the effects of carbon limitation on the expression of the *gabDTP* operon. The *gab* operon was previously shown to be regulated by catabolite repression (Zaboura and Halpern, 1978). A mutation in *cyaA* (the gene for adanylyl cyclase) prevents the production of cAMP during glucose starvation (reviewed in Saier *et al.* (1996)). The activator/repressor CRP binds cAMP and regulates the expression of many genes that allow the utilization of alternative carbon sources (Saier *et al.*, 1996). Preliminary studies with the Δ*cyaA* mutant strain exhibited poor growth on glucose-containing media, and no growth on media supplemented with other carbon souces (data not shown). The Δ*cyaA* stain was not used for further studies because the large difference in growth rates compared to wildtype strains would make interpretation of results difficult.

3.7 Regulation of the *gab* Operon in Response to Media pH

Previously, it has been shown that the *gabT* gene is dependent upon media pH for expression. Specifically, the gene is induced in media with pH 9 relative to pH 7. When cultures are grown in LB media (without glucose) the pH of the media becomes alkaline in late stationary phase (Stancik *et al.*, 2002). Thus, in our experiments, some of the induction of the *gabDTP* operon observed in stationary phase (Figure 6) may be due to the alkalization of the media. To test this, cultures of *E. coli* with operon *lacZ*-fusions to *ygaF* (HS1024), *gabD* (HS1057) and *gabP* (HS1010) were grown in buffered and

unbuffered LB media and expression of each of the gene fusions was examined. For the cultures grown in buffered media, sterile MOPS buffer was added to the LB after autoclaving, to a final concentration of 100 mM and the pH was adjusted to 7. No buffer was added to the control LB media. Cultures grown in unbuffered media to an OD_{600} of 1.6 (early stationary phase) showed similar expression of all three gene-fusions compared to cultures grown in buffered LB media (Table 5). The unbuffered cultures only reached an average pH of 8.4 after overnight incubation, compared to pH 7.3 of the buffered cultures (Table 5). Despite the difference in pH between the two, there was still no significant difference in the expression from any of the three fusions between buffered and unbuffered cultures (Table 5).

3.8 Utilization of GABA as a Carbon and Nitrogen Source

The original studies on the utilization of GABA revealed that *E. coli* could not use this compound as either a carbon or nitrogen source (Dover and Halpern, 1972). However, most of the work done on the pathway used cultures in exponential phase. More recently, data has shown that *E. coli* K-12 can grow on GABA as a nitrogen source, albeit very slowly (Schneider *et al.*, 2002). In light of this observation, the ability of *E. coli* to utilize GABA as a nitrogen or carbon source and the effect of *rpoS* was reexamined.

The operon *lacZ*-fusions to each of the genes in the region, *csiD/ygaF* (HS1024), *gabD* (HS1057), *gabDT-intergenic* (HS1037), and *gabP* (HS1010), were gridded onto

M9 minimal media plates supplemented with various carbon and nitrogen sources to assay the ability of *E. coli* to utilize them for growth. Glucose, glycerol and GABA were supplied as carbon sources and NH_4^+, GABA, glutamate and glutamine were supplied as nitrogen sources (results summarized in Table 6). Growth was most rapid on glucose and ammonia, as expected, which are the optimal carbon and nitrogen sources for *E. coli*.

When GABA is supplied as the sole nitrogen source, growth of the wildtype strain was very poor. However, an *rpoS*⁻ mutant derivative, GC122, had much better growth, resulting in larger, mucoid and white colonies (Figure 13). One possible explanation for the poor growth observed on GABA is that the high concentrations of GABA supplied may be toxic. To test this possibility the strains were gridded on both ammonia and GABA together as nitrogen sources. Growth on this media was comparable to that observed with ammonia as the sole nitrogen source, indicating that GABA is not toxic at these concentrations, but rather cannot be utilized well as a nitrogen source (Table 6).

To determine if the enhanced growth of the *rpoS*⁻ mutant is due to a difference in the growth rate between these cultures, the strains GC4468 (wildtype), GC122 (*rpoS*⁻), HS2529 (Δ*gabC*) and HS2530 (Δ*gabC rpoS*⁻) were grown in liquid M9 cultures with either 0.05% NH_4Cl or 1% GABA as the sole nitrogen sources (Table 3). The *rpoS*⁻ derivative had a generation time of 7.4 h compared to 16.7 h for the wildtype strain, growing on M9 with 0.5% glucose, 1% GABA. Likewise, the *gabC*⁻ strain had a generation time of 7.3 h in the same media. Thus, either mutation results in over a two-fold increase in the growth rate of the culture. The *rpoS*⁻ *gabC*⁻ double mutant had a

generation time of 5.7 h, which is faster than either of the single mutants (Table 3), suggesting that the effect of the mutations is, at least in part, independent of one another. Similar results were obtained using another sub-optimal carbon source, glycerol, instead of glucose (Table 3).

When GABA was supplied as the sole nitrogen source, the *gabD-lacZ* and *gabT-lacZ* strains did not grow, or grew extremely poorly, indicating that the *gab* operon is essential for GABA utilization and that there are no other genes that can complement their function in wildtype *E. coli* (Figure 13, Table 3). The first step in the enzymatic breakdown of GABA by GabD and GabT, for use as a nitrogen source, is the key step, where the amino group is transferred from GABA to α-ketoglutarate to yield glutamate and succinic semialdehyde (Figure 1). The product of the *gabT* gene catalyzes this reaction. Thus, glutamate is the next intermediate in the assimilation of nitrogen from GABA. Growth on glutamate as the sole nitrogen source was similar to that seen with GABA as the sole nitrogen source, where more growth is observed from *rpoS*⁻ derivatives relative to the wildtype strains (Figure 13, Table 6). These observations suggest that the poor growth seen on GABA may be due in part to the poor growth of *E.coli* with glutamate as the nitrogen source in these strains. As expected, growth of strains harbouring *gabD-lacZ* and *gabT-lacZ* fusions on glutamate was similar to wildtype.

Mutants with enhanced ability to grow on GABA can be isolated. Halpern and colleagues (Dover and Halpern, 1972; Metzer *et al.*, 1979) isolated such mutations and mapped them to the "*gabC*" locus, which is upstream of the *gabDTP* operon. Schneider

et al. (2002) showed that mutants of the *ygaE* gene show elevated growth on GABA as a sole carbon and nitrogen source, relative to wildtype strains. We were interested to see if plating cultures onto media with GABA as the sole source of carbon or nitrogen would result in spontaneous mutations giving rise to colonies with enhanced growth on GABA (Table 4). $GABA^+$ utilization mutants could be isolated on media containing GABA as the sole carbon source, the sole nitrogen source, or both the sole carbon and nitrogen source. Interestingly, these mutants could not be isolated from *rpoS*⁻ strains (Table 4). The nature of the mutations that allowed enhanced growth on GABA is unclear. Due to these mutants arising spontaneously during growth of cultures on GABA as a sole nitrogen or carbon source, previous growth measurements of cultures on GABA may have some error due to the potential presence of GABA utilizing mutants.

Table 3: Growth Rate of *E. coli* Cultures on Various Carbon and Nitrogen Sources

Media	Strain	Doubling time, h ± SE
Glucose NH_4	WT	1.36 ± 0.02
	rpoS::*Tn*10	1.18 ± 0.12
	Δ*ygaE*	1.34 ± 0.01
	Δ*ygaE rpoS*::*Tn*10	1.17 ± 0.04
Glucose GABA	WT	16.7 ± 0.27
	rpoS::*Tn*10	7.4 ± 0.92
	Δ*ygaE*	7.3 ± 1.79
	Δ*ygaE rpoS*::*Tn*10	5.7 ± 0.57
Glycerol NH_4	WT	1.36 ± 0.03
	rpoS::*Tn*10	1.24 ± 0.06
	Δ*ygaE*	1.33 ± 0.02
	Δ*ygaE rpoS*::*Tn*10	1.27 ± 0.1
Glycerol GABA	WT	31.5 ± 2.88
	rpoS::*Tn*10	11.9 ± 2.34
	Δ*ygaE*	11.0 ± 3.55
	Δ*ygaE rpoS*::*Tn*10	5.1 ± 0.49

Table 4: Isolation of GABA$^+$ Utilization Mutants

	GABA Suppression Mutation Frequency (x 10^{-8} CFU) ± SE	
Media	**MG1655 (*rpoS*$^+$)**	**HS2210 (*rpoS*$^-$)**
GABA	21.8 ± 1.9	No Growth
GABA + NH_4	12.1 ± 2.1	No Growth
Glucose + GABA	30.2 ± 0.13 [b]	Lawn [a]
Glycerol + GABA	38.6 ± 8.6 [bc]	Lawn [a]

a. Growth on this media resulted in a lawn of bacteria with no GABA$^+$ utilizing colonies
b. Colonies on this media were difficult to distinguish due to background growth on the plate of wildtype cells
c. GABA$^+$ utilizing colonies on the glycerol media were smaller than those on the glucose media

Table 5: Expression of the *gabDTP* Operon in Response to Media pH

Strain/ Media[a]	Specific activity ± SE (pH)		
	E phase (OD_{600} = 0.3)	S phase (OD_{600} = 1.5)	24 hours (OD_{600} = 2)
ygaF-lacZ / LB	0.51 ± 0.01	11.82 ± 0.49	13.93 ± 0.12 (8.5)[b]
ygaF-lacZ / LB-M	0.65 ± 0.04	13.48 ± 0.10	17.31 ± 0.03 (7.3)
gabD-lacZ / LB	1.40 ± 0.07	35.41 ± 2.39	72.45 ± 1.54 (8.6)
gabD-lacZ / LB-M	1.40 ± 0.03	39.63 ± 1.08	70.70 ± 0.59 (7.3)
gabP-lacZ / LB	0.93 ± 0.01	11.76 ± 0.15	14.49 ± 0.38 (8.4)
gabP-lacZ / LB-M	0.87 ± 0.03	13.76 ± 0.36	18.47 ± 0.18 (7.4)

a. LB, Luria Bertani broth; LB-M, MOPS buffered LB broth, pH 7.0
b. Final pH of the culture is shown in parentheses beside the 24-hour values

Table 6: Growth of *gab* Operon Mutants on Various Carbon and Nitrogen Sources

Nitrogen source	Relative Growth on Various Nitrogen Sources									
	GC4468 (WT)		**HS1024** (*csiD/ygaF-lacZ*)		**HS1057** (*gabD-lacZ*)		**HS1037** (*gabDT$_{intergenic}$-lacZ*)		**HS1010** (*gabP-lacZ*)	
RpoS:	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
NH_4	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
GABA	++	+	++	+	-	-	-	-	++	+
Glutamate	++	++	++	++	++	++	++	++	++	++
Glutamate + GABA	++	++	++	++	++	++	++	++	++	++
Glutamine	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Glutamine + GABA	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
NH_4 + GABA	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
NH_4 + Glutamate	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
NH_4 + Glutamine	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Relevant genotypes are given below the strain designation. M9 Minimal media plates were prepared as described in the Materials and Methods section. 0.5% Glucose was used as the nitrogen source for all plates. All nitrogen sources were supplied at 1% final concentrations, except NH_4Cl, which was supplied at 0.05%. ++++ indicates optimal growth (using growth on NH_4^+ as the standard); ++ indicates intermediate growth; + indicates poor growth, - indicates no growth. Abbreviations: GABA, γ-aminobutyric acid.

Table 7: GABA Transaminase (GSST) and Succinic Semialdehyde Dehydrogenase (SSDH) Activities in Wildtype and *rpoS*⁻ Cultures Grown in Rich LB Media

Strain	Activity (nmol min^{-1} mg $protein^{-1}$) ± SE	
	GSST	SSDH
Exponential phase		
GC4468 (wildtype)	None Detected	1.78 ± 0.17
GC122 (*rpoS*)	None Detected	1.91 ± 0.08
Stationary phase		
GC4468 (wildtype)	58.9 ± 4.55	78.1 ± 2.14
GC122 (*rpoS*)	2.49 ± 0.07	3.96 ± 0.11

Chapter 4 Figures

Figure 5: Expression of the Identified *gab* Operon *lacZ*-Fusions on LB Plates Supplemented with X-GAL.

Strains were grown for 1 day at 37°C and scanned. The position of each of the fusions is shown in Figure 2. rsd1057, *gabD-lacZ*; rsd1037, *gabDT*$_{\text{intergenic}}$-*lacZ*; rsd1010, *gabP-lacZ*; rsd1024, *csiD/ygaF-lacZ*

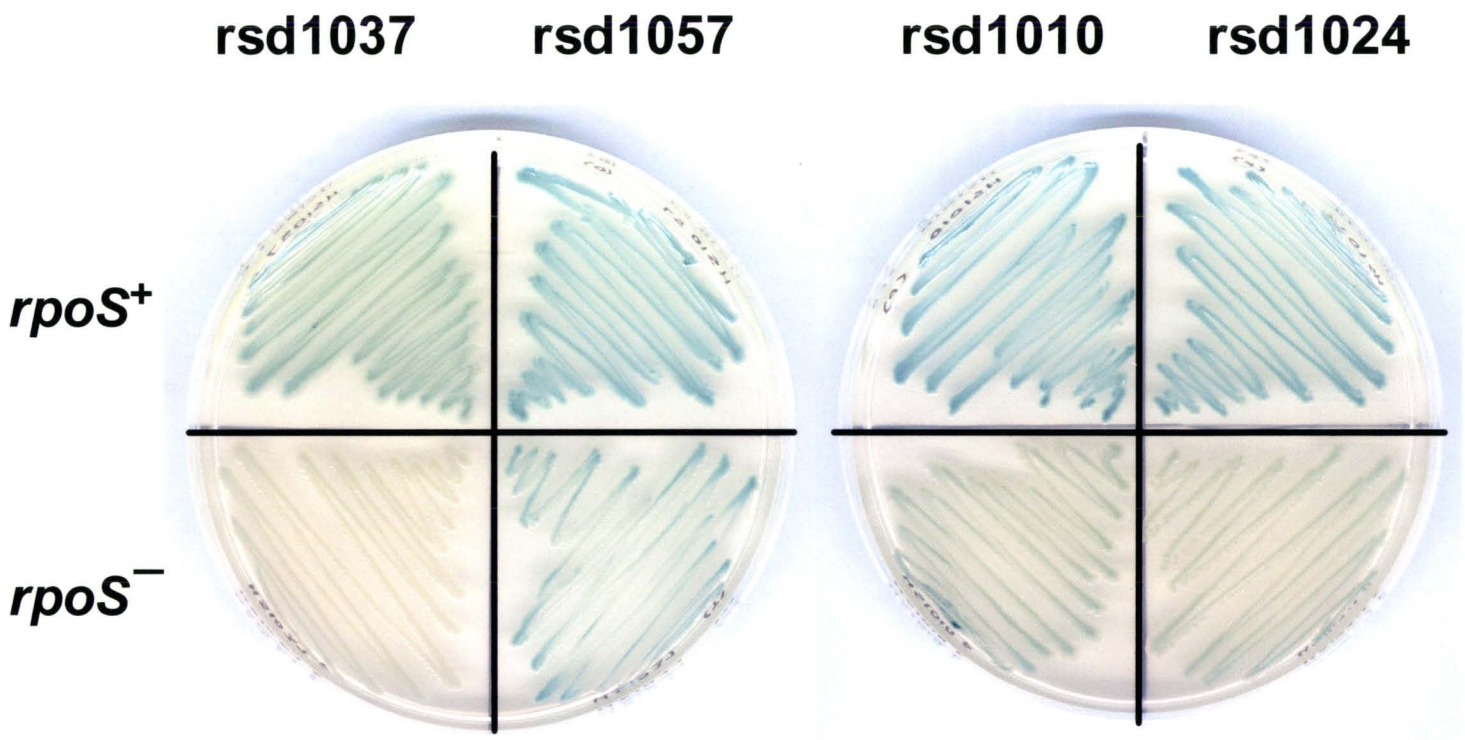
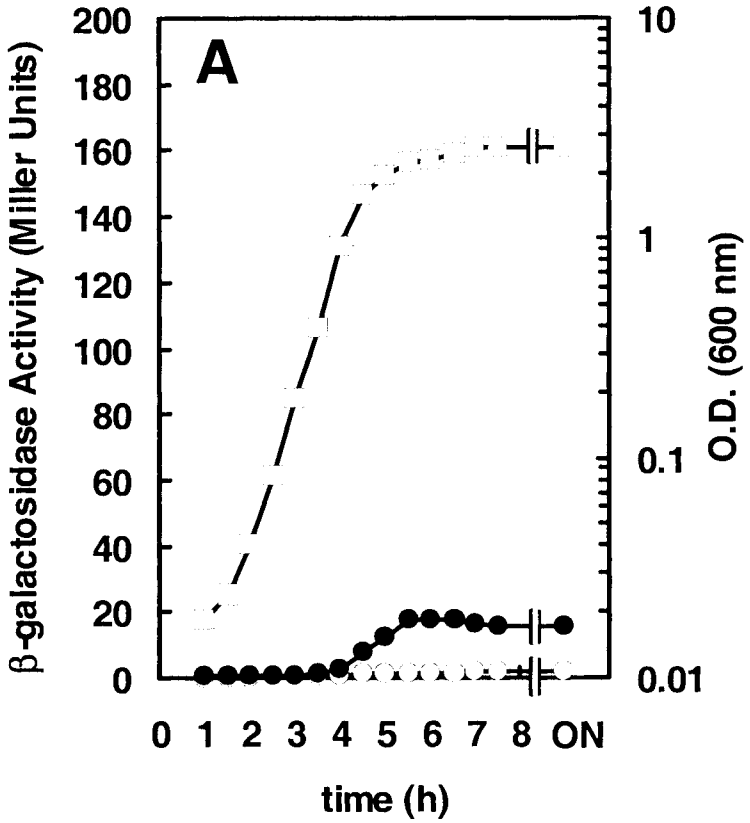
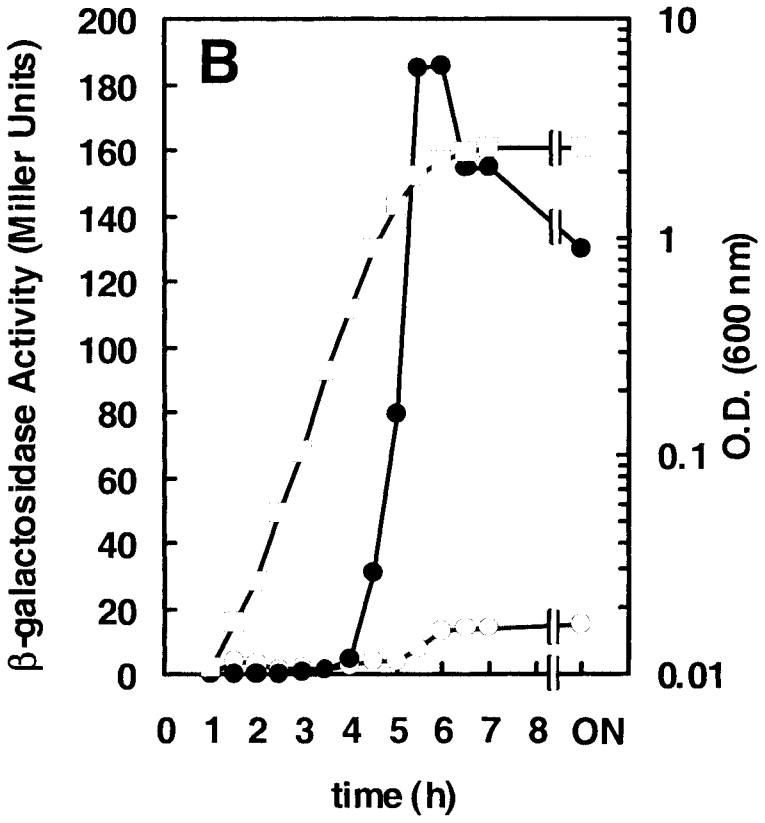

rsd1037
rsd1057
rsd1010
rsd1024
rpoS+
rpoS−

Figure 6: Expression of *ygaF* and the *gabDTP* Genes in Rich Media over the Growth Curve of *E. coli.*

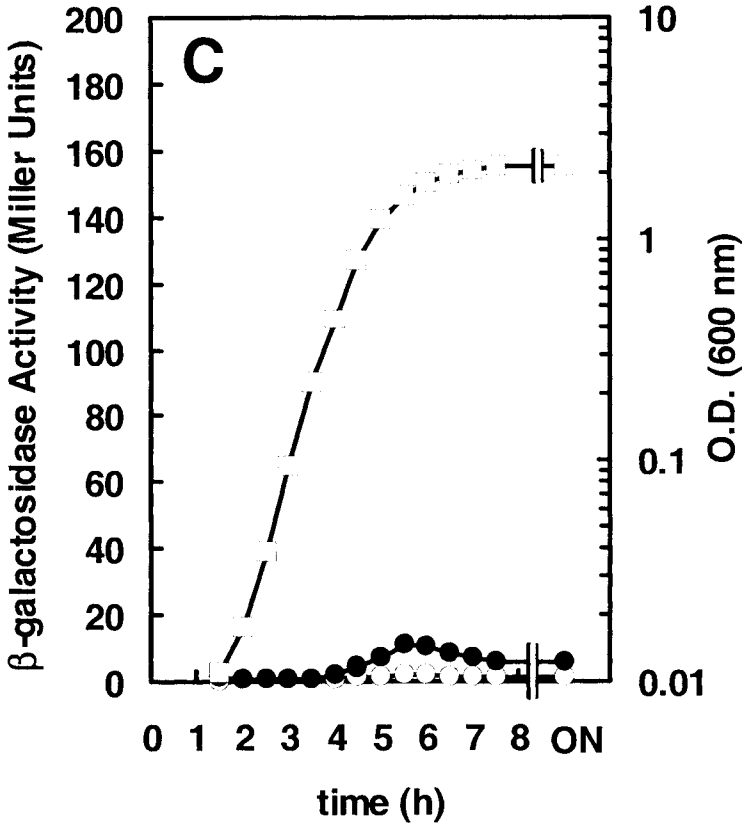
Expression is shown as specific activity from each of the fusions: *csiD/ygaF-lacZ* (A), *gabD-lacZ* (B), $gabDT_{intergenic}$-*lacZ* (C), and *gabP-lacZ* (D). β-Galactosidase activity was measured from triplicate cultures of each strain at the indicated timepoint. (□), O.D.600 of culture; (○), β-galactosidase activity of *rpoS*-negative strain; (●), β-galactosidase activity of *rpoS*-positive strain. Abbreviations: O.D., optical density; ON, overnight (approximately16 h)



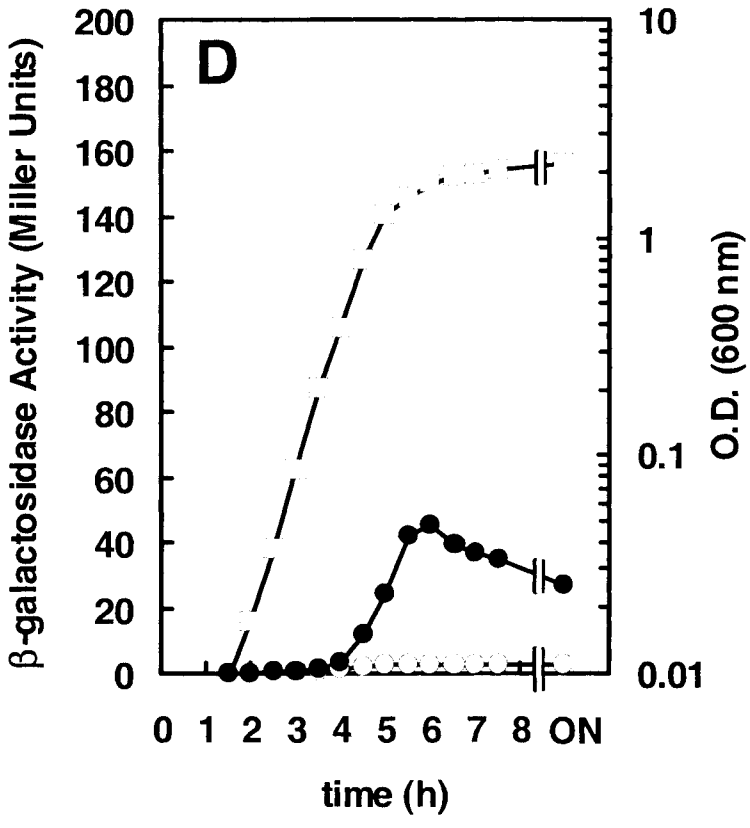
rsd1024 (*ygaF-lacZ*)



rsd1057 (*gabD-lacZ*)



rsd1037 (*gabDT-lacZ*)



rsd1010 (*gabP-lacZ*)

Figure 7: Northern Analysis of *ygaF* and *gabDTP* in Wildtype and *rpoS*⁻ Strains. The gene used to probe each of the blots is shown to the side of the pictures. The lower panels of each blot show equal amounts of RNA in each of the lanes. Abbreviations: *rpoS*⁻, *rpoS*-negative strain; *rpoS*⁺, *rpoS*-positive strain; E, exponential phase ($O.D._{600}$ of culture is 0.25-0.35); S, stationary phase ($O.D._{600}$ of the culture is 1.6-1.8).

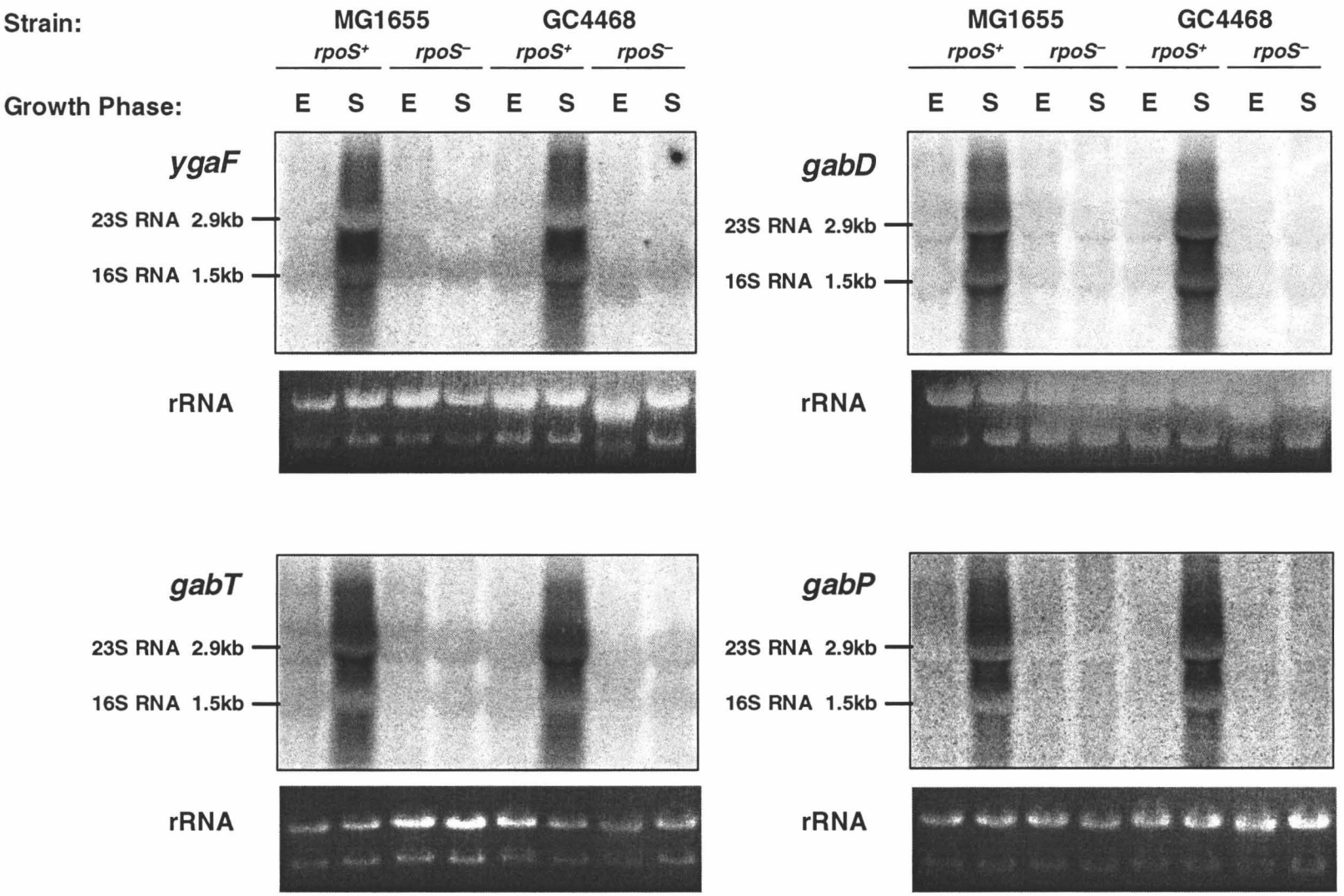

Strain:
MG1655
GC4468
rpoS+
rpoS−
Growth Phase:
E S E S E S E S
ygaF
23S RNA 2.9kb
16S RNA 1.5kb
rRNA
gabD
gabT
gabP

Figure 8: GABA Transaminase (GSST) and Succinic Semialdehyde Dehydrogenase (SSDH) Activities in Wildtype and *rpoS*⁻ Cultures Grown in Rich LB Media.

The error bars are the standard error of specific activity determined from triplicate cultures. Actual values used to construct the graph are given in Table 7. The enzyme activity was measured from each culture twice and the values averaged. No GSST enzyme activity above background could be detected in exponential phase. Abbreviations: E, exponential phase ($O.D._{600}$ = 0.25-0.35); S, stationary phase ($O.D._{600}$ = 1.6-1.8).

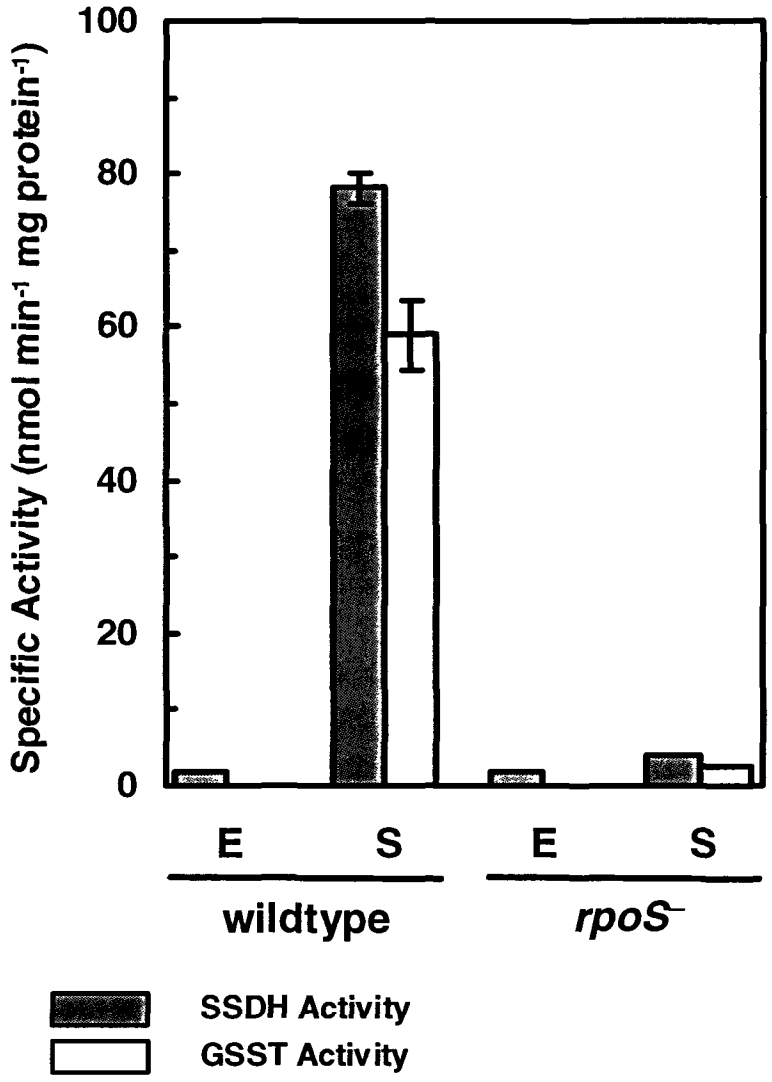

Specific Activity (nmol min-1 mg protein-1)
100
80
60
40
20
0
E
S
E
S
wildtype
rpoS–
SSDH Activity
GSST Activity

Figure 9: Northern Analysis of A) *gabD* and B) *gabP* Expression in Strains Carrying the RpoS-Dependent (rsd) *lacZ*-Fusions.
Total RNA was isolated from stationary phase ($O.D._{600}$ = 1.6-1.8) cultures of wildtype and strains carrying *lacZ* fusions to *gab* operon members in both $rpoS^+$ and $rpoS^-$ backgrounds. Each lane was loaded with 5 μg of total RNA. The top blot was probed for the *gabD* expression and the lower blot was probed for *gabP* gene expression. rRNA sizes are indicated on the left (in kilobases).

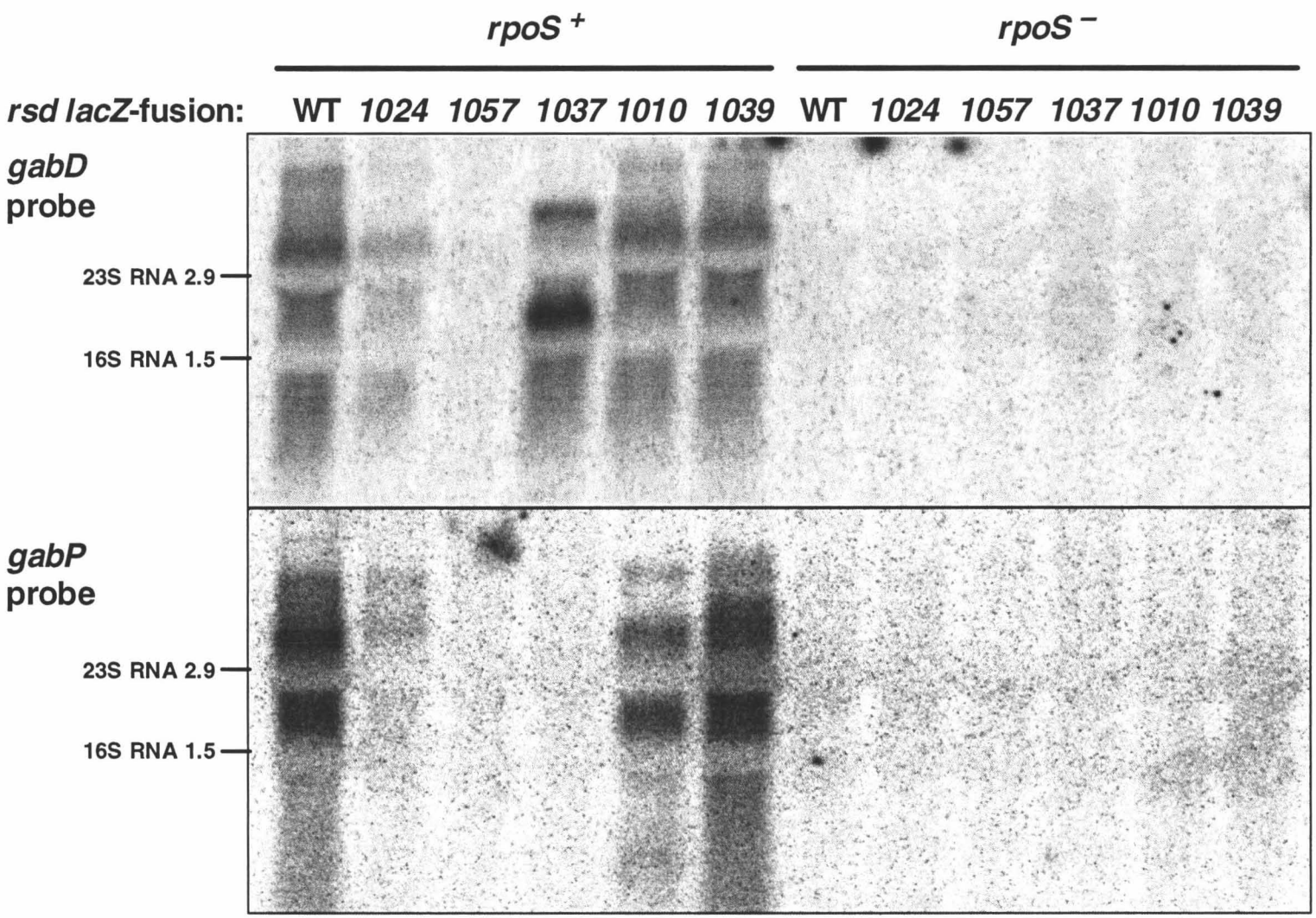

rpoS +
rpoS −
rsd lacZ-fusion:
WT 1024 1057 1037 1010 1039
WT 1024 1057 1037 1010 1039
gabD probe
23S RNA 2.9
16S RNA 1.5
gabP probe
23S RNA 2.9
16S RNA 1.5

Figure 10: Northern Analysis of *gabD* Expression in Strains Carrying Mutations in *rpoS* and *ygaE*.
mRNA was isolated from exponential (E) phase (O.D.600 = 0.25-0.35) and stationary (S) phase ($O.D._{600}$ = 1.6-1.8) cultures. Each lane was loaded with the mRNA equivalent to 3.5 μg of total RNA and the blot was probed for the *gabD* gene. RNA sizes are indicated on the left (in kilobases).

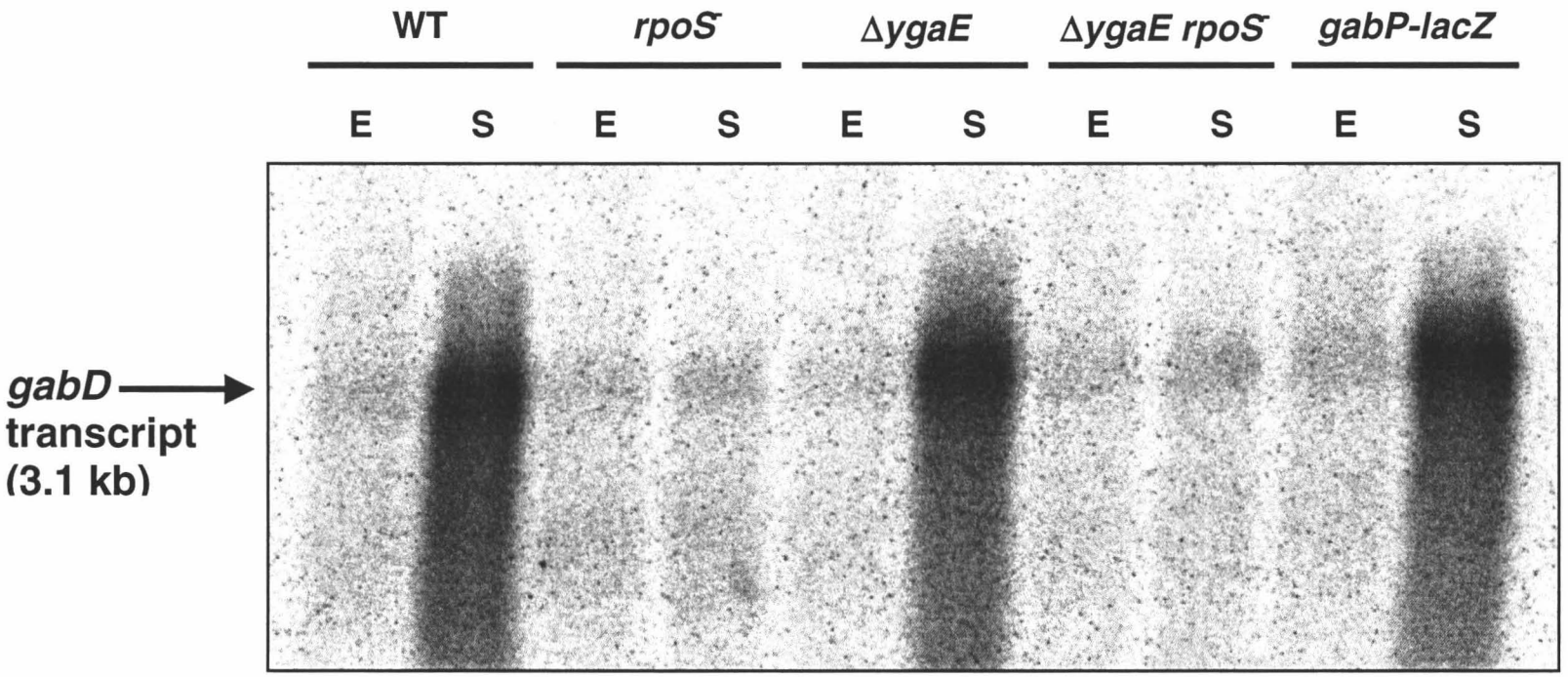

WT
rpoS⁻
ΔygaE
ΔygaE rpoS⁻
gabP-lacZ
E
S
E
S
E
S
E
S
E
S
gabD
transcript
(3.1 kb)

Figure 11: RT-PCR Analysis of *csiD*, *ygaF* and the *gabDTP* Operon.
Total RNA isolated from GC4468 (WT) cultured to stationary phase ($O.D._{600}$ = 1.6-1.8) was used to create cDNA by reverse transcription. PCRs with and without reverse transcriptase were run and the products separated on a 1% agarose gel. Lane M, 1 kb Ladder (Fermentas); Lanes 1-5, PCRs run with genomic DNA as positive controls; Lanes 6-10, PCRs run using cDNA as template; Lanes 11-15, PCRs run from RNA without reverse transcription to control for DNA contamination.

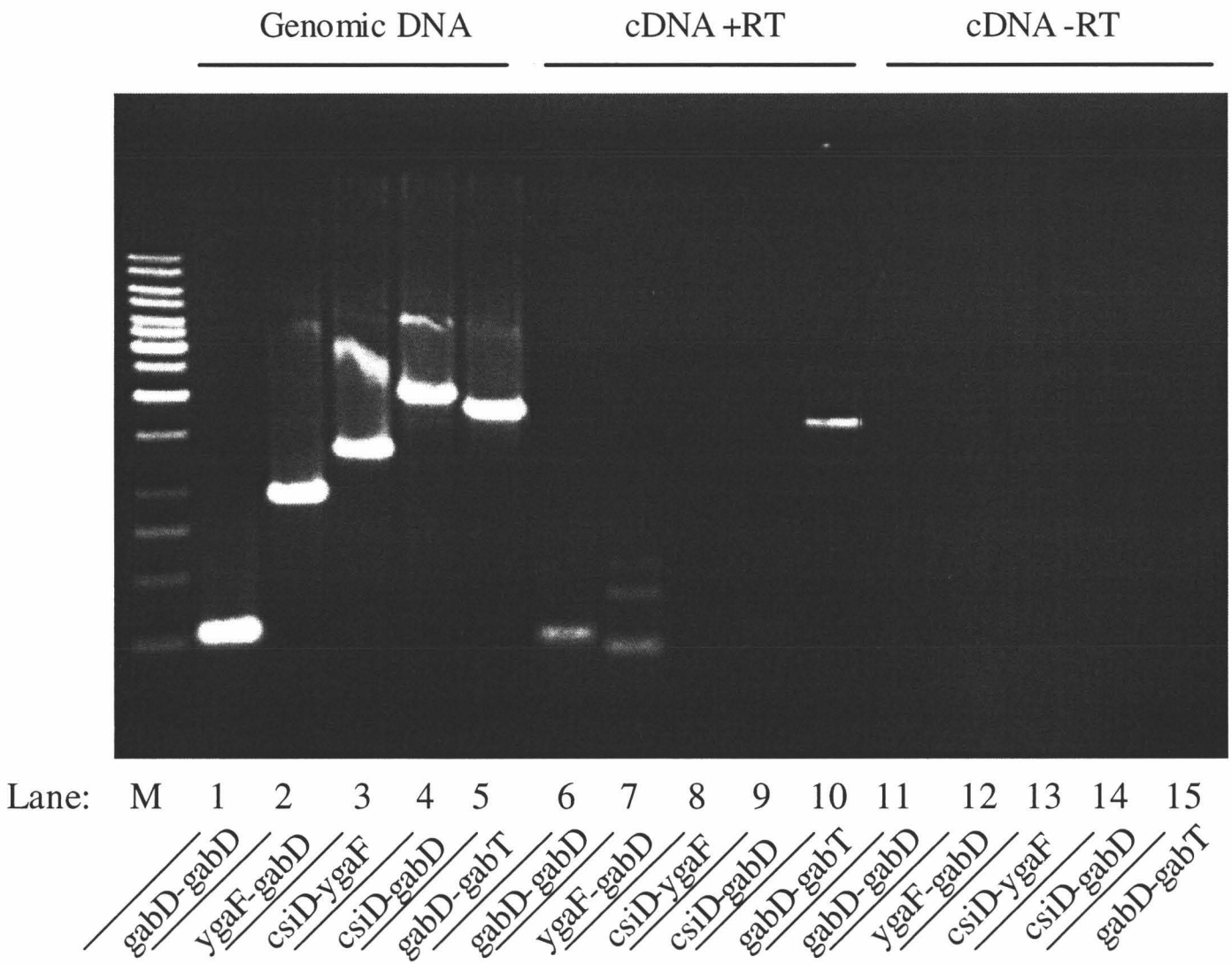

Genomic DNA
cDNA +RT
cDNA -RT
Lane: M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
gabD-gabD
ygaF-gabD
csiD-ygaF
csiD-gabD
gabD-gabT
gabD-gabD
ygaF-gabD
csiD-ygaF
csiD-gabD
gabD-gabT
gabD-gabD
ygaF-gabD
csiD-ygaF
csiD-gabD
gabD-gabT

Figure 12: Schematic Representation of RT-PCR Results from Figure 11.
The structure and position of the *csiD-ygaF-gabDT* genes are shown. Promoters are indicated above the gene map. Approximate binding position of the primers used are shown directly bellow the gene map (not to scale). The relative size of the expected RT-PCR products is shown on the bottom. Abbreviations: **+** , a PCR product was formed using the indicated primer set; **–**, indicates that no PCR product was formed using the primer set.

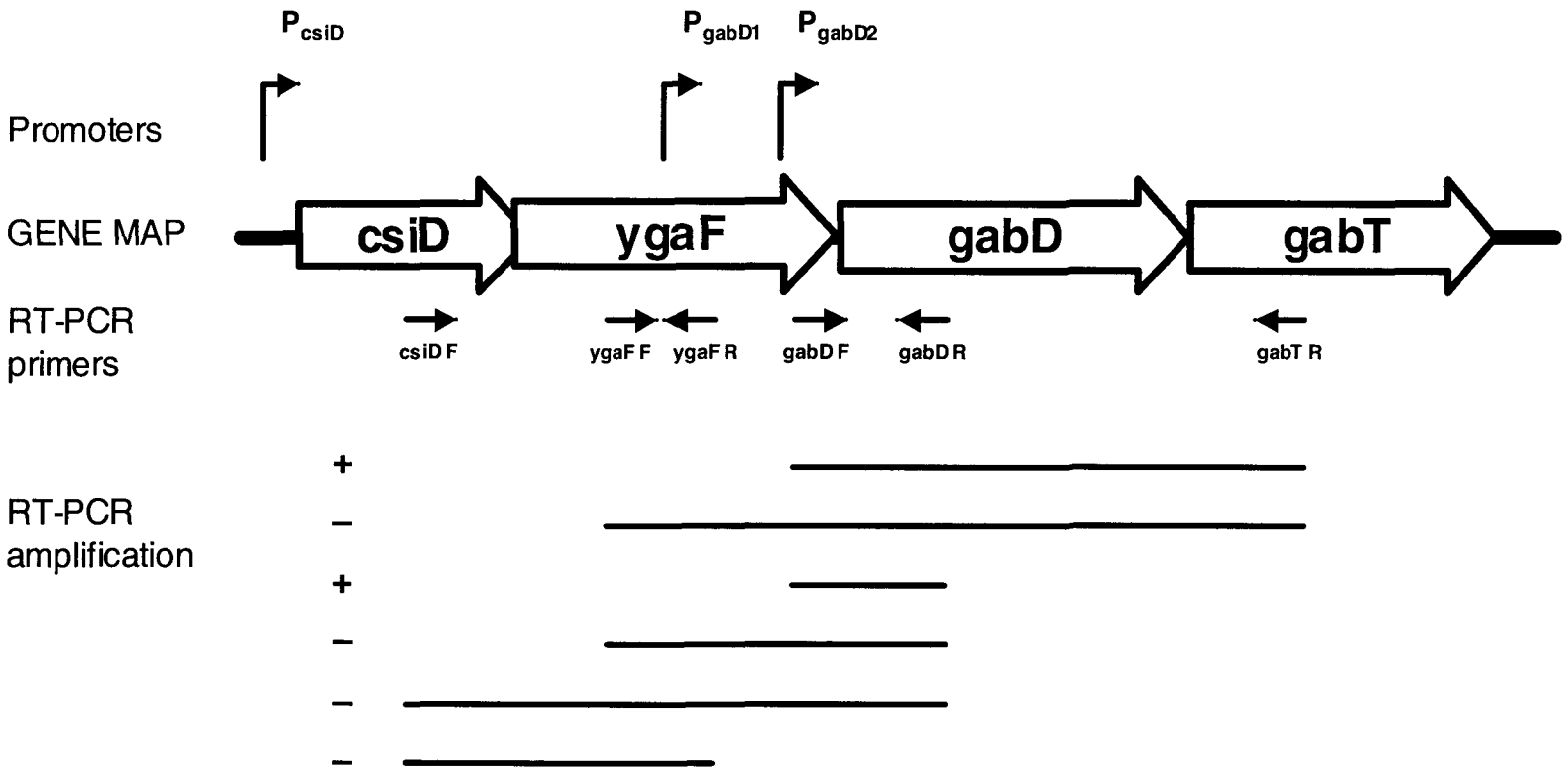
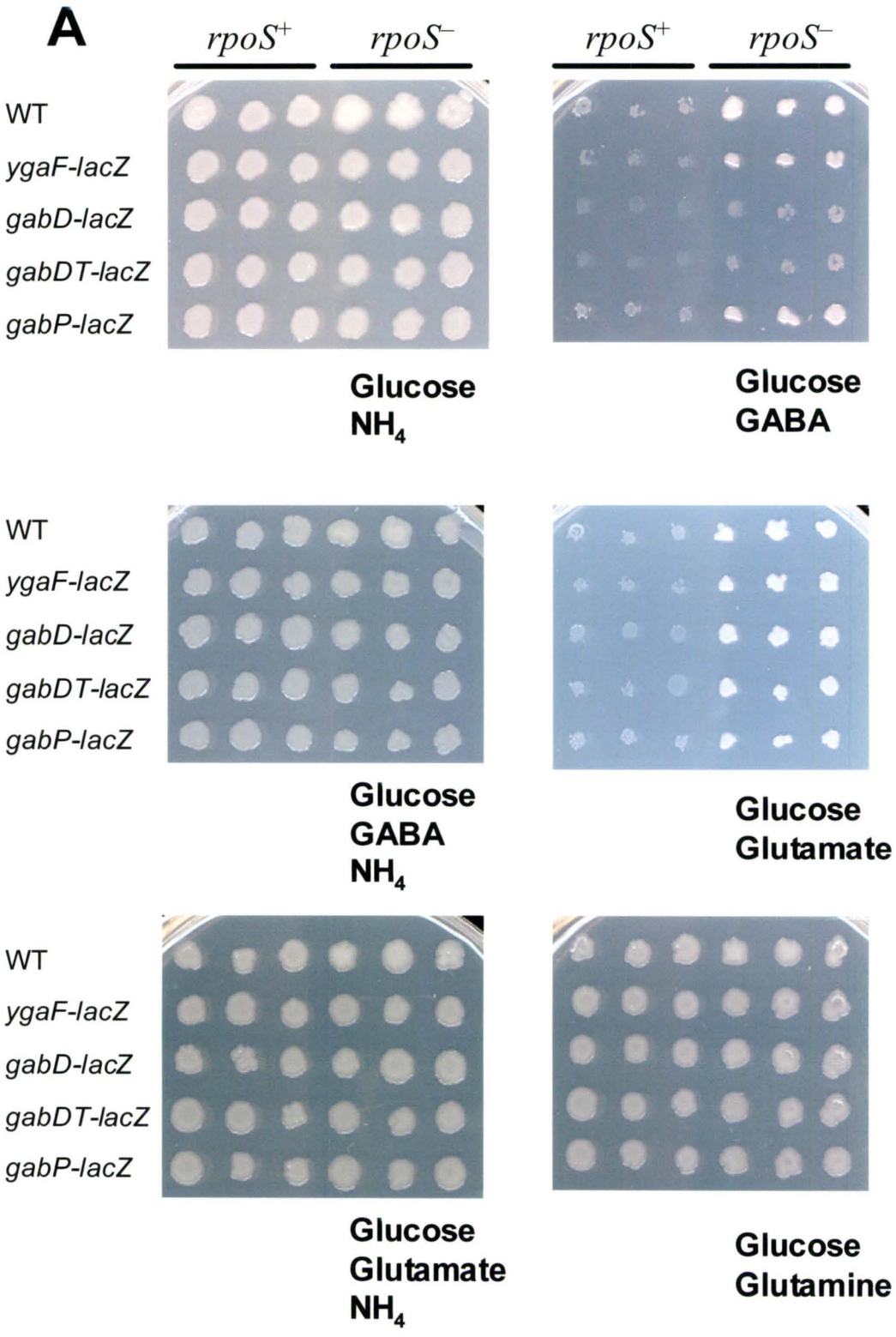

P csiD
P gabD1
P gabD2
Promoters
GENE MAP
csiD
ygaF
gabD
gabT
RT-PCR primers
csiD F
ygaF F
ygaF R
gabD F
gabD R
gabT R
RT-PCR amplification
+
–
+
–
–
–

Figure 13: Replica Plates of *gab* Operon *lacZ*-Fusion and Regulatory Mutant Strains on Various Nitrogen and Carbon Sources.
Strains were replica plated onto Minimal M9 media to test their ability to grow with 1% GABA, 0.05% ammonia, 1% glutamate, and 1% glutamine as sole nitrogen sources. 0.5% Glucose, 1% Glycerol and 1% GABA were tested as carbon sources. (A) Growth of strains carrying *gab* operon *lacZ*-fusions to test their ability to grow. (B) Growth of strains carrying *rpoS*, *nac* and *ygaE* mutations to assay their effects. (Note: A strain carrying a Δ*ygaE*, *gabP-lacZ* double mutation was not obtained and is absent from the plates in Part B). X-Gal was included in some plates at 25 μg/ml to qualitatively assay gene expression of *lacZ*-fusions.


A
rpoS+
rpoS–
rpoS+
rpoS–
WT
ygaF-lacZ
gabD-lacZ
gabDT-lacZ
gabP-lacZ
Glucose
NH4
Glucose
GABA
WT
ygaF-lacZ
gabD-lacZ
gabDT-lacZ
gabP-lacZ
Glucose
GABA
NH4
Glucose
Glutamate
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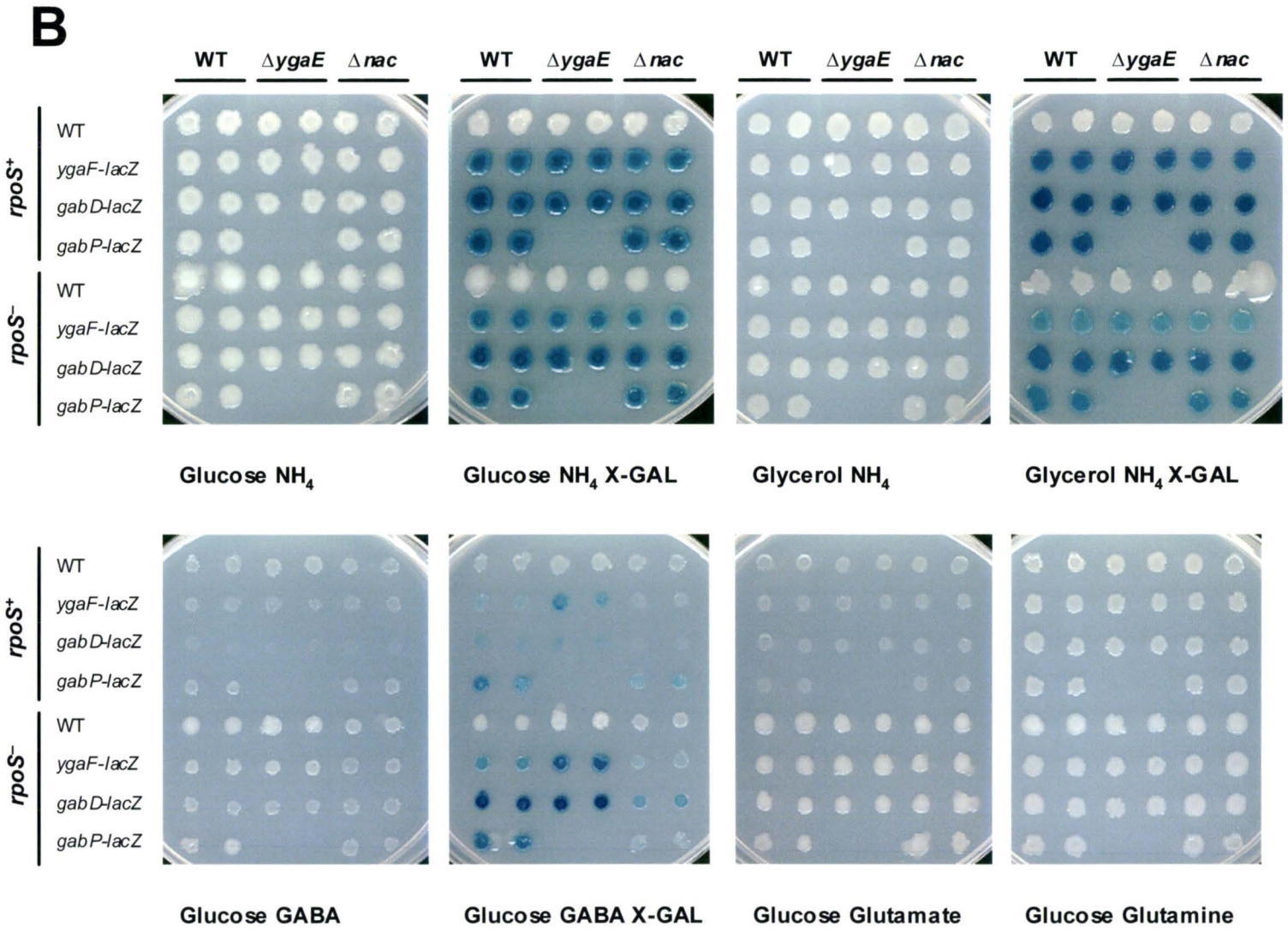
gabP-lacZ
Glucose
Glutamate
NH4
Glucose
Glutamine


B
WT
ΔygaE
Δnac
rpoS+
rpoS–
WT
ygaF-lacZ
gabD-lacZ
gabP-lacZ
Glucose NH4
Glucose NH4 X-GAL
Glycerol NH4
Glycerol NH4 X-GAL
Glucose GABA
Glucose GABA X-GAL
Glucose Glutamate
Glucose Glutamine

Chapter 5 Discussion

5.1 RpoS-Dependence of the *gab* Operon

Expression of the *gabDTP* operon was shown to be RpoS-dependent (Schellhorn *et al.*, 1998) using random operon *lacZ*-fusions to the *gabD* and *gabP* genes, which showed a 10-fold *rpoS*-dependence in rich media. Subsequently, Baca-DeLancey *et al.* (1999) also reported the isolation of a *lacZ*-fusion within the *gabT* gene that was upregulated by conditioned media. This fusion to *gabT* also showed an approximately 18-fold *rpoS*-dependence in stationary phase. The same *gabT* fusion is also up-regulated by growth in alkaline media (Stancik *et al.,* 2002), and has a 6-fold higher alkaline induction at OD_{600} = 0.5 compared to OD_{600} = 0.2 (Stancik *et al.,* 2002). In contrast, work done by Schneider *et al.*, (2002) revealed that the *gabDTP* operon is transcribed from a σ^{70}-dependent promoter, and found no evidence for regulation of the operon by RpoS. The σ^{70}-dependent promoter is positively regulated by Nac, in response to nitrogen sufficiency and is repressed by the GabC (YgaE) repressor (Schneider *et al.*, 2002). At the 3' end of the *gabDTP* operon is the gene *ygaE*, encoding a hypothetical regulator (Schneider *et al.*, 2002). YgaE is oriented in the same direction as the *gabDTP* operon but it is unclear whether it is actually part of the operon. If *ygaE* is transcribed as part of the operon, then polar insertions into the *gab* operon members should interfere with *ygaE* expression (Schneider *et al.*, 2002). This polar insertion in *gabT* results in an increase in SSDH (encoded by *gabD*) enzyme activity (Schneider *et al.*, 2002). Based on this

evidence, they concluded that the *ygaE* gene is part of the *gabDTP* operon, and the polar insertion in *gabT* prevents expression of the YgaE repressor, causing an increase in *gabD* expression (Schneider *et al.*, 2002). They also hypothesized (Schneider *et al.*, 2002) that the *rpoS*-dependent expression reported in previous studies was due to an artifact resulting from polar *lacZ*-fusions to the operon used in those studies (Schellhorn *et al.*, 1998; Baca-DeLancey *et al.*, 1999; Stancik *et al.*, 2002).

The work presented here shows conclusively that the *gabDTP* operon is highly RpoS-dependent in wildtype *Escherichia coli*. The *gab* operon *lacZ*-fusion data (Figure 6) is in good agreement with Northern analyses of the *gabD*, *gabT* and *gabP* genes (Figure 7). Additionally, enzyme activities of GSST (GabT) and SSDH (GabD) were found to be highly *rpoS*-dependent (Figure 8). There is a possibility that a mutation in the (*ygaE*) *gabC* gene could account for these elevated levels of activity; however, the northern blots (Figure 7) were done in two strain backgrounds, GC4468 and MG1655, so a mutation is unlikely. Additionally, this shows that regulation of the *gabDTP* operon by *rpoS* is not strain specific. Recent work by Metzner *et al.* (2004) is in general agreement with these results. They identified an *rpoS*-dependent promoter, within the *ygaF* gene, that drives transcription of the *gabDTP* operon. They also showed that the *gabDTP* operon is dependent on stimuli (nutrient limitation, osmotic stress) that are known to induce transcription of RpoS (Metzner *et al.*, 2004).

Northern analyses also reveal that transcription of the *ygaF* gene is dependent on *rpoS*. Little is known about the *ygaF* gene, with respect to either its regulation or its

function. The encoded protein is homologous to dehydrogenase enzymes (Schneider *et al.*, 2002). The *ygaF* gene is co-transcribed with *csiD* from a single promoter upstream of the *csiD* gene (Metzner *et al.*, 2004). Transcription from this promoter is dependent on RpoS and CRP (Marschall *et al.*, 1998). YgaF is not likely involved in GABA metabolism, because a mutation in the gene has a negligible effect on growth on GABA as a nitrogen source (Schneider *et al.*, 2002). The substrate and/or metabolic role for YgaF is currently unknown. The location of *ygaF* adjacent to the *csiD* gene and *gabDTP* operon suggests that YgaF is likely important for stress resistance and possibly nutrient scavenging, possibly for alternative carbon sources.

5.2 Relative Importance of Known Regulators of the Operon

The role of YgaE (GabC, CsiR) in regulation of the *gabDTP* operon is currently unclear. Previous studies report that the *ygaE* gene is part of the *gabDTP* operon (Schneider *et al.*, 2002) and thus any fusions in the operon should have a polar effect on its expression. However, both of the strains, HS1010 and HS1039, with identified fusions to *gabP*, have an equal level of *gabD* expression compared to the wildtype strain GC4468 as shown by northern analysis (Figure 9). Additionally a strain carrying a deletion in *gabC* exhibits the same level of transcription of *gabD* in stationary phase as the wildtype GC4468 (Figure 10). Together, these two observations suggest that *ygaE* (*gabC)* does not modulate transcription of the *gab* operon by RpoS. Additionally, the polar insertions in *gabP* appear to have no effect on the level of *gabD* transcription in contrast to

observations by Schneider *et al.* (2002), so *ygaE* is likely not part of the *gab* operon. This observation is consistent with another study (Metzner *et al.*, 2004), who reported that expression of *ygaE* is not RpoS-dependent or dependent on the promoter preceding *gabD*, which clearly indicates that *ygaE* is not part of the *gabDTP* operon.

Previous studies have shown that YgaE regulates the *gab* operon (Schneider *et al.*, 2002). The identified *gabDTP* promoter is regulated by Nac and σ^{70}, and has potential binding sites for YgaE (Schneider *et al.*, 2002). Metzner *et al.* (2004) recently showed that YgaE (renamed CsiR) regulates expression of the *csiD* promoter in stationary phase. Additionally, Metzner *et al.* (2004) showed, by primer extension and *lacZ*-fusion studies, that the *gabDTP* operon is transcribed from two *rpoS*-dependent promoters. The first is upstream of *csiD*, and is regulated by CRP, RpoS and YgaE (Marschall *et al.*, 1996; Metzner *et al.*, 2004). The second promoter is within the *ygaF* gene, approximately 360 bp upstream of the *gabD* open reading frame (Metzner *et al.*, 2004). Expression from this second promoter is regulated by RpoS alone, as sequences upstream of the core promoter do not modulate transcription from the promoter (Metzner *et al.*, 2004).

Previous studies have implicated YgaE (Schneider *et al.*, 2002), nitrogen limitation via Nac (Schneider *et al.*, 2002) and carbon starvation via CRP (Metzner *et al.*, 2004) as regulators of the *gabDTP* operon. Our results suggest that, based on growth on plates, *rpoS*$^-$ mutations appear to have the greatest effect on transcription of the operon when growing in the presence of ammonia as the nitrogen source (Figure 13B) relative to mutations in *ygaE* (*gabC)* or *nac*. This result was based on the phenotype of colonies

growing on plates and expression was qualitatively determined by degree of blue colour in the presence of X-gal in strains with *gab* operon *lacZ*-fusions (Figure 13). Under nitrogen limiting conditions (growth on GABA as the sole nitrogen source), expression of the *gabDTP* operon appeared to be dependent on Nac (Figure 13B), in agreement with a previous report (Schneider *et al.*, 2002). Unexpectedly, *rpoS*⁻ Δ*ygaE* double mutants had high levels of *csiD/ygaF-lacZ* expression relative to the *rpoS*⁻ mutant strain. Recently, Metzner *et al.* (2004) reported that YgaE represses transcription of the *csiD* gene, which is consistent with our results; however, expression of the operon should be low in *rpoS*⁻ mutants (Marschall *et al.*, 1998). The reason for this discrepancy is unclear. One possible explanation is that a promoter upstream of *csiD* can be recognized by a sigma factor other than RpoS and lead to transcription of the gene.

5.3 Role of *csiD* and *ygaF* in Regulation of the *gabDTP* Operon

Although *csiD* and *ygaF* are both *rpoS* dependent and directly upstream of the *gabDTP* operon in the same orientation, it is unclear whether they comprise a single transcript with the *gab* operon. A transcript of sufficiently large size was found in the northern analysis (Figure 9) although it is unclear whether this transcript was of the entire *gabDTP* operon or was comprised of *csiD-ygaF-gabDT*. Larger RNA molecules in an agarose gel are difficult to accurately size. Expression of the *gabP* gene was absolutely dependent on a promoter upstream of the *gabD* gene (Figure 9), so a transcript with the *gabDT* genes and *gabP* was expected to be present. The RT-PCR analysis of the *csiD-ygaF-gabD* region (Figure 11) suggested that the *csiD* and *gabDTP* genes were not co-

transcribed, as no PCR product could be obtained spanning from the midpoint of *ygaF* to the 5' end of *gabD*. Although this is a negative result, all controls run at the same time worked, so there is little reason to believe this was due to a failure in the reaction, but rather was due to the absence of an mRNA present (Figure 11). The result suggested that the *rpoS*-dependent promoter must be somewhere within the 3' end of the *ygaF* ORF. Metzner *et al.* (2004) showed, by primer extension, the existence of an *rpoS*-dependent promoter approximately 360-bp upstream of the *gabD* ORF, within the *ygaF* gene. However, in contrast to our results, Metzner *et al.* (2004) also reported that the *csiD-ygaF-gabDTP* genes comprise an entire operon, with the *csiD* promoter responsible for 60% of the *rpoS*-dependent transcription of the operon. To examine expression of the *gabDTP* operon, Metzner *et al.* (2004) constructed *lacZ*-fusions to a plasmid borne copy of the *gabDTP* operon. The *lacZ*-fusion constructs were crossed into a lambda phage derivative and inserted into the chromosome at the *attB* site to make a single copy insertion, ultimately yielding merodiploid strains for some members of the *gab* operon (Metzner *et al.*, 2004). Expression of the *gabD-lacZ* fusion was decreased in the strain with no *csiD* promoter relative to the strains with an intact *csiD* gene and promoter (Metzner *et al.*, 2004), which suggests expression of the *gabDTP* operon is dependent on the *csiD* promoter (Metzner *et al.*, 2004). However, this observation could also be explained in part by the elimination of the second copy of the *csiD* gene, whose product may be involved in regulation of the *gabDTP* operon (Metzner *et al.*, 2004). In this study, a dramatic decrease was observed in the expression of the *gabD* and *gabP* genes, in a

strain (HS1024) carrying a polar insertion in the *csiD* gene (Figure 9), however it is not clear whether the disruption in *csiD* has a polar effect on the transcription of the *gabDTP* operon, or if the disruption of the function of CsiD protein has a negative effect on the transcription of the *gab* operon (Metzner *et al.*, 2004; this study). This later hypothesis is supported by northern analyses of *gabD* expression in strain HS1024 (*csiD-lacZ*) showing a decrease in intensity of the shorter transcript, which likely originates from the promoter within *ygaF* (Figure 9). To resolve this, a non-polar deletion of the chromosomal *csiD* should be created and studied. Additionally, the effect of over-expression of CsiD from a plasmid could be examined to determine if the presence of the CsiD protein in *trans* regulates expression of the *gabDTP* operon.

5.4 Utilization of GABA as a Sole Nitrogen Source in *E. coli*

RpoS-negative mutants grow twice as fast as wildtype cells on GABA as the sole source of nitrogen in minimal media, although it has been established that the *gabDTP* operon is highly *rpoS*-dependent (this study). The increased growth rate occurs even in early exponential phase, at a point where *rpoS* expression is expected to be low (Lange and Hengge-Aronis, 1994). The elevated growth of *rpoS*$^-$ mutants on GABA as the sole nitrogen source is also dependent on the *gabDT* genes, because the *gabD-lacZ* and *gabDT-lacZ* mutations in HS1057p and HS1037p (respectively) prevent growth of these strains on GABA as the sole nitrogen source (Table 6). Mutations in *ygaE* (*gabC*) have been shown to allow for increased growth on GABA as a sole nitrogen source (Schneider *et al.*, 2002). However, the increased growth rate of *rpoS*$^-$ mutants is independent of

ygaE, because double mutants grow faster than either of the single mutants (Table 3). There are several hypotheses that could account for increased growth on GABA as a sole nitrogen source. The first is the possibility that the *rpoS*⁻ mutants have elevated transport ability of GABA. However, this is unlikely because the *gabP-lacZ* mutants (HS1010p and HS1039p) show the same phenotype as the wildtype *rpoS*⁻ parental strain GC122 (Figure 13, Table 6).

A second hypothesis is that there is enhanced degradation of GABA into glutamate in *rpoS*⁻ mutants. Results from this study contradict this hypothesis and make it unlikely. The enhanced utilization of GABA in *rpoS*⁻ mutants requires expression of the *gabDT* genes as discussed above (Table 6), but expression of the operon is undetectable by northern analysis and β-galactosidase assay of *gab*-operon *lacZ*-fusion strains in *rpoS*⁻ mutants (Figure 6, Figure 9). These results may not provide a complete explanation, because they were obtained using cultures growing in rich media, while the enhanced growth of *rpoS*⁻ mutants on GABA is observed on minimal media with GABA as the sole nitrogen source. The *gab* operon has a promoter that is Ntr regulated and thus should be expressed as a result of growth on GABA, a poor nitrogen source (Schneider *et al.*, 2002). However, this promoter is σ^{70}-dependent and is not recognized by RpoS (Schneider *et al.*, 2002).

A third hypothesis to explain the elevated growth of *rpoS*⁻ mutants is that these strains have an enhanced ability to utilize glutamate as a nitrogen source. This hypothesis is supported by the fact that wildtype strains and *rpoS*⁻ mutants show the same growth

phenotype on glutamate as on GABA, with the exception that the *gabDT* genes were not required for growth on glutamate (Table 6). Although this is a qualitative observation, it is a striking result. The ability to utilize glutamate, and other nitrogen sources that produce glutamate, is limited by the ability of *E. coli* to obtain ammonia for the synthesis of glutamine (Reitzer, 1996b). Because the nitrogen of GABA is transferred to glutamate by GSST (GabT) (Figure 1), the ability of *E. coli* to utilize the glutamate produced may be the limiting factor in growth on GABA. Ammonia could be produced from glutamate by the glutamate dehydrogenase enzyme (encoded by *gdhA*), but its expression is inhibited by glutamate (Reitzer, 1996a). Ammonia could also be produced by degradation of glutamate into aspartate (by Aspartate aminotransferase), followed by degradation of aspartate to fumarate and ammonia (by Aspartase) (McFall and Newman, 1996). These enzymes are vital for utilization of glutamate as a carbon source, but their role in nitrogen metabolism is unknown (Marcus and Halpern, 1969). Aspartase is repressed by growth on glucose by cAMP-CRP, and is regulated weakly by FNR in response to oxygen levels (Golby *et al.*, 1998). If expression of the pathway for glutamate degradation is negatively *rpoS*-dependent, then *rpoS*$^-$ cultures may be able to derive more ammonia from glutamate, leading to increased growth on glutamate as a sole nitrogen source. Coincidentally, the gene encoding Aspartate aminotransferase, *aspC*, is located directly downstream of the *ompF* gene (Koga-Ban *et al.*, 1983). Expression of *ompF* (a relatively well characterized gene) is 19-fold higher in *rpoS*-negative mutants during nitrogen limitation relative to wildtype strains (Liu and Ferenci, 2001). Interestingly, there is a

previous link between intracellular glutamate levels and RpoS activity. High glutamate levels stimulate the activity of σ^S-bound RNA Polymerase (Ding *et al.*, 1995), and the intracellular levels of glutamate are lower in *rpoS*$^-$ mutants (Tweeddale *et al.*, 1998). Dover and Halpern (1972) reported that isolation of GABA$^+$ utilizing mutants in an Aspartase$^-$, glutamate-nonutilizing *E. coli* strain, recovered Aspartase activity and the ability to grow with glutamate as a carbon source. However, no derect evidence was presented to support these statements (Dover and Halpern, 1972). Clearly, the ability to utilize GABA as a sole nitrogen source, and the ability of *E. coli* to utilize glutamate are closely connected.

A fourth hypothesis is that GABA may be toxic to *E. coli*, and GABA is less toxic to *rpoS*$^-$ mutants. Plates supplemented with ammonia in addition to GABA or glutamate grow as well as plates supplemented with only ammonia (Table 6), so the levels of GABA and glutamate tested are unlikely to be toxic to the cells.

5.5 Isolation of GABA$^+$ Mutants That can Utilize GABA as a Sole Carbon and Nitrogen Source and the Involvement of Other Genes

Is there an alternate pathway that allows the cell to utilize GABA for growth? Schneider *et al.* (2002) found that when cells are grown on media with putrescine as the sole source of nitrogen, a GABA Transaminase and a Succinic Semialdehyde Dehydrogenase are expressed that are independent of the *gabD* and *gabT* genes. When *E. coli* cultures are grown in high numbers on plates with GABA as the sole source of carbon and/or nitrogen spontaneous mutants arise which are capable of increased growth

on GABA (Table 4). These mutants cannot be isolated in an *rpoS*$^-$ background (Table 4), however they can be isolated in a *gabD*$^-$ background (S. Yousuf, M.Sc. thesis). These two observations suggest there is a second pathway for utilization of GABA, and this second pathway may also require RpoS for expression. The *sad* gene in *E. coli* encodes a second Succinic Semialdehyde Dehydrogenase (using NAD as a cofactor) (Donnelly and Cooper, 1981; Marek and Henson, 1988). However, little is known about the regulation or the metabolic role of this gene. The *goaG* gene encodes a putative GABA or ornithine Transaminase (Jovanovic and Model, 1997), but likewise, little is known about the regulation of the gene. Other enzymes may exist in the cell that are capable of degrading GABA that have yet to be discovered.

The nature of the isolated GABA$^+$ utilizing mutants is unclear (Table 4). Some mutants may have increased levels of GSST and SSDH from *gabT* and *gabD*, respectively, similar to the GABA utilizing mutants isolated by Dover and Halpern (1972). Due to these mutants arising spontaneously during growth of cultures on GABA as a sole nitrogen or carbon source, previous growth measurements of cultures on GABA may have some error due to the potential presence of GABA utilizing mutants.

5.6 Physiological Relevance of *gabDTP* Operon Expression in Stationary Phase

To date, the *gabDTP* operon has been shown to be upregulated in response to general stress via σ^S (Schellhorn *et al.*, 1998; Metzner *et al.*, 2004), carbon limitation (Zaboura and Halpern, 1978; Metzner *et al.*, 2004), cell-to-cell signaling (Baca-

DeLancey, *et al.*, 1999), alkaline pH (Stancik *et al.*, 2002) and nitrogen limitation (Schneider *et al.*, 2002). Given the large number of mechanisms identified that have a role in the regulation of the *gabDTP* operon, it is likely that the enzymatic breakdown of GABA has multiple functions in the cell, other than as a nitrogen source.

Glutamate Decarboxylases, encoded by the *gadA* and *gadB* genes in *E. coli*, are *rpoS*-dependent and produce GABA as a product of the decarboxylation of glutamate (Ma *et al.*, 2002). Expression and activity of these enzymes occurs primarily under acidic pH (Castanie-Cornet *et al.*, 1999; De Baise *et al.*, 1999; Capitani *et al.*, 2003), and is vital to survival of cells during stress due to extremely low pH (Castanie-Cornet *et al.*, 1999). The glutamate-GABA antiporter, encoded by the *gadC* gene, may export the GABA produced by this reaction out of the cell in exchange for glutamate (De Baise *et al.*, 1999). *gadC* is co-expressed with *gadB,* so that the GadC antiporter is present at the same time as Glutamate Decarboxylase (De Baise *et al.*, 1999). If no glutamate is present outside of the cell, the degradation of GABA by the products of the *gabDT* genes may serve as an alternative to export (Metzner *et al.*, 2004). So that this potential nitrogen source is not wasted in the low nutrient conditions of stationary phase, the *gab* operon may be up-regulated by RpoS to internalize GABA and potentially metabolize it as a nitrogen source.

Metzner *et al.*, (2004) suggested that a role for the *gab* operon may be to synthesize glutamate, and maintain high intracellular glutamate levels in stationary phase. High glutamate levels are important in stimulating RpoS activity in stationary phase

(Ding *et al.*, 1995), and in the absence of glutamate the activity of σ^S-containing RNA polymerase is low (Tweeddale *et al.*, 1998). By up-regulating pathways involved in the production of glutamate, including metabolism of GABA into glutamate by GabT, RpoS may stimulate its own activity (Metzner *et al.*, 2004).

5.7 Conclusions

The results from this study clearly demonstrate that the *gabDTP* operon is highly *rpoS*-dependent. Utilizing Northern analysis of wildtype *E. coli* strains without insertions in the *gabDTP* operon, expression of the *gabDTP* operon was found to be highly RpoS- and stationary phase-dependent. The activities of the corresponding enzymes, GabD (SSDH) and GabT (GSST), were 20 fold higher in stationary phase cultures of wildtype cells compared to isogenic *rpoS*-mutant strains. However, growth on GABA as a sole nitrogen source was better in *rpoS*⁻ mutants relative to wildtype strains. Similar growth patterns were observed when the strains were placed on L-glutamate as the nitrogen source, suggesting that the limitation for growth with GABA as the nitrogen source may be due to an inability to utilize glutamate efficiently. The expression of the *gab* operon was found to be independent of the negative regulator YgaE during entry into stationary phase in rich media. Although the *csiD* and *ygaF* genes are in close proximity to the *gabDTP* operon and are both highly *rpoS*-dependent, the *gabDTP* operon is transcribed independently from these genes. These results suggest the metabolism of GABA plays a role in stationary phase stress resistance; however, the role and relative importance of the *gabDTP* operon remains unknown.

Chapter 6 References

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Appendix A: Detailed Protocols

Media Preparation and Growth Conditions

For rich media experiments, all strains were grown in 1X Luria Bertani (LB) media obtained from GIBCO BRL or Invitrogen. The LB broth is created by adding 25 g powder (10g Peptone 140, 5g yeast extract, 10g NaCl)/L ddH_2O. The broth is sterilized by autoclaving at 121°C, 17psi for small quantities of media and 127°C, 17psi for large quantities of media. Unless otherwise indicated cultures were grown at 37°C in Erlenmeyer flasks at a 1:5 culture to flask ratio and 200 rpm agitation to ensure optimal growth temperature and aeration. All liquid LB cultures are inoculated from single isolated-colonies on LB plates. LB plates were prepared by mixing equal parts pre-prepared sterile 2X LB and molten 2X Agar. (1x Agar is 15 g/L of granulated agar from BBL). After growth, plates are stored at 4°C for up to 2 weeks. For long-term storage, frozen permanents are made by resuspending the colonies on an LB plate in 1X LB media with 10 % glycerol and storing the culture in 1.5 ml screw-capped tubes at –80°C. Where indicated the media is supplemented with antibiotics to select for the appropriate resistant strains at the following concentrations: Ampicillin 100 μg/ml, Chloramphenicol 25 μg/ml, Kanamycin 50 μg/ml.

β-Galactosidase assay

The β-Galactosidase assay allows quantification of expression levels from strains carrying *lacZ* fusions to a gene of interest. The assay is conducted as reported in Miller (1972). The product of the *lacZ* gene is the enzyme β-galactosidase, whose natural substrate is lactose. An artificial chromogenic substrate, o-nitrophenol-β-D-galactopyranoside (ONPG), can be cleaved by β-galactosidase to release a yellow coloured product, o-nitrophenol, which can be quantified spectrophometrically at 420 nm. The assay is conducted as an end-point assay, where the reaction is continued until the yellow enzymatic product is visible to the eye, and the reaction is halted by the addition of Na_2CO_3. The bacterial culture being assayed must be maintained in early exponential phase for at least 8 generations to ensure the turnover of any residual β-galactosidase produced in the stationary phase overnight culture. This ensures an accurate baseline β-galactosidase activity in exponential phase.

Protocol:

- Isolate a single colony and transfer it into 10 ml of LB media in a 50 ml flask at 37°C and 200 rpm
- Grow the culture overnight
- Perform a 1:1000 subculture by adding 10 µl of the overnight culture into 50 ml of LB media
- Grow the culture at 37°C and 200 rpm until the culture has reached an $O.D._{600}$ of 0.2-0.3
- Subculture 10 µl into 10 ml of fresh LB media in a 50 ml flask and continue to grow as before until the culture has reached an $O.D._{600}$ of 0.2-0.3. This second subculture

step ensures that the culture is maintained in early exponential phase for at least 8 generations prior to the start of the assay.

- Subculture 50 µl into 50 ml of fresh LB media in a 250 ml flask and continue to grow the culture as before. After 1 hour has elapsed, sample 1ml of culture into a borosilicate tube on ice containing 10 µl of 15 mg/ml Chloramphenicol (a final concentration of 150 µg/ml) to halt growth and protein synthesis.
- Continue to sample 1ml of culture as before every 30 min until the culture is in stationary phase, approximately 8 hours after the start.
- Measure the $O.D._{600}$ of each of the isolated samples. Cultures that have grown past an $O.D._{600}$ of 0.5 are diluted 1 in 10 with water to achieve an accurate reading.
- Culture tubes are covered with parafilm and stored in ice at 4oC in the refrigerator overnight.
- The next day the β-galactosidase assay is set up for each tube:
  1. Add 0.95 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) to duplicate borosilicate tubes for each culture time-point being assayed.
  2. Re-suspend the culture by vortexing the tubes and add 50 µl of bacterial culture into each of the two tubes containing the Z-buffer.
  3. Add 25 µl of 0.1 % SDS to the reaction tube
  4. Add 50 µl of Chloroform to each reaction tube
  5. Vortex the tubes for 10s to break open the cells
  6. Prepare a control tube with 50 µl of LB media in place of the culture.
  7. To start the reaction add 200 µl of 4 mM ONPG and START timing
  8. Cover the tubes with foil and monitor the reaction until a yellow colour is present
  9. STOP the reaction by adding 0.5 ml of 1M Na_2CO_3 to the tube. Record the time the reaction was stopped at.
  10. Measure the absorbance spectrophotometrically at 420 nm in a plastic cuvette with a 1 cm pathlength.

- For the early time-points the cultures will have a low concentration of cells. If no yellow-coloured end product is seen in the assay using 50 µl of culture, the volume of culture can be increased to 250 µl or to a maximum of 500 µl. The amount of Z-buffer in the assay must be adjusted so the total of Z-buffer + culture is always 1 ml. Separate controls need to be prepared for these reactions with a corresponding amount of LB buffer in the place of the culture.
- The specific activity is calculated with the formula:

$$\textbf{S.A. [Miller Units]} = \frac{1000 \times (O.D._{420})}{(O.D._{600}) \times (\text{culture volume (ml)}) \times (\text{time of reaction (min.)})}$$

pH-dependant Expression Assay

The pH of a culture grown in LB can change over the course of the growth curve. The media, which starts out at pH 7 becomes alkaline as the cell density of the culture increases (reference Slonzewski paper). This change in pH can affect the expression of genes, so to control for pH dependent effects on expression a β-galactosidase assay can be conducted using cultures that are grown in LB media buffered to pH 7. The β-galactosidase assay is conducted identically to that shown earlier, with a few changes:

Protocol:

- For each culture to be assayed (in triplicate) two types of media are used, unbuffered LB and LB buffered to pH 7.0
- To prepare the unbuffered LB, equal volumes of 2X LB and 1X ddH_2O are mixed together. Both are sterilized by autoclaving before mixing.
- To prepare buffered LB (per L), 500 ml of 2X LB (autoclaved to sterilize) and 300 ml is mixed with 100 ml of 1M 3-(N-Morpholino)propanesulfonic acid (MOPS) and pH is adjusted to 7.0 with 3M NaOH. After pH adjustment sterile ddH2O is added to a final volume of 1000 ml and the buffered LB is filter sterilized using a 60 ml syringe and a 0.22 μm Millipore filter.
- Triplicate cultures are grown overnight in 10 ml of LB media in 50 ml flasks, at 37°C and shaking at 200 rpm.
- Perform a 1:1000 subculture by adding 10 μl of the overnight culture into 50 ml of LB media
- Grow the culture at 37°C and 200 rpm until the culture has reached an $O.D._{600}$ of 0.2-0.3
- Subculture 10 μl into 10 ml of fresh LB media in a 50 ml flask and continue to grow as before until the culture has reached an $O.D._{600}$ of 0.2-0.3. This second subculture

step ensures that the culture is maintained in early exponential phase for at least 8 generations prior to the start of the assay.

- Subculture 50 µl into 50 ml of LB media and 50 ml MOPS-buffered LB media for each culture in a 250 ml flask and continue to grow the culture as before.
- Grow the culture until the O.D.600 reaches 0.3 and sample 1 ml of culture into 2 borosilicate tubes for each culture. Add 10 µl of 15 mg/ml Chloramphenicol and store the samples on ice.
- Continue to grow the culture to an O.D.600 of 1.6 and sample 1ml of culture into 2 borosilicate tubes for each culture. Add 10 µl of 15 mg/ml Chloramphenicol and store the samples on ice. Cover the isolated samples with parafilm and store the samples on ice at 4°C overnight.
- Continue to grow the culture overnight (approximately 16 h) and take a 1 ml sample of culture into 2 borosilicate tubes for each culture. Add 10 µl of 15 mg/ml Chloramphenicol and store the samples on ice.
- Pour the remaining culture into a sterile 15 ml polypropylene tube (Sarstedt) and measure the final pH of the cultures.
- Measure the $O.D._{600}$ of each of the isolated samples. Cultures that have grown past an $O.D._{600}$ of 0.5 are diluted 1 in 10 with water to achieve an accurate reading.
- Perform the β-galactosidase assay as described previously.

GSST and SSDH Enzyme Assays

The enzyme activity of Glutamate Succinic Semialdehyde Transaminase (GSST, GABA Transaminase), the product of the *gabT* gene and Succinic Semialdehyde Dehydrogenase (SSDH), the product of the *gabD* gene are assayed as described previously (Schneider *et al.,* 2002, Metzer and Halpern, 1979). Enzyme activity in both assays is determined by measuring either the reduction of NADP or the oxidation of NADH spectrophotometrically at 340 nm. Both NADH and NADPH have a Molar Extinction coefficient of 6220 $M^{-1}cm^{-1}$. The assay is conducted as a continuous assay, where the reaction is measured in real time for a specified length of time and the ΔA340/min is calculated. Due to a lack of a spectrophotometer that is temperature regulated and adjustable, both of the assays were conducted at 30°C.

Protocol:

A. Isolation of Crude Cellular Protein Extracts

- Isolate a single colony and transfer it into 10 ml of LB media in a 50 ml flask at 37°C with shaking at 200 rpm
- Grow the culture overnight
- Perform a 1:1000 subculture by adding 10 µl of the overnight culture into 50 ml of LB media
- Grow the culture at 37°C with 200 rpm agitation until the culture has reached an $O.D._{600}$ of 0.2-0.3
- Subculture 10 µl into 10 ml of fresh LB media in a 50 ml flask and continue to grow as before until the culture has reached an $O.D._{600}$ of 0.2-0.3. This second subculture step ensures that the culture is maintained in early exponential phase for at least 8

generations prior to the start of the assay.

- Subculture 150 µl into 150 ml of fresh LB media in a 1000 ml flask and continue to grow the culture as before. Once the culture has reached an $O.D._{600}$ of 0.3, pour 50 ml of culture into a 50 ml Falcon polypropylene tube on ice and add 500 µL of 15 mg/ml Chloramphenicol (final concentration of 150 µg/ml) to stop growth and protein synthesis. Incubate the culture on ice for 30 min. and centrifuge the cells at 3000 x g for 15 min. at 4°C. Resuspend the cell pellets in2 ml of 100 mM KPO4 buffer, pH 7.5, and re-centrifuge as before. Store the cell pellets at –80°C until ready to use.
- Continue to grow the culture until it has reached an $O.D._{600}$ of 2.0, pour 15 ml of culture into a 15 ml polypropylene tube (Sarsedt) on ice and add 150 µL of 15 mg/ml Chloramphenicol (final concentration of 150 µg/ml) to stop growth and protein synthesis. Prepare the cells identically to the exponential phase sample above and store the cell pellets at –80°C until ready to use.
- Remove the cell pellets from the freezer and resuspend them in 1 ml of resuspension buffer (100 mM KPO4 buffer, pH 7.5, 9% glycerol, 1 mM PMSF, 1 mM DTT). Store the cell pellets on ice and make sure they stay cold throughout the entire process.
- Lyse the cells using 3 bursts of sonication for 5s each for the exponential phase samples and 4 bursts of sonication for 5s each for the stationary phase samples. The samples are stored on ice for at least 2 minutes between each round of sonication. At the end of the sonication the samples should be clear and have a green coloured tint to them. Avoid introducing air bubbles into the liquid while sonicating.
- The crude cell extracts are added into a 13 ml centrifuge tube and 4 ml of resuspension buffer is added to each to make a final volume of 5 ml. The samples are spun at 35, 000 rpm in a pre-chilled Type 50Ti rotor (Beckman) for 90 minutes at 4°C in a L8-70M Ultracentrifuge (Beckman) to remove cell debris and membrane components which contain NADH oxidizing activity.
- Crude soluble protein extracts are stored at 4°C and are assayed for enzyme activity within two days.

B. Determination of Succinic Semialdehyde Dehydrogenase (SSDH) Activity

The SSDH enzyme is the product of the *gabD* gene and the determination of SSDH activity is based on the reduction of NADP as succinic semialdehyde is converted to succinate by the enzyme. Background activity of NADP reduction can be measured before addition of the substrate, succinic semialdehyde. The reactions are assayed in the UVICON spectrophotometer using the autorate assay. The maximum adsorption of light by NADPH occurs at 340nm. Readings are taken every 0.3 min for 10 readings in total. The slope of a regression line fitted to the reading points is calculated by the spectrophotometer program and is given as ΔA_{340}/min. The line should be linear to be meaningful. The reactions are performed in duplicate.

Protocol:

- Premix the reaction buffer by adding (per ml final reaction volume):
  - 100 µl of 1 M KPO4 buffer pH 7.8
  - 885 – X µl of ddH20 (where X is the volume of protein extract being assayed)
- Preheat the reaction buffer to 30°C in a heating block
- Add the reaction buffer to a clean quartz cuvette
- Add 10 µl of 28 mM NADP to the reaction mixture in the cuvette (the NADP stock is made in 0.1 M HEPES buffer pH 7.0 and is freshly prepared just before use)
- Add X µl of the crude protein extract and mix the reaction by placing a small square of parafilm over the mouth of the cuvette and mix by inversion.
- Place the cuvettes in the spectrophotometer and measure the level of background NADP reducing activity (the activity should be very small, near zero)
- Remove the cuvettes from the spectrophotometer and add 5 ul of 120 mM Succinic

semialdehyde as the substrate. Quickly mix the reaction as before and place it back into the spectrophotometer. Measure the level of specific SSDH activity.

- Subtract the background ΔA_{340}/min from the specific ΔA_{340}/min.
- The specific activity is calculated with the formula:

$$S.A. = \frac{1000 \times (\Delta A_{340}/min)}{(6.22\ nmol/ml) \times (protein\ extract\ volume\ (ml)) \times (protein\ concentration\ (mg/ml))}$$

C. Determination of Glutamate Succinic Semialdehyde Transaminase (GSST) Activity

The GSST enzyme is the product of the *gabT* gene and converts GABA + α-ketoglutarate into glutamate and succinic semialdehyde. The determination of GSST activity is more complex than the SSDH assay. The activity cannot be determined directly because none of the reactants or products can be assayed directly. Thus, one of the reactants of the reaction, α-ketoglutarate, is coupled to a second reaction based on the oxidation of NADH as α-ketoglutarate is converted to glutamate by the enzyme Glutamate Dehydrogenase (GDH). The reactants for the coupled reaction are supplied in excess so the rate of reaction is dependent on the production of α-ketoglutarate. Background activity of NADH oxidation can be measured in a control reaction without the addition of the substrate, succinic semialdehyde, run in parallel. The reactions are assayed in the UVICON spectrophotometer using the autorate assay as before in the SSDH assay. Readings are taken every 0.3 min for 10 readings in total at 340nm. The

start of the assay should be delayed for 3 min before taking readings to allow the reaction rate to become linear. The slope of a regression line fitted to the reading points is calculated by the spectrophotometer and is given as ΔA_{340}/min. The activity of each sample is determined in duplicate.

- Premix the reaction buffer by adding (per ml final reaction volume):
  - 100 µl of 1 M HEPES buffer pH 7.0
  - 685 – X µl of ddH20 (where X is the volume of protein extract being assayed)
  - 100 µl of 400 mM L-glutamate
  - 100 µl of 400 mM NH_4Cl
  - 1.6 U L-Glutamic Dehydrogenase (GDH) (Sigma Aldrich)
- Preheat the reaction buffer to 30°C in a heating block.
- Add the reaction buffer to a clean quartz cuvette
- Add 10 µl of 15 mM NADH to the reaction mixture in the cuvette (the NADH stock is made in 0.1 M HEPES buffer pH 7.0 and is freshly prepared just before use)
- Add X µl of the crude protein extract and mix the reaction by placing a small square of parafilm over the mouth of the cuvette and mix by inversion.
- Place the cuvettes in the spectrophotometer and measure the level of background NADP reducing activity (the activity should be very small, near zero)
- Remove the cuvettes from the spectrophotometer and add 5 µl of 120 mM Succinic semialdehyde as the substrate. Quickly mix the reaction as before and place it back into the spectrophotometer. Measure the level of specific SSDH activity.
- Subtract the background ΔA_{340}/min from the specific ΔA_{340}/min.
- The specific activity is calculated with the formula:

$$\text{S.A.} = \frac{1000 \times (\Delta A_{340}/\text{min})}{(6.22\ \text{nmol/ml}) \times (\text{protein extract volume (ml)}) \times (\text{protein concentration (mg/ml)})}$$

Growth Rate Determination

When the stains GC4468 (wildtype) and GC122 (*rpoS*$^-$) are grown on M9 plates containing GABA as the sole source of nitrogen, the *rpoS*$^-$ mutants appear to grow much better, forming mucoid white colonies. To determine if the increased growth is due to a faster growth rate on GABA as the sole source of nitrogen, the growth of a liquid culture was monitored. Likewise, the following procedure (with small modifications) can be used to measure the growth rate of any number of strains.

Protocol:

- Inoculate single isolated colonies of *E. coli* into 5 ml of M9 media supplemented with 0.5 % Glucose and 0.05% NH_4Cl. Grow the culture overnight at 37°C with shaking at 200 rpm.
- Dilute the culture 1:1000 into 10 ml of M9 media supplemented with various Carbon and nitrogen sources
- Allow the culture to grow at 37°C, 200 rpm for 2 hours.
- At regular intervals, sample 1 ml of culture into a borosilicate tube, on ice, and add 150 μg/ml chloramphenicol to halt growth of the culture. At least 3 samples should be taken to ensure the growth rate is constant.
- Measure the $O.D._{600}$ of the samples. Plot the $O.D._{600}$ values vs. time using Microsoft's Excel spreadsheet program. The program can draw an exponential line of best fit through the data points with the general equation:

  $y = y_o e^{mx}$ which is equivalent to $N = N_o e^{kt}$, where:

  N = the O.D.600 value at time t

  N_o = the starting O.D.600 value

  t = the time in min

  k = the growth rate constant

- A useful measure of the growth rate of a bacterial culture is its generation time (G) which is the time required for the bacterial cells to double in number. The generation time can be determined with the equation:

$$G = \frac{\ln 2}{k}$$

Isolation of GABA$^+$ Utilizing Mutants

When wildtype *E. coli* K-12, such as the strain GC4468, is grown on plates containing GABA as the sole source of nitrogen, the growth rate is extremely slow. When the same strain is grown on GABA as the sole Carbon source there is no apparent growth. However, growth of a lawn of *E. coli* on both of these media results in the appearance of colonies with increased ability to utilize GABA as a carbon source, nitrogen source or both. These are spontaneous mutants, which appear because they have a growth advantage when GABA is the sole carbon or nitrogen source. Mutants are isolated from actively growing cultures in LB media.

Protocol:

- Isolate a single colony and transfer it into 10 ml of LB media in a 50 ml flask at 37°C and 200 rpm
- Grow the culture overnight
- Perform a 1:1000 subculture by adding 50 µl of the overnight culture into 50 ml of LB media
- Grow the culture at 37°C and 200 rpm until the culture has reached an $O.D._{600}$ of 0.8
- Chill the cultures on ice for 30 min. and pour 15 ml of culture into a 15 ml polypropylene tube. Centrifuge the culture at 5000g for 15 min. at 4°C. Discard the supernatant.
- Resuspend the cells with 10 ml of 1 X M9 salts containing no nitrogen or carbon source. Centrifuge again as before and repeat the wash/centrifugation a second time. Resuspend the pellet in 1 X M9 salts containing no nitrogen or carbon source. The pellet should be resuspended so that there is a final concentration of approximately 1 x 10^8 cells in 750 µl of 1 X M9. Extra volume should be present so that the actual

concentration of the cells can be determined. The concentration of cells is determined using a 1 in 10 dilution of the cell suspension into ddH20 and measurement of the $O.D._{600}$. An $O.D._{600}$ reading of 1 corresponds to $1X10^9$ cells per ml.

- The following plates are prepared to select for growth of $GABA^+$ suppressor mutants: M9 media supplemented with 1% GABA as the sole carbon and nitrogen source are used to select for $GABA^+$ C and N utilization mutants, M9 media supplemented with 1% GABA and 0.05% NH4 to isolate $GABA^+$ C utilization mutants, and M9 media supplemented with 0.5% Glucose or 1% Glycerol and 1% GABA to isolate $GABA^+$ N utilization mutants.
- To each plate, in triplicate, 750 µl (corresponding to approximately 1 X 10^8 cells) is added to the surface of the plate and is allowed to dry into the plate in the Class II hood. The plates should be relatively dry so as to allow adsorption of the liquid into the plate, without the creation of localized pools of liquid on the plate. This ensures a smooth even lawn of growth, and well-isolated, evenly distributed colonies of $GABA^+$ utilization mutants, ensuring an accurate count.
- The plates are incubated at 37°C for two to three days to allow the formation and growth of mutants. The number of mutants per plate are counted and expressed per 10^8 cells

Replica Plating

Replica plating out cells to determine their phenotype on various carbon and nitrogen sources is a quick and easy way to determine the phenotype of a strain and do a qualitative test of β-Galactosidase activity on strains containing *lacZ* fusions. Stains can be patched into media in a microtitre dish and grown overnight. Using an appropriate stamping tool, the cultures can be patched onto solid media plates. The resulting colonies are uniformly spaced and are fairly round in shape. Additionally, the same amount of culture is spotted onto each plate, so the plates are easy to compare and cross contamination of media is easy to reduce. At least 3 patches of each strain should be placed into the microtitre dish to check for contaminants and observe any colony-to-colony variation in growth or expression.

Protocol:

- Streak out each of the strains being tested on appropriate media and incubate the plates overnight at 37°C.
- Fill each well of a 96-well microtitre dish with 200 µl of media (LB is sufficient for overnight growth of cultures).
- Cover the microtitre dish with a piece of parafilm; ensure the entire dish and all the wells are covered. Place the dish under UV light for 2 min to ensure the surface of the Parafilm is sterile.
- Isolate a single colony with a sterile toothpick and transfer it into one of the wells by poking the toothpick through the Parafilm.
- Repeat for the rest of the wells, ensuring that each strain has at least 3 isolated colonies inoculated into the dish.

- Grow the cultures in the microtitre dish overnight
- If the cultures are to be replica-plated onto LB plates, then they can be patched directly from the overnight cultures in the microtitre dish.
- If the cultures are to be plated on minimal media plates, then the cultures should be diluted to reduce media carry-over. This can be done in two ways: i) 5-10 μl of each culture can be transferred with a pipetman from the overnight microtitre dish into the wells of a new microtitre dish containing M9 salts (without a carbon or nitrogen source); ii) a small amount of the cultures from the overnight microtitre dish can be transferred using the stamping tool, into a new dish with M9 media as above. The first method results in a little more media carry-over, but also transfers more cells in general and is more reproducible. The second method results in less media carry-over but may result in cultures becoming too dilute, so patches on the replica-plates will not be consistent.
- To transfer the culture onto the plates, a replica-plating tool is used, which has 48 evenly spaced metal prongs that fit into the wells of the microtitre dish. The ends of the prongs are sterilized by soaking them in a dish containing 95% ethanol. The ethanol is removed from the prongs by passing them quickly through a Bunsen burner flame and allowing the ethanol to burn off. Care should be taken not to heat the tips too much or they will kill the cultures when inserted into the microtitre dish.
- The sterile replica-plating tool is inserted into the wells of the microtitre dish to contact the culture, removed, and pressed lightly and evenly on the surface of a plate to transfer a small amount of culture from the tips of the prongs to the surface of the plate.
- Plates are incubated for an appropriate amount of time and temperature (depending on the experiment and media used) and examined for growth. X-GAL may be included in the plate to a final concentration of 50 μg/ml to examine β-Galactosidase activity in the strains.

PCR

Polymerase Chain Reaction (PCR) is a key technique in the creation and analysis of deletions and the synthesis of radiolabelled DNA probes for Northern analysis discussed below. PCR is characterized by a sequential, exponentially increasing synthesis of a target DNA by a thermostable DNA polymerase (most frequently Taq polymerase). The specific amplicon that is created depends on the primers that are selected and optimized reaction conditions. For a good review of the PCR theory, useful techniques and considerations for primer design, see (Sambrook and Russell, 2001). Briefly, when designing primers, the two primers used should have similar %GC contents and differ in Tm by less than 5 degrees. There should be a low degree of complementarity between the two primers and low self-complementarity. I like to use the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to help select primers. All reagents should be stored at –20°C and thawed on ice just before use. Extra care should be taken not to contaminate any of the reagents. Be sure to include at least one negative control, containing no template, to ensure that any product formed is specific.

General Protocol

- Remove reagents from the freezer and thaw them on ice. Assemble the reaction according to the following table:

Reagent	Experimental Reaction		"No template" Negative Control	
	Volume (µl)	Final Concentration	Volume (µl)	Final Concentration
10 X PCR buffer	5	1X*	5	1X*
50 mM $MgSO_4$	2	2 mM	2	2 mM
10 mM dNTP	1	0.2 mM	1	0.2 mM
ddH_2O	33.5	-	35.5	-
25 µM Primer F	2	1 µM	2	1 µM
25 µM Primer R	2	1 µM	2	1 µM
Template DNA	2	10 ng	-	-
Taq Polymerase (1U/µL)	2.5	2.5 U	2.5	2.5 U
Total	50	-	50	-

1X reaction buffer is: 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20

- It is important to keep the reactions on ice once *Taq* polymerase has been added to reduce the formation of primer-dimers. I wait until the thermal cycler has heated to 80°C before placing the tubes inside. This acts like a "hot start", which improves the fidelity of the reaction. The following can be used as a general PCR reaction:

Step	**Time (min:sec)**	**Temperature (°C)**	**Cycles**
Initial denaturation	2:00	94	1
Denature	0:30	94	25
Anneal primers	0:30	Tm-5 [a]	
Elongation	1:00/kb [b]	72	
Final Elongation	7:00	72	1

a. the optimal annealing temperature must be determined empirically, but 5°C below the Tm of the primers is usually a good starting point

b. the amount of time allotted to elongation depends on the length of the expected amplicon (PCR fragment). As a general rule 1 minute of elongation per 1000 bp is sufficient.

Gene Knockout Creation

A. Preparation of Electrocompetent Cells

When transforming linear PCR fragments, which subsequently must be recombined into the chromosome, it is vital to have a high transformation efficiency. This is also important when working with strains that are not optimized for transformation, such as MG1655. Transformation of *E. coli* by electroporation potentially has a higher transformation efficiency than any other method, and so it is the method of choice when creating deletion mutants using PCR fragments (Datsenko and Wanner, 2000). Electroporation uses high voltage, low current conditions to make the membrane of *E coli* permeable so that DNA molecules may enter the cells. The efficiency of electroporation for transformation is proportional to the length of DNA that must be taken up by the cells; smaller DNA fragments will yield a higher transformation efficiency. In order for electroporation to work properly, arcing of electricity through the sample must be avoided. To prevent this the cells must be suspended in a medium that has a low concentration of ions, such as 10% glycerol. The following protocol can be scaled down to create less competent cells if required. The cultures of cells containing the pKD46 plasmid must be grown at 30°C because the replication of the plasmid is temperature sensitive, and the plasmid will be lost at 37°C without selection.

Protocol:

- Streak out the MG1655-pKD46 strain onto LB plates supplemented with ampicillin to select for the plasmid and incubate at 30°C overnight. Pick a single colony and inoculate 10 ml of LB media supplemented with ampicillin. Cultures are grown

overnight in 50 ml flasks, at 30°C and shaking at 200 rpm.

- Perform a subculture by adding 1 ml of the overnight culture into 500 ml of LB media in a 2 L flask. Grow up the culture at 30°C until it has reached an $OD_{600} = 0.1$.
- Add 10 ml of 1 M L-arabinose solution for a final concentration of 20mM L-arabinose in the culture [Arabinose is used to induce expression of the λ RED recombinase genes located on the pKD46 plasmid]. Continue to grow the culture to an $OD_{600} = 0.6$
- Pour the culture into a 500 ml centrifuge bottle and chill on ice for 30 min. Swirl the culture frequently to help it cool.
- Centrifuge the culture in a Beckman J6-MI centrifuge for 15 min. at 4200 rpm, 4°C. Decant the supernatant and resuspend the cells in 500 ml of ice-cold sterile 10% glycerol (in ddH_2O).
- Centrifuge as above and decant the supernatant by aspiration. It is vital to aspirate the supernatant because the cell pellet will be very loose, and decanting will result in significant cell loss.
- Resuspend the cells in another 500 ml of ice-cold sterile 10% glycerol (in ddH_2O).
- Centrifuge as above and aspirate off the media, being careful not to disturb the cell pellet
- Resuspend the cells in a 50 ml of ice-cold sterile 10% glycerol (in ddH_2O) and transfer the cell suspension to a 50 ml Falcon tube. Centrifuge as above. Remove the supernatant by aspiration.
- Resuspend the culture in 1ml of ice-cold sterile 10% glycerol (in ddH_2O). Aliquot 100 µl of the cells to chilled microfuge tubes and flash freeze the cells in liquid nitrogen. Store the cells at -80°C until ready to use. Alternatively, the cells can be used right away for transformation without freezing.

B. PCR Amplification of the *cat*-Containing Cassette

The first step to knock out a gene from *E. coli* is to replace the gene with a cassette containing a selectable marker, such as chloramphenicol acetyl transferase, *cat*, which confers resistance to chloramphenicol upon the cell. The *cat* cassette is located on the plasmid pKD3 (Datsenko and Wanner, 2000). There are primer sites on either side of the cassette that may be used to create a PCR product that contains the cassette.

1) Design primers to amplify the antibiotic resistance cassette.

Primers used for creating knockouts are generally 56 bp long and contain two distinct regions. The 5' end of the primer consists of 36 bp that are homologous to the boundary of the gene being deleted. This sequence may be longer than 36 bp to create a higher efficiency of recombination but 36 bp is the minimal length of DNA that can be recombined into the chromosome by the λRED recombinase system. (Datsenko and Wanner, 2000) The 3' end of the primer contains a sequence that is homologous to the pKD3 plasmid to allow amplification of the cassette. The sequence of the forward primer that binds to the plasmid is: 5'-GTGTAGGCTGGAGCTGCTTC-3' and the sequence of the reverse primer is 5'-CATATGAATATCCTCCTTAG- 3' (Datsenko and Wanner, 2000). Examples of these primers can be found in Table 2.

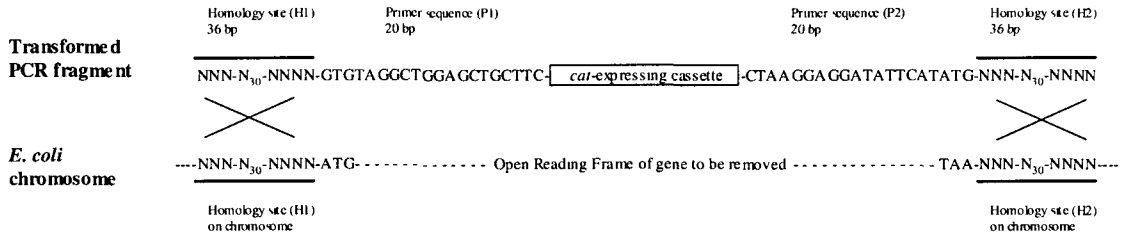


Figure A1: Recombination between the PCR fragment and the gene of interest to replace the open reading frame with the *cat* gene. Homologous recombination (indicated by crosses) occurs between the homologous regions synthesized on the ends of the PCR fragment and the corresponding sequence in the *E. coli* chromosome, which flanks the open reading frame of the gene of interest. This sequence varies depending on the gene being disrupted, and is indicated by a series of N (meaning any possible base) The representative start (ATG) and stop (TAA) codons of the open reading frame are shown.

2) PCR amplify the antibiotic resistance cassette as described above.

Purified pKD3 plasmid is used as template. The following two tables give the set up of the PCR reaction and cycling conditions. After the reaction is complete, run a sample of the PCR product on a 1% agarose gel to confirm that amplification has occurred. The resulting PCR product should be approximately 1000 bp long.

Reaction components for PCR amplification of *cat*-expressing cassette from pKD3

Reagent	Experimental Reaction		Negative Control	
	Volume (µl)	Final Concentration	Volume (µl)	Final Concentration
10 X PCR buffer	5	1X*	5	1X*
50 mM $MgSO_4$	2	2 mM	2	2 mM
10 mM dNTP	1	0.2 mM	1	0.2 mM
ddH_2O	34.5	-	35.5	-
25 µM Primer F	2	1 µM	2	1 µM
25 µM Primer R	2	1 µM	2	1 µM
10 ug/ml pKD3 plasmid DNA	1	10 ng	-	-
Taq Polymerase (1U/µL)	2.5	2.5 U	2.5	2.5 U
Total	50	-	50	-

1X reaction buffer is: 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20

Reaction conditions for PCR to create *cat*-expressing cassette from pKD3

Step	**Time (min:sec)**	**Temperature (°C)**	**Cycles**
Initial denaturation	2:00	94	1
Denature	0:30	94	25
Anneal primers	0:30	55	
Elongation	1:00	72	
Final Elongation	7:00	72	1

3) Purify the PCR product (optional).

The PCR product can be purified to remove any residual pKD3 plasmid, nucleotides and

primers/primer dimers. The PCR product can be purified by either gel extraction (QiaEX II Gel Extraction kit from Qiagen) or a simple PCR purification kit (PCR purification kit for Qiagen). The protocols supplied by the manufacturer should be followed as given. The gel extraction procedure is more time consuming but the resulting PCR fragment is of higher purity. The PCR purification kit will not remove any of the template pKD3 plasmid, but will remove the buffer, enzyme, nucleotides and some of the primers. In practice, I don't usually purify the reaction prior to transformation, because transformants carrying the pKD3 plasmid can be eliminated on the basis of phenotype (see below). If arcing is encountered during electroporation, due to the presence of salts from the PCR reaction, then the PCR reaction should be purified.

C. Isolation of Knockout Strains by Phenotype

The resulting *cat*-expressing cassette produced by PCR is transformed into the electrocompetent MG1655 pKD46 strain. Once inside the cells the PCR fragment can recombine into the chromosome at the sites of homology on the ends of the PCR products (see Figure A1; Datsenko and Wanner, 2000). Efficient recombination occurs in the presence of the λ RED recombinase components Gam, Bet, and Exo, which are produced from the pKD46 plasmid. Once the PCR product has recombined with the chromosome, the cat gene will be stably expressed and confer chloramphenicol resistance on the recombinants. Thus, the recombinants may be selected by growing the cells on plates with chloramphenicol and selecting Cm^R (chloramphenicol-resistant) colonies. At the

same time, the cells are grown at an elevated temperature, 37-42°C, to cure the temperature sensitive pKD46 plasmid from the strain.

Protocol:

- Use freshly prepared electrocompetent MG1655 pKD46 cells that have been induced with 20mM L-arabinose. Alternatively, previously prepared cells stored at -80°C may be used. Place the cells on ice and ensure they are kept cold.
- Chill some 2mm electroporation cuvettes on ice and place the PCR reaction on ice as well.
- Once the cells are thawed, use a pipetman (P200) to make sure the cells are thoroughly suspended by pipeting in-and-out repeatedly, and pipet 50 µl of the electrocompetent cells into the bottom of an electroporation cuvette. Return the cuvette to the ice.
- Add 2-4 µl of the PCR reaction into the cells in the cuvette and mix by pipeting in-and-out and gently shaking the tube side to side. Make sure the PCR reaction is thoroughly mixed into the cell suspension. Place the cuvette back on ice.
- Prepare the electroporation apparatus, the BioRad Gene Pulser II and the Pulse Controller Plus. The electroporation conditions are, 25 µF, 2.25 kV on the Gene pulser and X on the Pulse Controller Plus.
- Remove the cuvette from the ice and wipe the outside to ensure it is dry. Place the cuvette into the holder of the Gene Pulser so that the metal sides of the cuvette are in contact with the electrodes at the back of the holder. A pulse is delivered to the cell suspension by pressing both red buttons and releasing at the sound of the tone.
- Immediately add 1 ml of LB to the cell suspension in the cuvette and mix thoroughly by pipeting. Transfer the contents of the cuvette into a 1.5 ml microfuge tube.
- Resuspend the remaining 50 ml of electrocompetent cells in 1ml of LB as a negative control. Tape the tubes to the wheel in the 37°C incubator; rotate and incubate the cells for 1 hour to allow for recombination and expression of chloramphenicol resistance.

- Pellet the cells by centrifugation (maximum speed for 1 min in a desktop microfuge), remove the supernatant with a pipet and resuspend the cells in 100 µl of LB media.
- Place 50 µl of the cell suspension onto an LB plate supplemented with 25µg/ml chloramphenicol, and spread the liquid evenly on the surface of a plate using a sterile bent glass rod. Store the remaining 50 µl of cells in case no recombinants are isolated, to plate the next day.
- Incubate the plates at 37°C overnight. Check the plates the following day for chloramphenicol-resistant colonies.
- Re-streak several (5-10) colonies onto LB + 25µg/ml chloramphenicol plates and incubate at 37°C overnight. Re-streak each of the isolates a 3rd time onto the same media and incubate overnight at 37°C. The high temperature should cure the cells of pKD46 plasmids. Store the original plates at 4°C.
- To ensure there is no remaining pKD46 plasmid, OR pKD3 plasmid transformed from the PCR reaction, the cells can be patched onto LB + 25µg/ml chloramphenicol and onto LB + 25µg/ml chloramphenicol + 100µg/ml ampicillin plates. Any cells harbouring a plasmid will have ampicillin resistance and will grow on both media, while the desired recombinants cured of the pKD46 plasmid will only have chloramphenicol resistance. Patch single, isolated colonies with a sterile tooth pick onto both kinds of media, incubate overnight at 30°C, and check the plates the next day for bacterial growth. Re-streak any patches that are ampicillin sensitive onto fresh LB + 25 µg/ml chloramphenicol plates and incubate at 37°C overnight. Prepare frozen permanent stocks of each of the strains and confirm the gene replacement by PCR.

D. Confirmation of Gene Replacement by Colony PCR

PCR can be used to confirm the deletion of the gene and its replacement by the *cat-*

expressing cassette. In most cases this is sufficient to ensure that the gene of interest has in fact been replaced, based on the difference in size between the cassette and the original open reading frame (ORF). However, if the open reading frame and the cassette are the same size (1000 bp), then PCR alone will not be sufficient to ensure that the ORF has been replaced. In this case, chromosomal DNA isolated from the strain may be sequenced to ensure that the ORF has in fact been replaced. When designing primers for confirmation of the deletion, I usually select primers that will bind 250 bp upstream and downstream of the gene respectably, so that the resulting PCR product should be approximately 1500 bp in size if the cat-containing cassette has replaced the ORF of the gene of interest. Performing PCR with new primers that are homologous to the *E. coli* chromosome, ensures that: i) the cassette has recombined with the gene of interest and not elsewhere on the chromosome and, ii) that the amplified cassette is in the chromosome and the resulting PCR product is not from transformed pKD3 plasmid. PCR should be performed on both the parental wild type strain, and the newly created knockout strain. A size change in the PCR product from the two strains provides evidence of the deletion of the gene of interest and replacement with the cat-expression cassette. To save time, individual colonies can be used directly in PCR, without having first isolated chromosomal DNA from the strains. A procedure for performing Colony PCR follows:

Protocol:

- Streak out the wildtype parental strain and the gene knockout strain on LB plates and incubate the plates overnight at 37°C.

- Remove reagents from the freezer and thaw them on ice. Assemble the reaction according to the following table:

Reagent	Experimental Reaction		"No template" Negative Control	
	Volume (µl)	Final Concentration	Volume (µl)	Final Concentration
10 X PCR buffer	5	1X*	5	1X*
50 mM $MgSO_4$	2	2 mM	2	2 mM
10 mM dNTP	1	0.2 mM	1	0.2 mM
ddH_2O	31.5	-	35.5	-
25 µM Primer F	2	1 µM	2	1 µM
25 µM Primer R	2	1 µM	2	1 µM
10% Tween 20	2	0.4%	2	0.4%
Template colony	-	-	-	-
Taq Polymerase (1U/µL)	2.5	2.5 U	2.5	2.5 U
Total	50	-	50	-

1X reaction buffer is: 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20
10% Tween 20 should be prepares freshly beforehand from a pure stock. Tween 20 must be used to lyse the cells because it is not a strong detergent and will not denature the Taq polymerase.

- It is important to keep the reactions on ice once *Taq* polymerase has been added to reduce the formation of primer-dimers. To add the colony as a template, pick a single isolated colony from the plate with a sterile toothpick, stick the toothpick into the tube and swirl it until the reaction tube becomes slightly turbid. To aid in cell lysis, the reactions may be vortexed briefly. When performing the reaction, the initial denaturation time should be increased to 4 min to ensure the cells lyse to release the template DNA. The following may be used as for the PCR reaction:

Step	Time (min:sec)	Temperature (°C)	Cycles
Initial denaturation	4:00	94	1
Denature	0:30	94	25
Anneal primers	0:30	Tm-5 [a]	
Elongation	1:00/kb [b]	72	
Final Elongation	7:00	72	1

a. the optimal annealing temperature must be determined empirically, but 5°C below the Tm of the primers is usually a good starting point

b. the amount of time allotted to elongation depends on the length of the expected amplicon (PCR fragment). As a general rule of 1 minute of elongation per 1000 bp is sufficient. Two different size products are expected for the two reactions so set the time so that the longest fragment has sufficient extension time.

RNA Isolation 1 – Basic Procedure

The key to isolation of high quality RNA in *E. coli* is to work very quickly under conditions where the RNA is in the presence of heat and/or RNases. It is especially important to work quickly when cultures are grown into stationary phase, because the mRNA in the cells is usually found in a partially degraded state before RNA isolation begins. It is also important to work carefully so that no DNA from the bacterial chromosome is isolated along with the RNA. If the RNA is going to be used for Northern analysis, then a small amount of contaminating DNA will not prevent the experiment from performing correctly, however if the RNA will be reverse transcribed into cDNA, then it is vital that the RNA is pure and free from any contaminating genomic DNA. All materials used should be RNase free, including the tips and tubes. Many aqueous solutions can be made RNase free by treating with 0.1% diethyl pyrocarbonate (DEPC). This protocol was originally obtained from Sigma Genosys and is derived from the method used by Kohrer and Domdey (1991).

Protocol:

- Isolate a single colony and transfer it into 10 ml of LB media in a 50 ml flask at 37°C with shaking at 200 rpm
- Grow the culture overnight
- Perform a 1:1000 subculture by adding 10 µl of the overnight culture into 10 ml of LB media in a 50 ml flask
- Grow the culture at 37°C with 200 rpm agitation until the culture has reached an $O.D._{600}$ of 0.2-0.3
- Subculture 50 µl into 50 ml of fresh LB media in a 250 ml flask and continue to grow

as before until the culture has reached an $O.D._{600}$ of 0.2-0.3.

- Once the culture has reached an $O.D._{600}$ of 0.3, pipette 15 ml of culture into a 15 ml polypropylene tube (Sarstedt) on ice to stop growth and reduce RNase activity. Incubate the culture on ice for 5 min. and centrifuge the cells at 5000 x g for 10 min at 4°C. Quickly remove the supernatant and flash-freeze the pellets in liquid nitrogen and store the cell pellets at –80°C until ready to use.
- Continue to grow the culture until it has reached an $O.D._{600}$ of 1.8, pour 3-5 ml of culture into a 15 ml polypropylene tube (Sarsedt) on ice to stop growth. Prepare the cell pellets identically to the exponential phase samples above and store the cell pellets at –80°C until ready to use.
- When ready to isolate RNA, remove the cells from -80°C and place on ice to thaw. For each sample, treat as follows:
  1. Add 250 µl of ice cold Resuspension Buffer (0.3M sucrose, 10mM sodium acetate, pH 4.2) and 37.5 µl of ice cold 0.5M EDTA.
  2. Resuspend the pellet and add the entire sample to a 2 ml microfuge tube on ice.
  3. Add 375 µl of Lysis Buffer (2% SDS, 10mM sodium acetate pH 4.2, 1% β-Mercaptoethanol), which has been preheated to 65°C. Incubate the tubes in a heating block at 65°C for 3 min.
  4. Add 700 µl of acidic phenol (pH 4.2, Sigma P4682) pre-heated to 65°C, and mix the contents of the tube thoroughly by vortexing. Incubate the tubes at 65°C for 3 minutes. Cool the samples on ice for 3 min.
  5. Centrifuge the tubes for 5 min. at maximum speed. Transfer the upper aqueous layer to a new tube. Try to recover as much of the aqueous phase as possible, but do not transfer any of the white interface material. If the aqueous phase is too thick and gooey to transfer, it may help to cut the end of the pipetman tip off.
  6. Repeat the phenol extraction 2 more times (steps 4 – 6).
  7. Perform an extraction using 700 µl of a mixture of acidic phenol:chloroform:isoamyl alcohol (25:24:1). After adding the

phenol:chloroform, vortex to mix the two phases and centrifuge for 5 min at maximum speed. Transfer the aqueous phase to a new tube and repeat the phenol:chloroform extraction once more.

8. Perform one final extraction with 700 µl of chloroform:isoamyl alcohol (24:1). After adding the phenol:chloroform, vortex to mix the two phases and centrifuge for 5 min at maximum speed. Transfer the aqueous phase to a new 1.5 ml tube. It is important to ensure that none of the organic phase is transferred along with the aqueous phase.
9. To purify the RNA, add 0.1 volumes of RNase-free 3M Sodium Acetate (pH 5.2). Vortex to mix. Add 2.5 volumes of 100% ethanol and mix by inversion. Store the sample at -20°C for 1 hour to help precipitate the RNA. If the starting volume of RNA sample is too large (greater than 350 µl) then it should be divided between two tubes before precipitation.
10. Pellet the RNA by centrifugation at 4°C for 30 min (maximum speed). Remove the supernatant with a pipet and wash the pellet with 1 ml of RNase-free 70% ethanol. Centrifuge again at 4°C for 30 min (max. speed) and remove the supernatant with a pipet. Take care not to disturb the RNA pellet, which is often not very solid.
11. Air-dry the pellet for 10-15 minutes. Be careful not to over-dry the pellet, because RNA pellets are very difficult to re-dissolve if over-dried.
12. Resuspend the pellet in RNase-free ddH_2O or 0.1 mM EDTA, pH 8.0. It may be necessary to incubate the samples at 37°C briefly (5-10 min) to help dissolve the RNA. Keep the RNA samples on ice when in use and store at -80°C when not in use.

- The RNA samples should be quantified by measuring the absorbance at 260 nm in a spectophotometer. A dilution of 5 µl of the RNA sample into 1 ml of ddH_20 should be sufficient to give a reading. The concentration of RNA may be determined by the following equation:

[RNA] (μg/ml) = OD_{260} x dilution factor x 40 μg/ml

- The correct concentration calculations can be confirmed by loading equal amounts of RNA samples onto a 1% agarose gel (non-denaturing). The gel is also used to check the integrity of the RNA and for any genomic DNA contamination. Genomic DNA will appear as a very high molecular weight band after the gel is run. Good quality RNA (low degradation) should have two sharp bands corresponding to the 23S and 16S rRNA. The higher 23S band should be approximately twice as bright as the 16S band. There should be a minimal amount of smearing towards the bottom of the gel, which is indicative of RNA degradation.

RNA Isolation 2 – RNA*later* Preserved Cells (Ambion)

The most difficult part of RNA isolation in *E. coli* is the ability to preserve the RNA from degradation. RNA degradation in E. coli is very rapid; the average half-life of an mRNA is about 1 minute. Due to the rapid turnover of mRNA a method to preserve the RNA at the time of sampling is critical for obtaining high quality RNA. Most commercial kits and protocols call for a centrifugation step after sampling cells, to remove the growth media. The time required for centrifugation allows the mRNA to be degraded within the cells, so a means is required to preserve the RNA at the time of sampling. A number of methods have been devised to accomplish the preservation of RNA, including the addition of the reagent RNA*later* (Ambion), which preserves RNA within the cell and inactivates RNases present within the cells. The reagent also protects RNA at elevated temperatures, preventing its degradation. The following protocol was used to preserve cells with RNA*later* prior to isolation of total cellular RNA.

Protocol:

- Isolate a single colony and transfer it into 10 ml of LB media in a 50 ml flask at 37°C with shaking at 200 rpm
- Grow the culture overnight
- Perform a 1:1000 subculture by adding 10 µl of the overnight culture into 10 ml of LB media in a 50 ml flask
- Grow the culture at 37°C with 200 rpm agitation until the culture has reached an OD_{600} of 0.2-0.3
- Subculture 50 µl into 50 ml of fresh LB media in a 250 ml flask and continue to grow as before until the culture has reached an OD_{600} of 0.2-0.3.
- Once the culture has reached an OD_{600} of 0.3, pipette 5 ml of culture into a 15 ml polypropylene tube (Sarstedt) containing 10 ml of RNA*later*, on ice to stop growth and reduce RNase activity. Incubate the culture on ice for 30 min. and centrifuge the cells at 5000 x g for 10 min at 4°C. Quickly remove the supernatant and flash-freeze the pellets in liquid nitrogen and store the cell pellets at –80°C until ready to use.
- Continue to grow the culture until it has reached an OD_{600} of 1.8, pipette 2 ml of culture into a 15 ml polypropylene tube (Sarsedt) containing 4 ml of RNA*later*, on ice. Prepare the cell pellets identically to the exponential phase samples above and store the cell pellets at –80°C until ready to use.
- When ready to isolate RNA, remove the cells from -80°C and place on ice to thaw.
- Isolate total RNA using the hot phenol method outline above.

mRNA Purification

Northern analysis in *Escherichia coli* is usually performed on total cellular RNA instead of mRNA. The main reason for this is that mRNA in *E. coli* is much more difficult to isolate from other RNA, than in most higher organisms. Eukaryotic cells have

polyA chains on the 3'ends of their mRNA, while *E. coli* cells do not. As a consequence, eukaryotic mRNA can be isolated using a column of oligo-dT coated beads. The presence of other RNA species, such as 16S rRNA, 23S rRNA, 5S RNA and various tRNAs can pose a problem for Northern analysis because rRNA comprises over 70% of the RNA in total RNA preparations from *E. coli*. The abundance of these rRNAs can saturate the membrane during blotting, and prevent mRNA species of similar size from binding to the membrane. This may make it difficult to determine both the number of bands present on a blot, or their size. For these reasons the removal of the rRNA should improve Northern analysis. Until recently a technique did not exist for *E. coli* mRNA isolation. Now there are a few different methods available, including the Microbe*Express* kit (Ambion).

Protocol:

- Total RNA should be isolated using the hot phenol method as described in the previous section.
- For the procedure to function properly the RNA must be free of any contaminating salts, and should be re-precipitated.
- Adjust the volume of the RNA sample to 100 µl using RNase-free ddH_2O in a 1.5 ml microfuge tube.
- Add 0.1 volumes (10 µl) of RNase-free 3M Sodium Acetate, pH 4.2, and mix by pipetting
- Add 2.5 volumes (250 µl) of 100% ice-cold ethanol, close the tube tightly, and mix by inversion.
- Store the sample at -20°C for 30 min to aid in precipitation of the RNA
- Pellet the RNA by centrifugation at 4°C for 30 min (maximum speed in microfuge). Remove the supernatant with a pipet and wash the pellet with 1 ml of RNase-free 70% ethanol. Centrifuge again at 4°C for 15 min (max. speed) and remove the

supernatant with a pipet. Take care not to disturb the RNA pellet, which is often not very solid.

- Air-dry the pellet for 10-15 minutes. Be careful not to over-dry the pellet, because RNA pellets are very difficult to re-dissolve if over-dried.
- Resuspend the pellet in 0.1 mM EDTA, pH 8.0. The EDTA is important for the mRNA isolation procedure because the RNA will be heated up to 37°C. Divalent cations, such as Mg^{2+} and Ca^{2+}, can cause cleavage of RNA at elevated temperatures. EDTA will chelate these ions and prevent the degradation of the RNA
- Follow the procedure provided with the Microbe*Express* kit precisely. The maximum RNA that may be purified is 10 µg in a 15 µl volume.
- It will be difficult to assay the quality of the RNA after purification because no rRNA bands will be present. Thus, it is important to work RNase-free and start with a total RNA sample of very high quality.

Northern Analysis

A. Probe Preparation

Probes for Northern analysis can be made by PCR using radioactive α-^{32}P dCTP in the PCR reaction (Sambrook and Russell, 2001). The starting point for making a probe is to amplify a segment of the gene of interest using PCR. This PCR product can then be labeled and used as a probe. The advantages of using PCR to also label the probes is that the reagents are mostly the same as those needed to make the original PCR product, minimizing expense, the reaction generates a large amount of labeled probe with a high activity, the incorporation of label into the probe is quite high, and the amount of starting material, template PCR product, doesn't need to be as high as for other methods. The main disadvantage is that the PCR reaction must have a high fidelity, if primer dimers or other unintended PCR products are created, they will incorporate radionucleotides and may lead to artifacts in the Northern analysis. The PCR reaction can't be run on a gel to confirm proper amplification, so the reaction conditions must be optimized beforehand. Usually I run a non-radioactive control (with regular dCTP) at the same time to ensure no primer dimers or other unwanted fragments are created.

Protocol:

- Amplify a section of the gene of interest by PCR as described above (PCR section).
- Run 5 µl of the PCR reaction on a 1% agarose gel to confirm that the proper sized fragment has been amplified and that there are no side reactions, including primer dimers.
- Run 15 µl of the PCR reaction on a 0.6% agarose gel. Cut the band from the PCR out

of the gel using a sterile scalpel blade and transfer the piece of agarose to a pre-weighed 1.5 ml microfuge tube. Minimize the exposure of the DNA to the UV light to prevent damage.

- Purify the DNA by gel-extraction using the QiaEX II Gel Extraction kit (Qiagen). The protocol supplied by the manufacturer should be followed as given.
- Run 1 µl of the purified DNA on a 1% agarose gel to confirm its purification and get a rough measure of the quantity.
- Assemble the radiolabelling reactions using the following table:

Reaction components for PCR labeling radioactive probes

Reagent	Experimental Reaction		Positive Control	
	Volume (µl)	Final Concentration	Volume (µl)	Final Concentration
10 X PCR buffer	5	1X*	5	1X*
50 mM $MgSO_4$	2	2 mM	2	2 mM
10 mM dATP,dGTP,dTTP	1	0.2 mM	1	0.2 mM
0.1 mM dCTP	1	0.002 mM	-	-
10 mM dCTP	-	-	1	0.2 mM
α-^{32}P dCTP (10µCi/µl)	5	50 µCi	-	-
ddH_2O	29	-	34	-
25 µM Primer F	2	1 µM	2	1 µM
25 µM Primer R	2	1 µM	2	1 µM
Gel-purified PCR fragment	0.5	varies	0.5	varies
Taq Polymerase (1U/µL)	2.5	2.5 U	2.5	2.5 U
Total	50	-	50	-

1X reaction buffer is: 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20

When assembling the reactions, the radioactive dCTP should be added last to

minimize exposure to radioactivity. The reaction conditions used for the radiolabelling should be identical to those used for amplification of the template fragment. Following the radiolabelling reaction the positive control reaction should be run on a 1% agarose gel to confirm proper amplification. The probe must be quantified and purified before it can be hybridized to the blot. The unincorporated radioactive nucleotides will cause excess background exposure on the blot if they are not removed.

Protocol:

- Remove the radioactive labeled probe from the thermal cycler and store it in a beta-shielded plastic block.
- Prepare a Probe-Quant G50 micro spin-column by inverting and shaking the column to resuspend the gel beads inside.
- Snap the bottom off of the column and loosen the cap ¼-turn. Place the column into a 2 ml lidless microfuge tube.
- Centrifuge the column in a Fisher Marathon 16KM microfuge at 2800 rpm (730 x g) for 1 min.
- Add 5 µl of ddH_2O to the radiolabelling PCR reaction, for a total of 55 µl. Mix by pipeting. (I recommend using an aerosol barrier pipetman tip to prevent contamination of the pipetman).
- Add exactly 50 µl of the radioactive PCR reaction to the top of the spin-column. Place the spin column into a new 1.5 ml microfuge tube.
- Centrifuge the column at 2800 rpm for 2 min to elute the radiolabelled DNA. The nucleotides will remain trapped in the column.
- Discard the column. Determine the volume of elutate in the tube using a pipetman. Usually, about 56-60 µl comes out of the column after centrifugation.
- Add 10 ml of biodegradable scintillation fluid (company) into 2 scintillation vials. Label one vial "Total Counts" and label the other vial "Purified".

- Add 1 µl of the original PCR reaction (before purification) to the "Total Counts" vial.
- Add 1 µl of the purified probe (column elutate) to the "Purified" vial.
- Determine the Counts per min (cpm) of both samples in the Beckman LS 1801 scintillation counter (Beckman Coulter). Read the cpm from channel 3 for ^{32}P.
- The percent incorporation is calculated by:

$$\% \text{ incorporation} = \frac{(\text{cpm from "purified"})(50)}{(\text{cpm from"Total"}) \times (\text{volume of purified probe from column})}$$

The purified probe can be stored at -20°C until ready to used. The probe should be hybridized to the blot as soon as possible because radiolysis will cleave the probe and the probe will also loose activity over time.

B. Separating RNA by Denaturing Agarose Gel-Electrophoresis

When running a RNA samples on an agarose gel, it is important to ensure the RNA is denatured. Because RNA is single stranded, it can bind to itself or to other RNA species at sequences of (partial) homology, thus the RNA may not run on the gel according to its actual size. Formaldehyde may be included in the gel and samples to keep the RNA denatured as it runs through the gel. When running a formaldehyde gel, all work should be done in the fume hood because the formaldehyde used is toxic. The gel should also have a LOW concentration of Ethidium bromide included because the chemical will inhibit the transfer of RNA onto the membrane during blotting.

Protocol:

- Pre-heat 15 ml of 10X MOPS running buffer and X ml of formaldehyde to 65°C in a

water bath.

- Prepare the gel tray and a 250 ml Erlenmeyer flask so that they are RNase-free. Wipe each with a RNase Zap wipe (Ambion) and rinse thoroughly twice with RNase-free ddH_2O.
- Weigh out 1.5 g of agarose and add it to 125 ml of DEPC-treated ddH_2O in the RNase-free flask. Dissolve the agarose by boiling the solution in the microwave.
- Place the agarose solution into the 65°C water bath and allow it to cool.
- Pour the pre-heated 15 ml of 10X MOPS buffer and 8.75 ml of formaldehyde into the cooled agarose and mix by swirling the flask. Add 30 µl of a 0.1 mg/ml stock of Ethidium bromide to the gel solution for a final concentration of 0.02 µg/ml.
- Pour the gel into the gel tray and allow it to solidify.
- Prepare the RNA samples according to the following table:

Component[a]	Volume (µl)	Concentration
RNA	X[b]	
ddH2O	8-X	
Formaldehyde	5.5	2.2 M
Formamide	15	50%
10x MOPS buffer	1.5	0.5x
Total	30	

a. All components should be RNase-free
b. The volume of RNA added depends on the concentration of each sample. All lanes should be loaded with an equal amount of RNA

- Heat the RNA samples at 55°C for 15 min to denature. After denaturing the RNA add 3 µl of 10X loading buffer (60% glycerol, 10mM EDTA pH 8.0, 0.25% bromophenol blue) to each of the samples.
- Once the gel has solidified, submerge the gel in an electrophoresis tank with 1X MOPS buffer. (add 0.02 µg/ml Ethidium bromide to the buffer)

- Load the samples into the gel. Run the gel at 5V/cm (50V) for three hours.
- Once the gel has finished running, photograph the RNA bands on the transilluminator. The rRNA bands will be faint, because Ethidium bromide does not bind well to single stranded RNA. Leave the gel on the gel tray when photographing to minimize exposure of the gel to unclean (RNase contaminated) surfaces.

C. Transfer of the RNA to a Nylon Membrane

Once the gel has finished running, and a photograph of the rRNA bands has been taken, the RNA is transferred to a nylon membrane. The simplest method is to place the gel into a transfer solution (usually SSC) and place the membrane on top of the gel with many layers of absorbent paper. The capillary action of the solution into the absorbent paper will carry the RNA up, out of the gel, onto the membrane.

Protocol:

- Place the gel on a piece of aluminum foil and cut off any unused lanes from the gel with a clean scalpel blade. Measure the dimensions of the gel with a ruler.
- Place the gel in a plastic container and rinse the gel with 500 ml of DEPC-treated ddH_2O. Place the gel on a shaker platform and allow the gel to soak for 15 min. with gentle agitation.
- Remove the water and soak the gel in 500 ml of 10X SSC for 15 min. with gentle agitation. Remove the buffer and repeat with another 500 ml of 10X SSC for 15 min.
- Cut a piece of Hybond-N^+ (Amersham) nylon membrane that is 3mm shorter in length and width than the gel.
- Cut 10 pieces of blotting paper to 7 mm shorter than the length and width of the nylon membrane.
- Cut a 5-6 cm high stack of brown paper towel that is 3 mm shorter in length and width than the blotting paper.

- Cut a wick out of blotting paper. The wick should be at least 2 cm wider than the gel and long enough so that the ends are submerged in the buffer reservoir. The wick should be 2 sheets thick.
- Assemble the capillary transfer apparatus as shown in Figure A2.

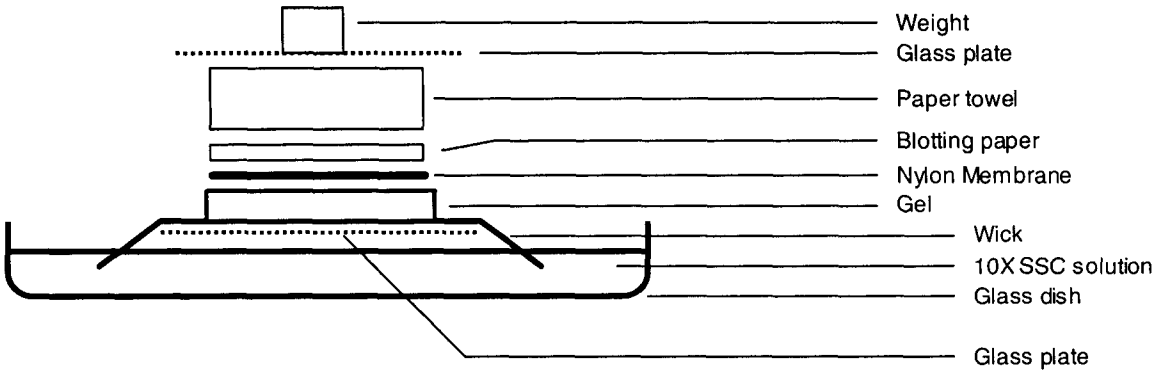


Figure A2: Transfer apparatus for Northern Analysis. The transfer apparatus allows SSC transfer buffer to flow from the reservoir, up the wick, through the gel and membrane and into the absorbent paper above. The flow of buffer pulls the RNA from the gel into the membrane where it is trapped. The weight on top is a small 100 ml Gibco bottle with 50 ml of water inside.

- Place 500 ml of 20X SSC solution into glass dish. Rest a glass plate on top of the dish. The glass plate should be longer then the width of the dish so that it can rest securely on top, but should be shorter than the length so that there is space for the wick to dip into the buffer reservoir.
- Wet the wick in the 20X SSC buffer and place it on top of the glass plate so that the ends are in the buffer in the glass dish. Roll a pipet over the surface of the wick to remove any air bubbles trapped underneath.
- Place the gel on the center of the wick. Roll a pipet over the surface of the gel to remove any air bubbles trapped underneath.
- Place the nylon membrane on the surface of the gel. Once the membrane has been placed, it should not be lifted or moved.

- Wet one of the pieces of blotting paper in the 20X SSC and place it on top of the nylon membrane. Roll a pipet over the surface of the blotting paper to remove any air bubbles trapped underneath. Place the rest of the blotting paper on top of the first piece. Add the entire stack of brown paper towel on top of the blotting paper.
- Balance a second glass plate on top of the paper towel. Cover the entire apparatus with plastic wrap to keep everything moist and help hold the top glass plate in place.
- Place a small weight on top of the glass plate to press all the paper together. I usually use a small glass bottle with 50 ml of water inside.
- Leave the apparatus overnight for about 16 hours.
- After the transfer is complete unassembled the apparatus and discard the absorbent paper.
- With the membrane still on the gel mark the location of the wells with a ballpoint pen. Cut the right hand corner off with scissors to help identify the orientation of the membrane.
- Gently peel the membrane off of the gel and place it RNA-side up on a piece of blotting paper.
- Fix the RNA to the membrane by UV crosslinking using a Stratalinker 2400 (Stratagene). Place the blot into the UV crosslinker, RNA-side up, press the "auto crosslink" button, and "START". The crosslinker will expose the blot to a fixed amount of UV light, chemically bonding the RNA to the membrane.
- Rinse the blot briefly in ddH_2O to remove any SSC salts on the membrane
- Proceed to the hybridization if ready, other wise the blot may be stored at -80°C for a short time, if it is well wrapped in plastic and sealed up.

D. Hybridization of Radiolabelled Probe to the Blot

Once the RNA blot has been prepared, and the probe has been labeled, the next step is to hybridize the probe to the RNA on the membrane. Hybridization in bottles is the best methods because it allows the hybridization buffer to constantly move over the

surface of the blot, a minimal amount of hybridization buffer is needed and it keeps all of the radioactive probe contained inside the bottle, reducing contamination. There are a number of different recipes for hybridization buffer commonly used, and several companies offer their own hybridization buffers. The two most common are Denhart's buffer and Church buffer. The hybridization buffer usually contains several blocking agents, which prevent binding of the probe to the membrane instead of the complementary RNA on the blot. The following protocol uses a Modified Church buffer, which is less complex than Denhart's buffer and is recommended by the manufacturer of the nylon membrane (Amersham). The blocking agent in this buffer is a high concentration of SDS.

Protocol:

- Preheat the Church hybridization buffer to 65°C in a water bath to fully dissolve the SDS. Church buffer is made of 0.5 M phosphate buffer, pH 7.2, 7% SDS, 10 mM EDTA pH 8.0. When the buffer is first prepared the SDS will not go into solution, it must be heated before the SDS will dissolve. The hybridization buffer may be stored at room temperature. During storage the SDS will likely precipitate out of solution.
- Preheat the hybridization oven to 68°C.
- Place the blot into a small hybridization bottle, with the RNA facing the centre of the bottle. Add 20 ml of 0.5 M phosphate buffer pH 7.2. Rinse the blot thoroughly with the buffer for 15 min.
- Remove the phosphate buffer and add 10 ml of the preheated hybridization solution to the bottle. Place the hybridization bottle in the oven on the rotisserie and pre-hybridize for 1-2 hours at 68°C and 4 rpm.
- The purified probe must be denatured before adding it to the hybridization solution. Use 0.5-2 x 10^6 cpm of probe per ml of hybridization buffer. Add the appropriate

volume of probe (usually about 20 µl) to a 1.5 ml microfuge tube. Add 0.1 volumes of 3M NaOH to the probe and mix thoroughly by pipeting. Incubate the probe for 5 minutes at room temperature. The probe should now be denatured.

- Remove the hybridization bottle from the oven and add the entire denatured probe directly to the hybridization buffer in the bottom of the tube. Do not put any directly onto the blot as this will cause high levels of background. Swirl the buffer in the bottom gently to mix the probe into the buffer. Return the bottle to the hybridization oven. Hybridize the probe to the blot overnight, approximately 16-20 hours, at 68°C and 4 rpm.

E. Washing, Exposure, and Scanning the Exposed Phosphorscreen

After hybridization is complete, the excess, unbound probe must be washed off the blot to reduce the level of background radioactivity on the membrane. Afterwards the blot is wrapped tightly in plastic to prevent it from drying out, and to prevent contamination of the Phosphorscreen. If the blot is allowed to dry, the probe cannot be stripped off. Exposure of the blot to a Phosphorscreen instead of film has several advantages. The Phosphorscreen is much less sensitive to light, so a dark room is not required, the exposure to radioactivity creates an image much faster (up to 7X) than exposure to film, the linear range of exposure is several magnitudes greater than film and the phosphorscreen can be easily scanned to create a high resolution digital image.

Protocol:

- Prepare three wash solutions, a low, medium and high stringency wash solution
  - Low stringency wash solution is 2X SSC, 0.1% SDS
  - Medium stringency wash solution is 1X SSC, 0.1% SDS

  - High stringency wash solution is 0.5X SSC, 0.1% SDS
- Remove the blot from the hybridization bottle and quickly place it into 100 ml of the low stringency wash solution in a small plastic dish. Briefly rinse the blot and discard the rinse solution.
- Rinse the blot with gentle agitation (using the platform in the hybridization oven) twice with 100 ml of low stringency wash, twice with the medium stringency wash and then twice with the high stringency wash for 15 minutes each at 30°C. Care should be taken to prevent the low stringency wash from precipitating, as this will lead to elevated levels of background radioactivity on the blot.
- Cut out a piece of plastic "overhead projector" film to 1 cm larger in length and width than the size of the membrane. Lay the plastic film on a few pieces of brown paper towel. Place the membrane, RNA up, onto the piece of plastic. Place a large piece of Saran wrap on top of the membrane. Use a pipet to roll out excess fluid from the blot and remove any air bubbles and creases in the plastic wrap. The excess fluid should be absorbed into the paper towels. Carefully wrap the plastic wrap around the back of the plastic overhead film.
- Tape the wrapped up blot into the exposure cassette with the RNA side facing up. Place the phosphorscreen into the cassette, so that the white side is pressed against the blot. Close the cassette.
- Expose the blot to the phosphorscreen for 15 min (high copy message, such as rRNA), or 4 hours (low copy message). The phosphorscreen may be exposed overnight, up to 24h, if the signal from the blot is very low. If there is no signal after 24 hours, it is unlikely that any signal is present).
- Remove the phosphorscreen from the cassette and place it into a STORM 280 Phosphorimager. The phosphorimager will scan the screen and create a digital image on the computer. Use the grid inside the cassette to align the screen on the bed of the phosphorimager and scan the appropriate area of the screen.
- To erase the phosphorscreen, expose it to fluorescent light for 20 min.

RT-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is similar to PCR as described previously, but the template used is cDNA generated from reverse transcription of mRNA. Reverse transcription uses a single primer and the enzyme Reverse Transcriptase, isolated from retroviruses, that makes a single stranded DNA copy (cDNA) from an RNA template. This cDNA can then be used as template in a PCR reaction. It is vital to have a highly pure starting RNA sample as template. Any genomic DNA contamination can be amplified in the PCR step, leading to synthesis of bands from genomic DNA and not from cDNA. The RNA samples can be digested with DNaseI beforehand to ensure there is no contaminating DNA, but there will likely be a small amount of DNA contamination. An RT^- control should be run at the same time, using template that did not have any reverse transcriptase added, so there will be no cDNA in the template. Any PCR bands resulting from this control are most likely due to genomic DNA contamination.

Protocol:

- Isolate total RNA from *E. coli* cells expressing the gene of interest. There should not be any genomic DNA contamination visible when the RNA is run on a non-denaturing agarose gel.
- To ensure there is as little genomic DNA contamination as possible, the RNA sample should be treated with DNaseI. The DNA*free* kit (Ambion) works well for digesting the DNA with DNaseI and removal of the DNaseI and divalent cations. The kit should be followed according to the manufacturers instructions. The RNA samples may be run on an agarose gel a second time to ensure the RNA has not been degraded.

- The reverse transcription reaction is set up as follows:

Component[a]	RT reaction		RT⁻ Control	
	Volume (µl)	Concentration	Volume (µl)	Concentration
RNA	X	1 µg	X	1 µg
ddH2O	10-X		10-X	
Reverse Primer	1 µl	2 pmol	1 µl	2 pmol
10 mM dNTP	1 µl	10 nmol	1 µl	10 nmol
Incubate at 65°C for 5 min, then chill on ice				
5X First-Strand buffer [b]	4 µl	1X	4 µl	1X
0.1 M DTT	2 µl	10 mM	2 µl	10 mM
RNase OUT [c]	1 µl	40U	1 µl	40U
Incubate at 42°C for 2 min				
Superscript II RT [d]	1 µl	200U	-	-
ddH_2O	-	-	1 µl	-
Incubate 42°C for 50 min				
Incubate at 70°C for 15 min to inactivate the reaction				
Total	20 µl	-	20 µl	-

a. All components should be RNase-free
b. 1X First-Strand buffer is 50 mM Tris-HCl, 75 mM KCl, and 3 mM $MgCl_2$
c. RNase OUT Ribonuclease Inhibitor (Invitrogen)
d. Superscript II Reverse Transcriptase, an engineered version of M-MLV RT (Invitrogen)

- After the reaction is complete, the RNA of the RNA-DNA hybrids must be digested for efficient PCR to occur. Add 1 µl of RNaseH (Invitrogen) and incubate at 37°C for 20 min.
- The cDNA reaction may now be used as template for the PCR reaction. No more than 10% of the CDNA should be used per PCR, as this will give lower yields of PCR product.
- The PCR reaction is set up as follows:

Reagent	Experimental Reaction		RT⁻ Negative Control	
	Volume (µl)	Final Concentration	Volume (µl)	Final Concentration
10 X PCR buffer	5	1X	5	1X
50 mM $MgSO_4$	2	2 mM	2	2 mM
10 mM dNTP	1	0.2 mM	1	0.2 mM
ddH_2O	33.5	-	35.5	-
25 µM Primer F	2	1 µM	2	1 µM
25 µM Primer R	2	1 µM	2	1 µM
cDNA reaction	2	-	2 [a]	-
Taq Polymerase (1U/µL)	2.5	2.5 U	2.5	2.5 U
Total	50	-	50	-

a. The RT- cDNA reaction is used as template in the RT- PCR negative control

- Reaction conditions are similar to a general PCR reaction. Due to small amount of contaminating DNA, which is difficult to eliminate, the PCR reaction should include no more than 25 cycles, or bands from genomic DNA may start to appear. Additionally, positive controls should be included that use genomic DNA as template to see the expected amplified fragments. The reaction conditions used are:

Step	**Time (min:sec)**	**Temperature (°C)**	**Cycles**
Initial denaturation	4:00	94	1
Denature	0:30	94	25
Anneal primers	0:30	Tm-5 [a]	
Elongation	1:00/kb [b]	72	
Final Elongation	7:00	72	1

a. The annealing temperature depends on the melting temperature of the primers being used
b. The elongation time depends on the length of the expected product. 1 minute pre 1000 bp is sufficient. The elongation time should be set for the longest fragment being amplified in a series of reactions.

- After the PCR reactions are complete, all reaction may be run on a 1% agarose gel to visualize the products of the reactions.
- The positive genomic controls should have bands present, while the negative controls should not have any bands present. If there are bands in the RT^- control, then there is too much genomic DNA contamination in the RNA starting material.

Appendix B – Macroarray Analysis of the *rpoS* Regulon in *E. coli*

Introduction

Use of microarray technology has allowed large steps to be taken in the area of genomics and analysis of gene expression in both prokaryotes and eukaryotes. Arrays have been used to examine changes in expression caused by regulators (Barbosa and Levy, 2000), changes caused by different growth conditions (Tao, *et al.*, 1999) and assigned adjacent genes into operons (Sabatti, *et al.*, 2002). As confirmation of the *rpoS*-dependent genes already identified and to gain a more complete picture of the *rpoS* regulon, we are employing macro-array technology to identify *rpoS* and stationary phase dependent genes in *E. coli*.

The Panorama *E. coli* macroarray (Sigma Genosys) is a nylon membrane with probes for all 4,290 ORFs in *E. coli* (based on the first published draft of the *E. coli* MG1655 genome annotated by Blattner *et al.* (1997)). The probes are 10 μg of PCR amplified DNA for each ORF. Hybridization of cDNA generated from Total RNA, allows simultaneous assaying of expression for all ORFs.

Materials and Methods

Media and Growth Conditions

All bacterial strains were grown in Luria-Bertani (LB) broth. Strains were grown overnight in LB media, subcultured into fresh media and maintained in the early exponential-phase (OD_{600} of <0.2) for at least 8 generations prior to the start of the experiment. Cultures were grown in flasks at 37°C with aeration at 200 rpm.

Isolation of Total RNA

Cultures were grown as described above, and aliquots were removed from five points along the growth curve to examine expression changes in different growth phases (OD_{600} = 0.2, 0.4, 0.8, 1.6, 3.0). Total RNA was extracted from wild type MG1655 and

rpoS$^-$ HS2210 strains using a modification of the hot-phenol method supplied with the Sigma-Genosys Panorama *E. coli* Gene Arrays (Sigma-Genosys). Samples were suspended in 250 µl Resuspension buffer (0.3 M sucrose, 10 mM sodium acetate, pH 4.2) and 37.5 µl of 0.5 M EDTA and the samples were incubated on ice for 5 min. To each sample, 375 µl of Lysis buffer (2% SDS, 10 mM sodium acetate, pH 4.2) was added; the tubes were vortexed and incubated at 65°C for 3 min. The samples were extracted three times with 700 µl acidic phenol (Sigma Aldrich) heated to 65°C. The samples were extracted with 700 µl of acidic phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature followed by a final extraction with 700 µl of chloroform:isoamyl alcohol (24:1). The RNA samples were precipitated using 0.1 volumes of 3 M sodium acetate and 2.5 volumes absolute ethanol. The RNA was pelleted by centrifugation at ~12, 000 rpm for 30 min. at 4°C. The RNA was rinsed with 70% ethanol, allowed to air dry and resuspended in 50 µl of sterile DEPC-treated water. To remove DNA contamination the RNA was treated with DNase I from the DNAfree kit according to the supplied instructions (Ambion). The RNA was quantified spectrophotometrically by measuring OD_{260nm}. RNA samples were stored at -80°C until used.

Probe Preparation and Northern Analysis

To check the quality of the RNA, Northern analysis was performed using an *rpoS*-dependent gene *osmY*. Oligonucleotide primers were synthesized to generate PCR-amplified probes to detect the specific mRNA transcripts of *osmY*: forward, 5'-CTGCTGGCTGTAATGTTGACCTC-3', and reverse, 5'-CATCTACCGCTTTGGCGATACTT-3'. The PCR tube contained 1x PCR buffer, 50 pmol of each of the forward and reverse primers, 0.2 mM each of the four dNTPs, 2 mM MgSO4, ~10ng *E. coli* chromosomal DNA and 2.5 U of Taq polymerase in a final volume of 50 µl. Reactions were run for 30 cycles under the following conditions: (i) 95°C for 15s; (ii) 61°C for 30s; and (iii) 72°C for 60s. The PCR product was purified

from a 1% agarose gel using a QIAex II Gel Extraction Kit (Qiagen) and was radiolabelled with [α-^{32}P] –dCTP (NEN Life Science Products) by PCR labeling (Sambrook, 2001) using identical reaction conditions as above. Radiolabelled probes were purified from unincorporated nucleotides prior to use using a Sephadex G-50 microspin column (Amersham Pharmacia).

Northern analysis was performed on 3.3 µg of the total RNA samples. The samples were prepared, run on a formaldehyde gel and transferred, overnight, onto a Hybond N+ Nylon Membrane (Amersham Pharmacia) according to the instructions provided with the membrane. RNA was fixed to the membrane by UV crosslinking in a Stratalinker 2400 (Stratagene). Prehybridization and hybridization were performed at 60°C with gentle agitation.

Probe Preparation and Panorama *E. coli* Genome Array Analysis

^{33}P-labeled cDNA was prepared as probe for the arrays using an optimized mixture of open reading frame (ORF)-specific *E. coli* primers according to the manufacturers instructions (Sigma Genosys). The cDNA synthesis tube contained 1x RT buffer, 50 pmol of the ORF-specific primers, 0.33 mM each of dATP, dTTP and dGTP, 20 µCi of ^{33}P-dCTP, 1ug of total RNA and 50 U of AMV reverse transcriptase in a final volume of 30 µl. Reactions were run for 3 hours and cDNA was purified from residual primers and dNTPs using a Sephadex G-50 microspin column (Amersham Pharmacia).

Radiolabelled cDNA was hybridized to the Panorama *E. coli* DNA macroarrays (Sigma Genosys) according to the manufacturer's instructions. Briefly, the purified cDNA was added to 5 ml of hybridization buffer (Sigma Genosys) and heated to 95°C for 10 min to denature the cDNA. The hybridization buffer with the probe was added directly to the array membrane after prehybridization using the same buffer. Hybridization continued for 18 hours after which the arrays were washed in Wash Solution (0.5x SSPE, 0.2% SDS) twice at room temperature for 5 min and twice at 65°C for 20 min with agitation. The arrays were exposed to Storage Phosphor screens and scanned on a Storm 280 Phosphorimager (Molecular Dynamics), and quantification of all 4,290 PCR-

amplified ORFs of the *E. coli* K-12 genome was performed using ImageQuant (Molecular Dynamics).

The volume reported for the duplicate spots was averaged to obtain the expression signal for each ORF on the array. Background was determined by the ImageQuant program using duplicate background spots on the membrane where no gene spots were present. The background value was automatically subtracted from the volume of the ORF spots. Normalization between the two arrays was performed by calculation of the total volume of all spots on the array and dividing the values from the two arrays. This ratio was then multiplied to the value of each ORF spot before dividing the expression signal from the two ORF spots on each array to obtain the differential expression (DE) of each gene.

Statistical analysis of the data is not possible because only 1 trial was performed on the data. The threshold value set by the manufacturer of 2-fold difference in expression was used to determine if an ORF was significantly changed in expression.

Results/Discussion

Northern Analysis of the *osmY* Gene

The most critical step in performing array experiments is isolation of clean, intact RNA. *E. coli* rapidly metabolizes the RNA with in the cell, so methods of RNA isolation must denature the native RNases found in *E. coli* rapidly (Tao, *et al.*, 1999). The hot acidic-phenol extraction protocol provided by the array manufacturer (Sigma Genosys) utilizes phenol and high temperatures to denature the RNases and remove them from the liquid phase. The low pH of the phenol removes DNA but leaves the RNA in the aqueous phase. The RNA isolated in this study had sharp rRNA bands with no downward smearing, but had a small amount of contaminating DNA. The DNA was removed using DNase I and the quality of the mRNA transcripts as well as the phenotype of the strains used was tested by performing Northern blot analysis on the *osmY* gene with the RNA samples obtained (Figure 1). *osmY* is highly expressed in stationary phase and is known

to be *rpoS*-dependent (Weichart *et al.*, 1993). The Northern reveals the presence of the *osmY* transcript in the wild type (MG1655) but not the control strain (*ΔrpoS*). *osmY* is expressed in a growth phase dependent manner, with the highest expression in stationary phase. The quality of the RNA is good, there is relatively little smearing of the transcript and the 16S rRNA control shows only a small amount of smearing as well.

Macroarray Analysis of *rpoS*-Dependent Genes Expressed in Stationary Phase

The RNA isolated from cultures grown to early stationary phase (OD_{600}=1.6) in the wildtype and *ΔrpoS* backgrounds was used to probe the arrays. Labeled cDNA was prepared with an incorporation of 40% for the wildtype RNA sample and 30% for the *ΔrpoS* sample. Scans of the arrays are shown in Figure B2. The spots on the membrane were quantitified and the differential expression (DE) of each ORF between the two arrays was calculated. A number of positively and negatively *rpoS*-dependent genes were found and identified (Tables B1 and B2).

There were only a limited number of *rpoS* dependent genes identified in the array which showed significantly different expression (Table B1, B2). Of the 9 genes found, only one, *dps*, has been previously shown as *rpoS*-dependent. *dps* is a highly abundant DNA binding protein in stationary phase important for DNA protection (Altuvia, *et al.*, 1994). A large number of highly expressed and highly *rpoS*-dependent genes were not identified including *katE, gabD* and *osmY*. This suggests that the array is not sensitive enough to determine changes in the mRNA expression of *rpoS*-dependent genes, or that the cDNA did not hybridize properly to the array. Also, the cDNA prepared may not be of sufficient quality to show the true expression levels of the *rpoS* regulon.

There was also a number of genes found that were negatively regulated by *rpoS* (Table 2). At least 4 genes involved in the synthesis of flagella are negatively regulated by *rpoS*. Among these is *fliC*, which had the highest DE value of all genes on the array. The *fliC* gene encodes flagellin, a key major part of the flagella complex. Adjacent to *fliC* is *fliA*, the gene for the flagellar sigma factor RpoF or σ^F (Mytelka and Chamberlin,

1996). *fliA* also shows higher expression in the *ΔrpoS* strain. RpoF regulon consists of many of the structural and regulatory genes for flagellar synthesis. Part of the up-regulation of flagellar genes in the *ΔrpoS* strain may be due to a lack of sigma factor competition. When there is no RpoS present, there is more RNA polymerase core enzyme available for other sigma factors, so expression of their respective regulons would be higher (Farewell, *et al.*, 1998; Ishihama, 2000).

The three genes *ogt*, *fnr* and b1332 are located adjacent to each other and show significant up-regulation in the *ΔrpoS* strain. *ogt* encodes a O-methylguanine transferase which is important in DNA repair (Rebeck and Samson, 1991) It is known that the spontaneous mutation rate increases in stationary phase (Siegele and Kolter, 1992). The *mutS* and *mutH* genes play an important role in DNA repair and are negatively regulated by *rpoS* (Tsui, *et al.*, 1997). The down regulation of *ogt* by *rpoS* may also play a role in increasing the mutation rate observed in stationary phase.

Caveats of the Macroarray Data

- The hybridization is inconsistent across the MG1655 array, possibly due in part to the large size of the arrays and minimal volume of hybridization solution. There is clearly more signal overall for the probes near the top of the array compared to the bottom of the array. The difference in hybridization makes normalization very difficult.
- It is difficult to gauge the degree of non-specific hybridization to each of the spots on the array. This is potentially a large problem, because a significant amount of hybridization is detected on the *rpoS* probe using the HS2210 (*ΔrpoS*) strain.
- Very few of the previously identified positively *rpoS*-dependent genes were identified as being differentially expressed on the array. This casts serious doubt on the validity of the data.

Table 1: Positively Regulated *rpoS*-Dependent Genes Identified by DNA Macroarray Analysis

Gene	Function	Fold-dependence
ydbD	Hypothetical, unknown function	2.06
rpoS	Stationary phase sigma factor	2.26
b1525	Putative enzyme, primary structure similar to *gabD*	2.03
b0619	Putative regulatory protein, similar to *yjdh*	2.43
b0960	Hypothetical, unknown function	2.13
b2656	Hypothetical, unknown function	2.35
dps	DNA protection during starvation	2.25
chpA	PemK-like protein 1	2.05
phnL	phosphonates transport ATP binding protein	2.01

Table 2: Negatively Regulated *rpoS*-Dependent Genes Identified by DNA Macroarray Analysis

Gene	Function	Fold-dependence
fliC	Flagellin: structural subunit of flagella	0.065
fliA	Sigma factor for flagellar operon (sigma F)	0.41
fliZ	Not required for motility; may regulate FliA	0.33
flgB	putative flagellar basal-body rod protein FlgB	0.32
yiaC	Hypothetical, unknown function	0.16
ogt	O-methylguanine transferase; DNA repair	0.27
fnr	Regulatory gene for nitrite and nitrate reductases	0.30
b1332	Hypothetical, unknown function	0.32
ompF	Outer membrane porin	0.54
yraQ	Hypothetical, unknown function	0.19
b2354	Hypothetical, unknown function	0.28

Figure B1: Northern Blot Analysis of the *osmY* and *rrnA* Genes. *osmY* is an RpoS and stationary phase dependent gene, *rrnA* is independent of stationary phase or RpoS. The quality of the RNA is good as both the *rrnA* and *osmY* bands are reasonably sharp. (+ = RNA samples were digested with DNase I once for 30 min, ++ = RNA samples were digested for a total of 60min to eliminate residual DNA).

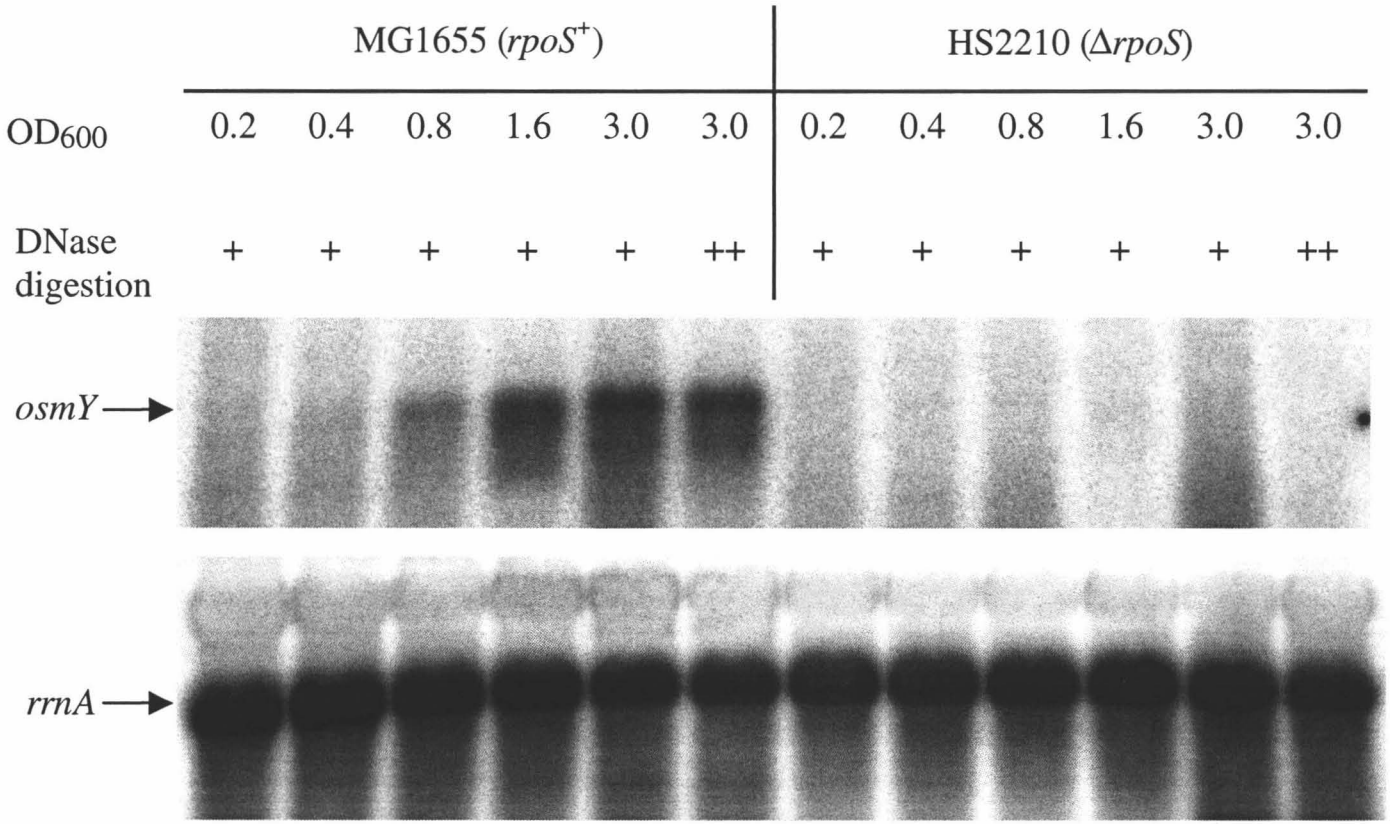
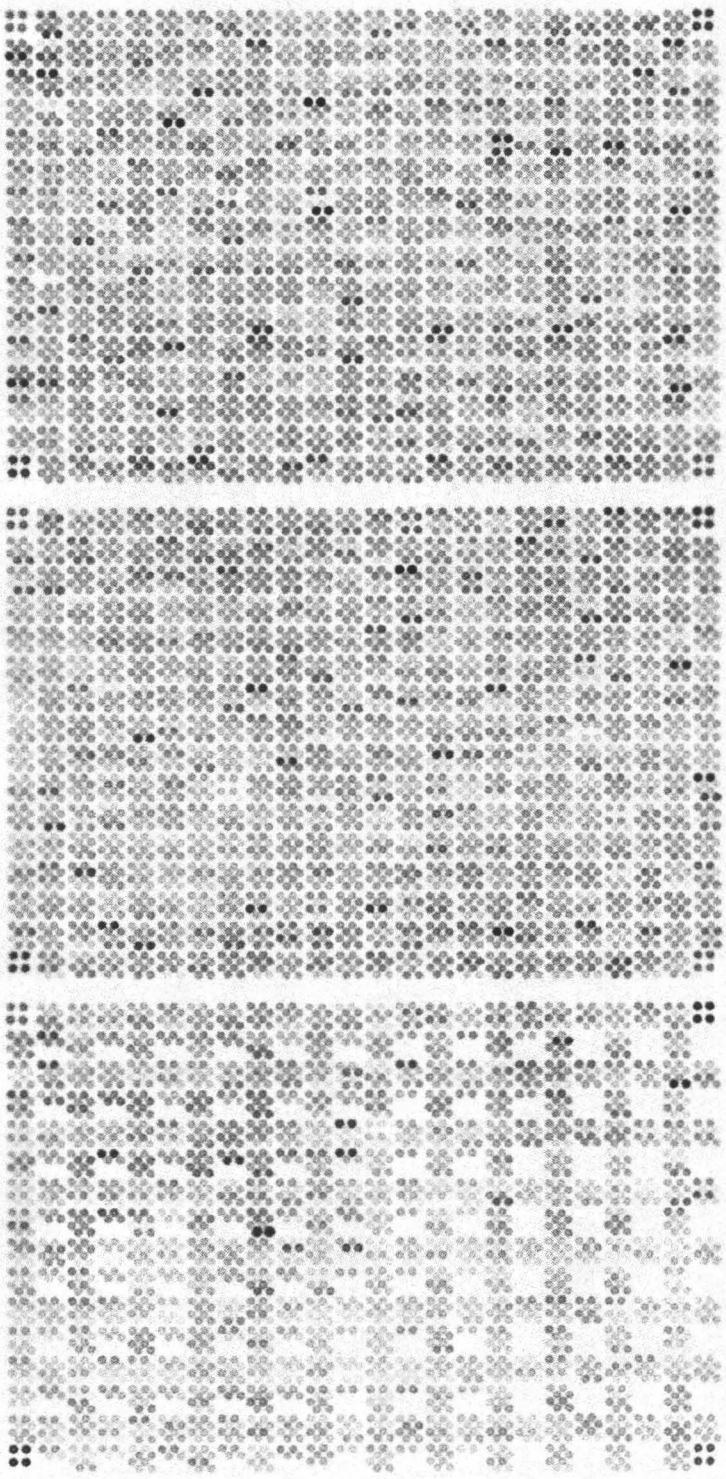

MG1655 (rpoS+)
HS2210 (ΔrpoS)
OD600
0.2 0.4 0.8 1.6 3.0 3.0 0.2 0.4 0.8 1.6 3.0 3.0
DNase digestion
+ + + + + ++ + + + + + ++
osmY
rrnA

Figure B2: Panorama Macroarray Analysis of Gene Expression. The Panorama macroarrays (Sigma Genosys) were prepared as described in the materials and methods section of Appendix B. P^{33}-labeled cDNA prepared from total RNA isolated from A) MG1655 (wildtype) or B) HS2210 (ΔrpoS) cultures grown to an OD_{600} of 1.6 were hybridized to the arrays, respectively.

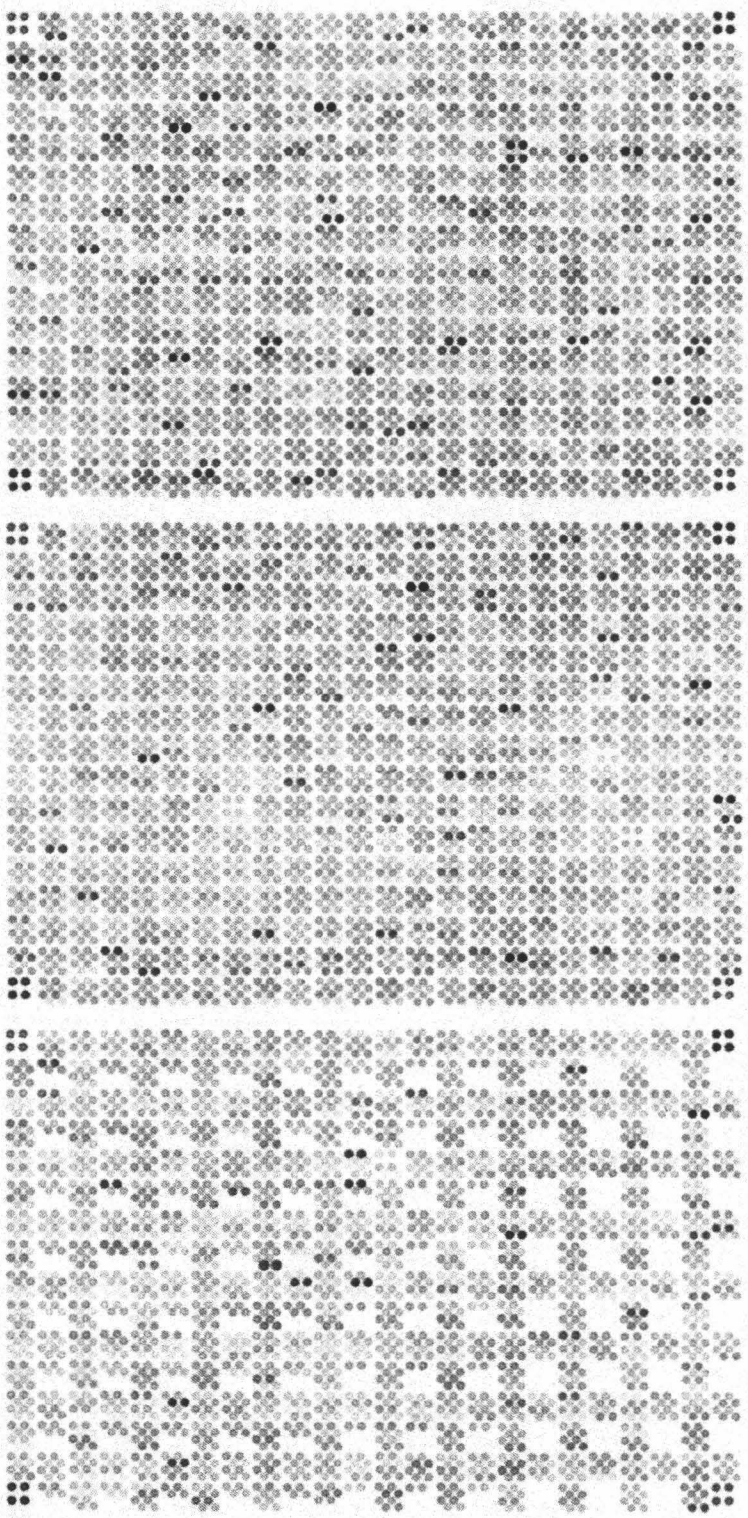
A

MG1655



B

HS2210



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Appendix C – Powerpoint Slides from Oral Defence Presentation

Regulation of the *gabDTP* operon and utilization of GABA as a sole nitrogen source in *E. coli*

Michael Schertzberg

December, 2004

Introduction

1) Nitrogen metabolism in *E. coli*

- Ammonia is the optimal nitrogen source for *Escherichia coli*
- At high ammonia concentrations, it is assimilated via glutamate dehydrogenase, directly into glutamate
- At low concentrations of ammonia, *E. coli* induces the expression of a nitrogen scavenging regulon, which is termed the Ntr response
- This allows the bacteria to use other sources of nitrogen, mainly organic compounds with amine groups, such as amino acids
- GABA (γ-aminobutyric acid) is one of the alternative nitrogen sources that can be utilized by *E. coli*

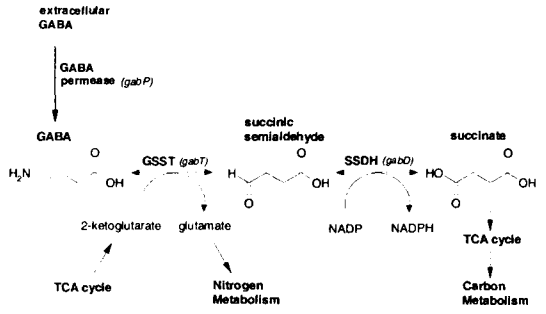
Introduction

2) Early work on GABA metabolism by the Halpern Lab

- It was originally reported that *E. coli* K-12 could not use GABA as a carbon or nitrogen source without the creation of mutants, which were not fully characterized, and all assays were performed in exponential phase
- Mutations mapped to a locus upstream of the operon, named *gabC*
- The structure of the operon was solved, and named *gabDTP*
- *gabD* encodes SSDH (succinic semialdehyde dehydrogenase)
- *gabT* encodes GSST (glutamate-succinic semialdehyde transaminase)
- *gabP* encodes a GABA permease
- The *gabDTP* operon is sensitive to catabolite repression where expression is repressed during growth on glucose and this repression is relieved by growth on poor nitrogen sources including GABA itself

Introduction

Pathway of GABA metabolism in *E. coli*



Introduction

3) Other work related to *gabDTP*

- Expression of the *gabDTP* operon has also been shown to be regulated by a number of conditions, including:
- Entry into stationary phase through the *rpoS* sigma factor (Schellhorn *et al.*, 1998; Baca-DeLancey *et al.*, 1999)
- Growth in conditioned rich media (cell-to-cell signaling) (Baca-DeLancey *et al.*, 1999)
- Alkaline pH in the media (Stancik *et al.*, 2002)

4) Identification of rsd operon *lacZ*-fusions to the *gab* region

- Schellhorn *et al.* (1998) identified 100 independent, randomly inserted operon *lacZ*-fusions that were *rpoS*-dependent. Six of these mapped to the *gab* operon region. Three of the fusions were reported, which all showed a high degree of *rpoS*-dependence.

Introduction

5) New information on GABA metabolism from Schneider *et al.* (2002)

- It is now known that wildtype *E. coli* K-12 can use GABA as a sole source of nitrogen with a doubling time of 6-7 hours.
- The fourth gene in the *gab* operon, *ygaE*, encodes a GntR-like transcriptional repressor that represses expression of the entire GABA operon
- This new downstream regulator was renamed *gabC*
- The *gab* operon is also regulated by nitrogen limitation by the Ntr response via the *nac* transcriptional activator
- GABA itself does not specifically induce expression of the *gab* operon

Schneider, B.L., S. Ruback, A.K. Kiupakis, H. Kasbarian, C. Pybus, and L. Reitzer. (2002) The *Escherichia coli gabDTPC* Operon: Specific γ-Aminobutyrate Catabolism and Nonspecific Induction. *J. Bacteriol.* **184**: 6976-86

Introduction

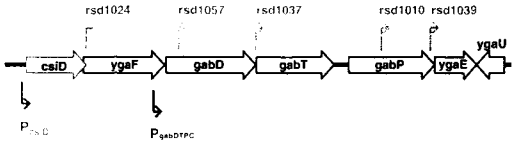
5) New information on GABA metabolism from Schneider *et al.* (2002)

- The *gabDTP* operon is transcribed from a promoter 101 bp upstream of *gabD*.
- Transcription from this promoter is sigma70 dependent and is positively regulated by Nac
- It reported that *rpoS*-dependent expression of the *gabDTP* operon is only seen in *gabC* mutant derivatives including strains containing the polar *gab* operon-*lacZ* fusions used in many studies

"Previous studies showing σ^S-dependent *gab* expression in stationary phase involved *gabC* mutants, which suggests that such expression does not occur in wild-type strains"

Introduction

Organization of the *csiD-ygaF-gabDTP-ygaE* locus



Blue arrows are locations of the rsd *lacZ*-fusions used in this study

The *csiD* gene and its promoter are identified in green

The brown arrow indicates the *gabDTP* operon promoter (Schneider *et al.*, 2002). The promoter is Nac and YgaE-dependent and is transcribed by σ^{70}-bound RNA Polymerase

Goals of this Study

1. Examine the regulation of the operon and its *rpoS*-dependence

- Is the *gabDTP* operon *rpoS*-dependent in wildtype strains?
- Is *ygaF* also *rpoS*-dependent?
- Are the enzymatic activities of GSST and SSDH dependent on *rpoS*?
- Where is the *rpoS*-dependent promoter? Is it the *csiD* promoter?
- Is this *rpoS*-dependent promoter dependent on YgaE (GabC)?

2. Examine the utilization of GABA as a sole nitrogen source

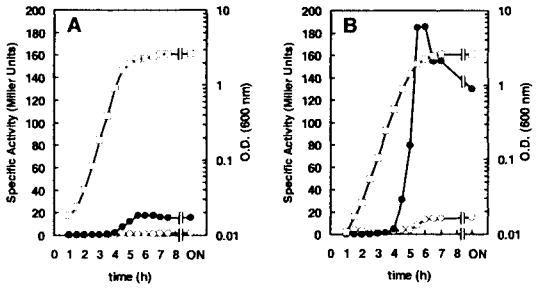
- Do wildtype cells grow better than *rpoS*-mutants on GABA as a sole nitrogen source?

Initial observations: Transcription of the *gabDTP* operon is *rpoS*-dependent

- Operon *lacZ* transcriptional fusions that were mapped to the *gab* operon were identified as being *rpoS*-dependent (Schellhorn *et al.* 1998).
- β-galactosidase activity of strains carrying the fusions was assayed in rich media over the growth curve of *E. coli* to determine the induction point and the degree of *rpoS*-dependence.

The fusions show a high stationary phase and *rpoS*-dependence

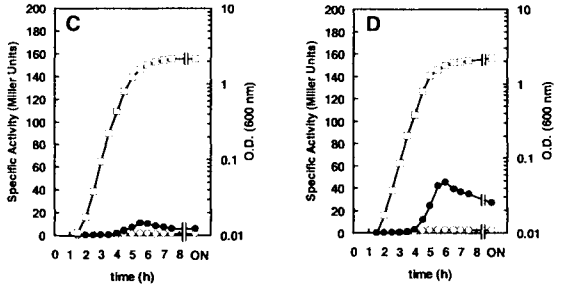
- Maximal expression occurs in early stationary phase and requires RpoS



rsd1024 (*ygaF-lacZ*) **rsd1057 (*gabD-lacZ*)**

The fusions show a high stationary phase and *rpoS*-dependence

- Maximal expression occurs in early stationary phase and requires RpoS

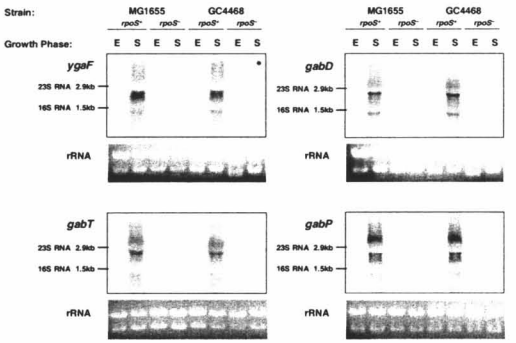


rsd1037 (*gabDT-lacZ*) **rsd1010 (*gabP-lacZ*)**

Is the *gabDTP* operon *rpoS*-dependent in wildtype strains?

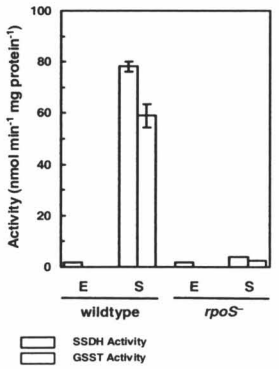
- One criticism raised about the use of *lacZ* fusions to study the expression of the operon, was that the *lacZ* fusions would have a polar effect on the expression of downstream genes.
- The *ygaE* gene is immediately downstream of the *gabDTP* operon and the *lacZ* fusions used could abolish its expression if it is part of the operon.
- This could affect expression of the operon, as YgaE was identified as a potential negative regulator of the operon (Schneider *et al.*, 2002).
- Little is known about the regulation and function of *ygaF*, its proximity to the *gabDTP* operon suggests it may also be *rpoS*-dependent and play a role in GABA metabolism.
- Hypothesis: Expression of the the operon is still *rpoS*-dependent in wildtype strains. Expression of *ygaF* is also highly *rpoS*-dependent.
- Experiment: 1) Perform Northern analysis on each of the *gabDTP* operon members and *ygaF* with wildtype strains to determine if transcription is dependent on *rpoS*. 2) Test the enzyme activities of GabD and GabT for *rpoS* dependence.

Result: Northern analysis of the *ygaF*, *gabD*, *gabT* and *gabP* genes confirms their *rpoS*-dependence in wild-type cultures



Results: The *rpoS*-dependence of the operon is consistent with an *rpoS*-dependent increase in GSST and SSDH enzyme activities

- Protein isolated from wildtype, stationary phase cultures has approximately 20-fold higher levels of both SSDH (GabD) and GSST (GabT) enzyme activity
- No GSST activity could be detected in exponential phase cultures



Is the *gabDTP* operon *rpoS*-dependent in wildtype strains?

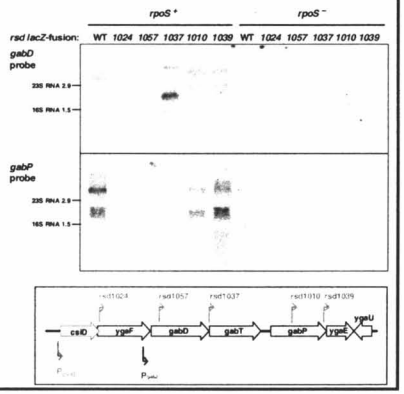
- The results of the Northern analysis and the enzymatic assays suggests that the *gabDTP* operon and the *ygaF* gene are highly *rpoS*-dependent
- It is unclear from these results whether the regulation by *rpoS* is **direct** (*rpoS* recognizing a promoter for *ygaF* and the *gabDTP* operon) or **indirect** (*rpoS* may positively regulate a transcription factor for the operon)
- A previous study reported that the *gabDTP* operon is not *rpoS*-dependent (Schneider *et al.*, 2002) , which does not agree with these results. It is unclear why this discrepancy exists. Some possible explanations:
  - Schneider *et al.* (2002) utilized the strain W3110, some strains are *rpoS*-negative. The strains we used (GC4468, MG1655) are both *rpoS*-positive
  - Schneider *et al.* (2002) performed their experiments in minimal media using exponential phase cultures. RpoS-dependence of the operon is observed upon entry into stationary phase

Where are the RpoS-dependent promoters for the operon?

- The location of the RpoS-dependent promoter(s) for the *gabDTP* operon are unknown. A promoter was identified by Schneider *et al.* (2002), however this promoter was dependent on σ^{70}-bound RNA polymerase for transcription.
- The *csiD* gene is located directly upstream of *ygaF* and the *gabDTP* operon and is relatively well characterized as *rpoS*-dependent. Is the promoter for *csiD* the *rpoS*-dependent *gabDTP* promoter?
- It is also unclear whether *gabP* has its own promoter, circumstantial evidence from Schneider *et al.* (2002) suggests that it does not.
- Hypothesis: There is a separate *rpoS*-dependent promoter for the *gabDTP* operon. There will be a single transcript from this promoter for the entire operon.
- Experiment: 1) The number and approximate size of transcripts produced for the *gabDTP* operon can be determined by northern analysis. Northern analysis of RNA from cultures harbouring the **polar** rsd *gab* operon *lacZ*-fusions will help to delineate where the promoters are. 2) RT-PCR of the *csiD-ygaF-gabDTP* region should identify any transcripts spanning all the genes.

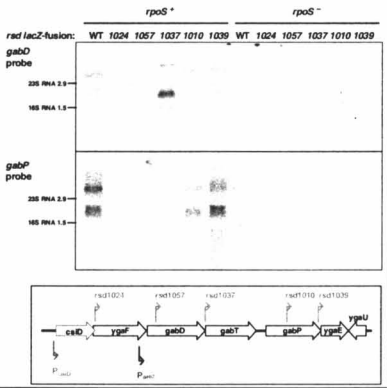
Results: Northern analysis of fusion strains and size of the *gab* operon transcripts

- There are two transcripts observed that include the *gabD* gene; a smaller transcript consistent with the *gabDT* genes, and a larger transcript consistent with the entire *gabDTP* operon.
- The presence of two distinct transcripts suggests there is either a specific RNA cleavage site or, alternatively, there may be a weak terminator located between *gabT* and *gabP*



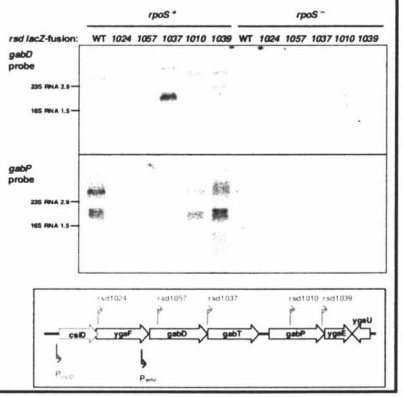
Results: Northern analysis of fusion strains and size of the *gab* operon transcripts

- The two fusions in *gabD* and the *gabDT* intergenic region (rsd1057 and rsd1037 respectively) both have a polar effect on *gabP* expression, which conclusively shows that *gabP* doesn't have a separate promoter



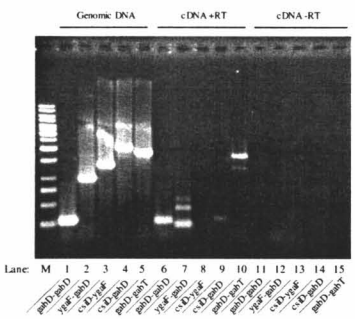
Results: Northern analysis of fusion strains and size of the *gab* operon transcripts

- Both fusions located in the *gabP* gene show a similar level of transcription of the operon compared to the wildtype strain.
- This indicates that either *ygaE* is not part of the operon, or *ygaE* does not regulate expression at the *rpoS*-dependent promoter.



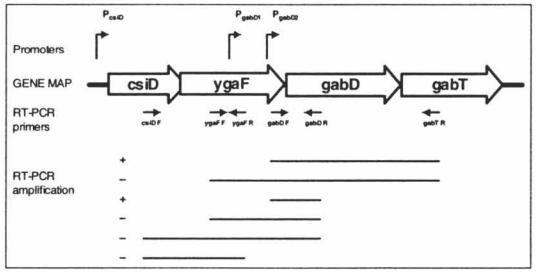
Results: *csiD* and *ygaF* are not part of the *gabDTP* operon

- RT-PCR of the *csiD-ygaF-gabDTP* region
- Although, all of the positive and negative controls showed the expected results, no RT-PCR product was observed spanning the midpoint of *ygaF* and the 5' end of *gabD*
- the *gabDTP* operon and *csiD-ygaF* comprise separate transcripts



Results: *csiD* and *ygaF* are not part of the *gabDTP* operon

- Although, all of the positive and negative controls showed the expected results, no RT-PCR product was observed spanning the midpoint of *ygaF* and the 5' end of *gabD*
- the *gabDTP* operon and *csiD-ygaF* comprise separate transcripts



Where are the RpoS-dependent promoters for the operon?

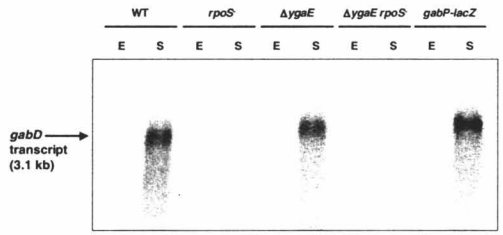
- These results suggest that there is an RpoS-dependent promoter upstream of the *gabD* gene (at the 3' end of the *ygaF* open reading frame) that transcribes the entire *gabDTP* operon. The RT-PCR analysis suggests that the *csiD* promoter is not responsible for transcription of the *gabDTP* operon.
- A recent study by Metzner *et al.* (2004) identified an RpoS-dependent promoter for the *gab* operon in the 3' region of the *ygaF* gene. Using primer extension the promoter was localized approximately 360 bp upstream of the *gabD* start codon.
- However, in contrast to our results, Metzner *et al.* (2004) showed that the *csiD* promoter drives transcription of the entire *csiD-ygaF-gabDTP* operon. The reason for this discrepancy is unknown.
- Interestingly, the transcription of the *gabDTP* operon may be stimulated by the CsiD protein. CsiD is likely an enzyme, and it may be involved in the metabolism of a compound that regulates the expression of the operon. This compound is not GABA.

Does YgaE (GabC) repress transcription from the *rpoS*-dependent promoter?

- YgaE encodes a putative transcriptional repressor of the *gabDTP* operon at the σ^{70}-dependent promoter for the *gabDTP* operon (Schneider *et al.*, 2002).
- From our results, Northern analysis of *gabD* expression was similar in WT and strains with a polar *gabP-lacZ* insertion. If the *ygaE* gene is part of the *gab* operon we would expect *gabD* expression to be higher in the *gabP-lacZ* strain. This suggests that the YgaE repressor does not repress transcription from the promoter recognized by RpoS.
- Hypothesis: The YgaE repressor does not regulate the *rpoS*-dependent transcription of the operon, thus a Δ*ygaE* mutant will have similar levels of *gabDTP* expression as a WT strain.
- Experiment: Northern analysis of *gabD* expression in WT, *rpoS*$^-$, Δ*ygaE* and Δ*ygaE rpoS*$^-$ mutants.

Results: Regulation of *gabDTP* by RpoS and YgaE are independent

- No significant difference was observed between wildtype and *ygaE* mutants in the levels of transcription of *gabD* in stationary phase
- In contrast, both of the *rpoS*⁻ strains show a much lower level of transcription in stationary phase



Does YgaE (GabC) repress transcription from the *rpoS*-dependent promoter?

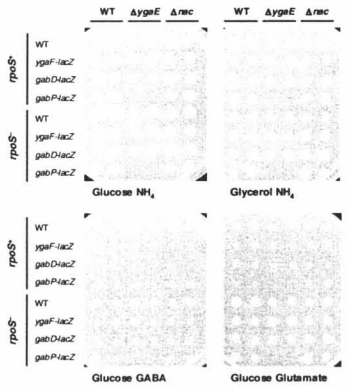
- These results indicate that YgaE does not repress the transcription of the *gabDTP* operon under the conditions tested (stationary phase in rich media).
- YgaE was implicated as a regulator of the operon because mutations in the gene result in higher levels of GabD and GabT enzyme activity (Schneider *et al.*, 2002).
- Metzner *et al.* (2004) reported that the *ygaE* gene is not part of the operon as previously predicted (Schneider *et al.*, 2002), and that YgaE is a negative regulator of the the *csiD* gene.
- YgaE is stationary phase-dependent but does not require *rpoS* for expression (Metzner *et al.*, 2004).

Are *rpoS*⁻ mutants impaired for growth on GABA as a sole nitrogen source?

- Expression of the *gabDTP* operon is highly *rpoS*-dependent and the levels of GSST and SSDH enzyme activity are also highly *rpoS*-dependent.
- Wildtype cultures of *E. coli* are able to utilize GABA as a sole nitrogen source (Schneider *et al.*, 2002), albeit very slowly (6-7 hours generation time).
- Hypothesis: RpoS mutants will be impaired in growth on GABA as a sole nitrogen source
- Experiment: Grow *E. coli* cultures on plates to qualitatively examine growth and determine generation time of WT and *rpoS*⁻ mutants in liquid media.

Results: *rpoS*⁻ mutants have a growth advantage on GABA as the sole nitrogen source

- When GABA is supplied as the sole nitrogen source, growth is very poor. However, in an *rpoS*⁻ mutant derivative, GC122, the growth is better, resulting in larger colonies
- Growth of *ygaE* mutants is also better than WT colonies, as expected
- Growth on glutamate is similar to growth on GABA, *rpoS* mutants grow better



Results: *rpoS*⁻ mutants have a growth advantage on GABA as the sole nitrogen source

- When growth rate was quantified in liquid media, both *rpoS* mutants and *ygaE* mutants grew 2-fold faster on GABA as the nitrogen source, relative to WT cultures
- Double mutants grew faster than the single mutants, suggesting that the effect of *rpoS* mutation and *ygaE* mutation are independent

Media	Strain	Doubling time, h ± SE
Glucose NH_4	WT	1.36 ± 0.02
	rpoS::*Tn10*	1.18 ± 0.12
	Δ*ygaE*	1.34 ± 0.01
	Δ*ygaE rpoS*::*Tn10*	1.17 ± 0.04
Glucose GABA	WT	16.7 ± 0.27
	rpoS::*Tn10*	7.4 ± 0.92
	Δ*ygaE*	7.3 ± 1.79
	Δ*ygaE rpoS*::*Tn10*	5.7 ± 0.57
Glycerol NH_4	WT	1.36 ± 0.03
	rpoS::*Tn10*	1.24 ± 0.06
	Δ*ygaE*	1.33 ± 0.02
	Δ*ygaE rpoS*::*Tn10*	1.27 ± 0.1
Glycerol GABA	WT	31.5 ± 2.88
	rpoS::*Tn10*	11.9 ± 2.34
	Δ*ygaE*	11.0 ± 3.55
	Δ*ygaE rpoS*::*Tn10*	5.1 ± 0.49

Are *rpoS*⁻ mutants impaired for growth on GABA as a sole nitrogen source?

- Unexpectedly, the results show that growth on GABA as a sole nitrogen source is better for *rpoS*⁻ mutants, despite the fact that expression of the *gabDTP* operon was highly RpoS-dependent.
- Mutants in the *gabP* gene show similar growth levels to wildtype strains, suggesting that the transport of GABA is not the limiting factor in its utilization
- Growth of colonies is similar on glutamate suggesting that the utilization of GABA as a nitrogen source may be limited by the ability of *E. coli* to utilize the glutamate produced from GABA breakdown.
- If *rpoS*⁻ mutants are better able to use glutamate as a sole nitrogen source, this could explain why the *rpoS*⁻ mutants can grow better on GABA.
- Glutamate pools are decreased in cultures of *rpoS*⁻ strains compared to wildtype strains (Tweeddale *et al.*, 1998), which supports this finding.

Are *rpoS*⁻ mutants impaired for growth on GABA as a sole nitrogen source?

- It is unclear how ammonia is derived from glutamate, one possible pathway is as follows:

Glutamate $\xrightarrow{\text{AspC}}$ Aspartate $\xrightarrow{\text{AspA}}$ Fumarate + NH_4

- Evidence suggests that this pathway may be negatively regulated by RpoS:
  - The *aspA* gene is regulated by FNR (Golby *et al.*, 1998), and *fnr* was identified as a negatively *rpoS*-dependent gene (Patten *et al.*, 2004)
  - The *aspC* gene is located directly downstream of the *ompF* gene, which is expressed 19-fold higher by *rpoS* mutants during nitrogen limitation.

Physiological relevance of the *gabDTP* operon

- **What is the role of the *gabDTP* operon in *E. coli*?**
  - GABA can be used as a nitrogen source by *E. coli*. The *gabDTP* operon is regulated by Nac in response to nitrogen limitation. Many genes upregulated by nitrogen limitation are involved in the transport and utilization of putrescine, a polyamine, including the *gab* operon (Schneider *et al.*, 1998).
  - The *gab* operon has been implicated in resistance to alkaline media (Stancik *et al.* 2002) The metabolism of GABA (a neutral molecule) into succinic acid could help keep the internal pH of the cell neutral in an alkaline environment.
  - Metabolism of GABA by the products of the *gabDTP* operon could aid in acid resistance. *E. coli* has three acid resistance systems. One system uses glutamate dehydrogenases (encoded by the *gadA* and *gadB* genes) to metabolize glutamate into CO_2 and GABA This reaction consumes protons and helps keep the internal pH neutral. Metabolism of GABA by the products of the *gab* operon could regenerate glutamate in the absence of an external glutamate source

Overall Conclusions

- The *gabDTP* operon is a complex operon regulated by a number of physiological conditions, including nitrogen limitation and entry into stationary phase.
- The *gab* operon is highly *rpoS*-dependent, at the transcriptional level. Little is known about how GabD, GabT and GabP are regulated at the post-transcriptional level, if at all
- The *gab* operon is transcribed from at least two promoters The first is RpoS-dependent, while the second is RpoD and Nac dependent. Both promoters lie within the *ygaF* coding sequence Previous studies suggest that the *gabDTP* genes are part of a larger operon with *csiD* and *ygaF*; however, our data suggested that the operon is not transcribed from the *csiD* promoter.
- The *rpoS*-dependent transcription of the operon was not repressed by YgaE
- Utilization of GABA as a nitrogen source is better in *rpoS* mutants. Growth is similar to that observed with glutamate as the nitrogen source, suggesting that the metabolism of glutamate by *E coli* may be a limiting factor for GABA utilization