ISOLATION OF γ-AMINOBUTYRATE (GABA) UTILIZING MUTANT STRAINS AND CHARACTERIZATION OF FACTORS SUFFICIENT FOR GABA UTILIZATION AS A CARBON AND NITROGEN SOURCE.

ISOLATION OF γ-AMINOBUTYRATE (GABA) UTILIZING MUTANT STRAINS AND CHARACTERIZATION OF FACTORS SUFFICIENT FOR GABA UTILIZATION AS A CARBON AND NITROGEN SOURCE

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE
(Biology)

McMaster University
Hamilton, Ontario

TITLE: Isolation of γ-aminobutyrate (GABA) utilizing mutant strains and characterization of factors sufficient for GABA utilization as a carbon and nitrogen source.

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NUMBER OF PAGES: xi, 94

Abstract

We have previously found that the *gab* operon of *Escherichia coli* is RpoS regulated suggesting that it plays an important role during stationary phase growth. The specific role of the *gab* operon is metabolism of γ-aminobutyrate (GABA). GABA, a four carbon amino acid, is the product of the glutamate decarboxylase reaction in *E. coli* and can serve as a source of carbon and nitrogen for members of the *Enterobacteriaceae*. Interestingly, we found that we could isolate GABA utilizing mutants even when they carried mutations that were predicted to abolish *gab* function. However, we were only able to isolate GABA utilizing mutants in the *rpoS*$^{+}$ background. This suggests two additional features of GABA metabolism in *E. coli*. One, that the *gab* operon in *Escherichia coli* is indeed dispensable for GABA utilization. Secondly, other RpoS regulated functions, independent of *gab*-encoded functions, may allow the cell to metabolize GABA. Finally, we have also investigated the inducing effects of GABA on *gab* expression in minimal and rich media and the role of glutamate in osmo-tolerance and acid adaptation.

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor, Dr. H.E. Schellhorn for providing me with the opportunity to study at McMaster University in pursuing a M.Sc. degree in his laboratory. I am grateful to him for his constructive criticism, patience, and overall support throughout the course of this project. I am also grateful to Dr. Dick Morton for his comments and suggestions during the thesis write-up process and for sitting on my committee.

My sincere thanks to my fellow lab members, both past and present, namely Jonathon P. Audia, Chantalle K. D' Souza, Lily Chang, Galen Chen, Dr. Rouha Kasra and of course all the summer students for all their technical advice, support and the laughs. Many thanks to all the friends I have made in the Biology Department. All this has helped make my stay here at McMaster University an enjoyable and memorable one.

Finally, I would like to thank my parents for their unconditional love and their continued support in every way possible. Also, I would like to thank God for putting me in the position to make something out of my life.

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List of Abbreviations

ADC	Arginine decarboxylase-initiated pathway
AST	Arginine succinyltransferase pathway
Ca^{2+}	Calcium
csiD	Carbon-starvation inducible gene D
CNS	Central nervous system
cAMP-CRP	cyclic adenosine monophosphate receptor protein
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
GABA	γ-aminobutyrate
Group I (CN)	Carbon and nitrogen utilizing GABA mutant
Group II (N)	Nitrogen utilizing GABA mutant
Group III (C)	Carbon utilizing GABA mutant
LB	Luria-Bertani
LXS	Luria-Bertani-5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside-Streptomycin
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Milliliter
mM	Millimolar
µm	Micromolar
MF	Mutational frequency
ng	Nanogram
nm	Nanometer
OD	Optical density
O/N	Overnight
ONPG	o-nitrophenyl-β-D-galactopyranoside
PCR	Polymerase chain reaction
REP	Repetitive extragenic palindromic elements
rpm	Revolutions per minute
RNA	Ribonucleic acid
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1. INTRODUCTION

1.1 Overview

Adaptation to different environmental stresses is critical for the survival of microorganisms. In their natural habitats, bacteria frequently encounter suboptimal growth conditions and in order to cope with these unfavourable circumstances, most microbes have developed complex stress response mechanisms. For instance, gram positive bacteria such as *Bacillus subtilis* respond to stresses by differentiating into highly resistant spores, while gram-negative species such as *Escherichia coli* and *Salmonella typhimurium* make use of stationary phase response mechanisms (Hengge-Aronis, 1996b).

Stationary phase cultures are easily distinguishable from those existing in exponential phase growth. This is because cells go from a state of maximal cell division and proliferation in exponential phase (generation time for *E. coli* in LB media is 25 minutes) to one of minimal cell growth and dormancy associated with stationary phase. Interestingly, this shift from exponential to stationary phase is accompanied by many changes in cellular physiology and morphology of the bacterial cultures with the purpose to offer longevity and increased resistance to the stresses associated with post-exponential phase growth. For instance, *E. coli* cells, normally rod-shaped during exponential phase growth, assume a smaller and more spherical shape in stationary phase (Lange et al., 1991a; Reeve et al., 1984). Moreover, the cytoplasm of the cells

condenses, the volume of the periplasm increases (Lange et al., 1991a; Reeve et al., 1984), the cell wall becomes cross linked and there is an increase in the degree of DNA supercoiling (Balke et al., 1987).

1.2 RpoS as a regulator

The end of exponential phase is marked by increased expression of genes which seem to help bacteria combat multiple stresses. Expression of these stationary phase genes is under the control of the *rpoS* encoded σ^S subunit (also called σ^{38} or RpoS) (Lange et al., 1991b; Mulvey et al., 1989) .This sigma S subunit of RNA polymerase is a master regulator that recognizes a set of promoters poorly recognized by the "housekeeping sigma factor" σ^{70}.

The RpoS regulon is quite large. Over forty genes and/or operons encoding a diversity of functions are known to be regulated by σ^S (Summarized in Table 1). These functions include DNA protection and repair (encoded by *katE, katG, aidB and xthA*) (Hengge-Aronis, 1996b), thermotolerance (*otsBA, htrE*) (Kaasen et al., 1992; Hengge-Aronis et al., 1991), osmotolerance (*osmY, proP, otsBA*) (Lange et al., 1993; Manna et al., 1994; Kaasen et al., 1992; Hengge-Aronis et al., 1991) and glycogen synthesis (*glgS*) (Hengge-Aronis et al., 1992). In some cases, RpoS also contributes to the virulence and pathogenesis of bacteria. RpoS is important for acid tolerance in *Salmonella typhimurium* (Lee et al., 1995) and in *Shigella flexineri* (Small et al., 1994). Likewise, RpoS regulates the expression of *spvR* in *S. typhimurium* (Kowarz et al., 1994). SpvR activates the expression of the plasmid borne virulence genes (*spvABCD*) (Kowarz et al., 1994) which are required for intracellular growth. Furthermore, in *Yersinia*

enterocolitica, an RpoS homologue regulates the enterotoxin gene *yst* which causes diarrhea (Iriarte et al., 1995).

The important role of RpoS in the *E. coli* stress response has led to the examination of similar RpoS systems in other bacteria. Homologues of *rpoS* have been discovered in *Salmonella typhimurium* (Kowarz et al., 1994), *Shigella flexineri* (Small et al., 1994), *Pseudomonas aeruginosa* (Fujita et al., 1994), *Yersinia entercolitica* (Iriarte et al., 1995), *Erwinia carotovora* (Becker-Hapak et al., 1997) and *Vibrio cholerae* (Yildiz et al., 1998). Furthermore, sigma factors that are structurally and functionally homologous to RpoS have been identified *in Acetobacter methanolicus, Xanthomonas camprestris, Pseudomonas putida*, and *Rhizobium meliloti* (Miksch et al., 1995). The role of RpoS in multiple stress protection seems to be fairly conserved amongst bacteria.

Regulation of RpoS expression is controlled at the levels of transcription, translation and protein stability (Lange et al., 1994b). While RpoS may be the predominant sigma factor in stationary phase, RpoS and many RpoS-dependent genes are also induced under various stress conditions including osmotic stress (Lange et al., 1994b), acid stress (Lee et al., 1995) and heat shock (Jishage et al., 1996) during exponential phase growth. Moreover, expression of many RpoS-dependent genes is far more complex than a simple activation by RpoS in that additional regulators are often involved either through binding near promoter regions or through cascade-like regulation. For example, *csiE* and *csiD* are co-regulated by RpoS and cAMP-CRP (Marschall et al., 1995; Marshall et al., 1998), while *osmY* (Lange et al., 1993) and *osmC* (Bouvier et al., 1998) are co-regulated by the DNA-binding protein Lrp (leucine responsive protein). Fis

(Xu et al., 1995), IHF (Lange et al., 1993) and H-NS (Barth et al., 1995) have also been identified as modulators of RpoS-dependent genes. Needless to say, more research needs to be done to learn more about this global stress response regulator known as RpoS and the complex network of genes it regulates. Doing so will undoubtedly have practical applications in the fields of pharmacology and medicine.

1.3 GABA: an inhibitory neurotransmitter in eukaryotes

GABA, or γ-aminobutyrate, is an amino acid. It is a four carbon compound and its chemical structure is as follows: NH_2-$(CH_2)_3$-COOH. Gamma-aminobutyric acid is the product of the glutamate decarboxylase reaction in *E. coli* (Marcus et al., 1969). It is also an intermediate in the pathway of L-arginine and putrescine (Figure 3) catabolism in *E. coli* and *K. aerogenes* (Friedrich et al., 1979; Shaibe et al., 1985). GABA can serve as a source of carbon and nitrogen for members of the *Enterobacteriaceae* and it is likely that small amounts of this amino acid are present in the natural environments of the members of the *Enterobacteriaceae* as a result of various degradative processes.

In 1950, GABA was independently identified and reported to be present in the vertebrate brain by Roberts and Frankel (1950) and Awapara et al. (1950). Early electrophysiological work carried out primarily in crustaceans firmly established GABA as an inhibitory neurotransmitter in invertebrates. By the early 1970s, GABA had been shown to satisfy all of the classical criteria of a neurotransmitter.

Amino acids are amongst the most abundant of all neurotransmitters present within the central nervous system (CNS). The majority of neurons in the mammalian brain utilize either glutamate or GABA as their primary neurotransmitters (Guyton and

Hall, 1996). In fact, GABA and glutamate have opposing inhibitory and excitory actions, respectively, and as a result, control to a large degree the overall "balance" in the CNS. In this way, these two neurotransmitters serve to regulate the excitability of virtually all neurons in the brain and have been implicated as important mediators of brain function or dysfunction (Hertz, 1983).

GABA is the major inhibitory neurotransmitter in the central nervous system. It is estimated that 30-40% of all CNS neurons utilize GABA as their primary neurotransmitter (Hertz, 1983). It is secreted by neurons in the spinal cord, basal ganglia, cerebellum, and many areas of the cortex. Like most neurotransmitters, GABA is stored in synaptic vesicles and is released in a Ca^{2+}-dependent manner upon depolarization of the presynaptic membrane. Following release into the synaptic cleft, GABA's actions may be terminated by selective uptake into presynaptic terminals and/or surrounding glia (the supporting cells of the nervous system that produce myelin sheath) where it gets metabolized. The world of pharmacology is heavily involved in creating drugs antagonistic to various parts of the GABA degradation pathway. For instance, GABA transaminase inhibitors cause high concentrations of GABA in tissues which is beneficial in lowering anxiety, preventing panic attacks, and protection against epileptic seizures (Guyton et al., 1996).

1.4 The *Gab* Operon:

1.4.1 Organization of the *gab* cluster

The specific role of the *gab* gene cluster is metabolism of γ-aminobutyrate (GABA) in *Escherichia coli* (Dover and Halpern, 1972a, 1972b, 1974). This cluster

consists of four genes, one regulatory gene and three structural genes (Figure 1). The three structural genes in this operon are *gabD*, *gabT* and *gabP*, where *gabD* and *gabT* encode the two metabolic enzymes, succinic semialdehyde dehydrogenase and glutamate succinic semialdehyde transaminase, respectively, involved in GABA catabolism; *gabP* codes for the γ-aminobutyrate transporter (Metzer et al., 1979; Niegemann et al., 1993). The control gene, *gabC*, specifies a *gab* repressor and is therefore believed to be a negative regulator of *gab* operon expression (Metzer et al., 1990). Previous studies (Kahane et al., 1978) seem to support this hypothesis where strains carrying the wild-type allele of *gabC* exhibit low expression of the *gab* genes but *gabC* constitutive strains showcase a derepression of the synthesis of enzymes involved in GABA utilization. The *gab* operon has been shown to be highly RpoS dependent (Schellhorn et al., 1998). The map position for the *gab* loci on the *E. coli* K-12 chromosome is at 57.5 min (Metzer et al., 1979).

The nucleotide sequences of the structural genes of the *gab* operon have been determined (Niegemann et al., 1993; Bartsch et al., 1990b). The three structural genes in the *gab* operon are transcribed unidirectionally from a common promoter upstream of *gabD* and their orientation within the cluster is 5'-*gabD-gabT-gabP*-3' (Figure 1) (Bartsch et al., 1990b; Metzer et al., 1990; Niegemann et al., 1993).

There lies an intergenic region of 234 base pairs between *gabT* and *gabP*, which contains three repetitive extragenic palindromic (REP) sequences with a length of 35-bp each (Niegemann et al., 1993). REP sequences of this type have been detected in the intergenic regions of a large number of operons from *E. coli* and *Salmonella typhimurium*

and are believed to play a role in regulating expression of genes within these operons (Gilson et al., 1984; Stern et al., 1984). In fact, REP units are found between genes with a several-fold step-down in expression of the distal gene compared to the promoter proximal gene (Gilson et al., 1984; Stern et al., 1984). These elements are possibly involved in downstream gene regulation within an operon by mechanisms like transcription termination via formation of stable hairpin loops, RNA cleavage or degradation arrest (Niegemann et al., 1993). Nevertheless, differences in relative expression of the *gab* genes are also suggested by codon usage. The calculated codon bias index of 0.34 and 0.27 for *gabD* and *gabT*, respectively, corresponds with the value of medium expressed *E. coli* genes, while the bias index of *gabP* (0.12) indicates a low expression rate of the gene (Niegemann et al., 1993).

1.4.2 Amino acid homologies of the *gab* gene products

The *gabD* gene consists of 1,449 nucleotides encoding a protein of 482 amino acids with a molecular mass of 51.7 kDa (Niegemann et al., 1993). The product of *gabD*, succinic semialdehyde dehydrogenase, shows significant homologies to the NAD^+-dependent aldehyde dehydrogenase from *Aspergillus nidulans* (Pickett et al., 1987) and several mammals (Hsu et al., 1988; Farres et al., 1989) and to the tumor-associated $NADP^+$-dependent aldehyde dehydrogenase from rats (Jones et al., 1988). The amino acid identity score was 35-38% between succinic semialdehyde dehydrogenase and the NAD^+-dependent aldehyde dehydrogenases and 29% between succinic semialdehyde dehydrogenase and the $NADP^+$-dependent tumor associated aldehyde dehydrogenase.

The *gabT* gene consists of 1,281 nucleotides encoding a 426 amino acid protein with a calculated molecular mass of 45.76 kDa (Bartsch et al., 1990b). The product of *gabT*, GABA transaminase, shows significant homologies to the ornithine transaminases (OAT) from *Saccharomyces cerevisiae* (SCOAT), from rat and from human mitochondria. The amino acid identity score was 28.2% between GABA transaminase and OAT from human mitochondria, 27.9% between GABA transaminase and OAT from rat mitochondria, and 27.5% between GABA transaminase and SCOAT (Bartsch et al., 1990b). These findings are in accordance with the nucleotide sequence for *S. cerevisiae* GABA transaminase (Andre et al., 1990). The fact that ornithine is accepted as a substrate by several GABA transaminases (Schulz et al., 1990; Yonaha et al., 1985; Yonaha et al., 1980), suggests that the structural relatedness of these aminotransferases may reflect functional similarities of these proteins.

The *gabP* gene consists of 1,401 nucleotides encoding a highly hydrophobic protein of 466 amino acids with a molecular mass of 51.1 kDa (Niegemann et al., 1993). The polypeptide consists of 12 highly hydrophobic regions of 20 amino acids each, connected by short hydrophilic sequences. The N- and C-terminal ends of the protein (15 amino acids each) are hydrophilic (Niegemann et al., 1993). The *gabP* gene product, GABA permease, shows strong resemblance to the proline, arginine and histidine permeases from *Saccharomyces cerevisiae* (Hoffmann, 1985; Tanaka et al., 1985; Vandenbol et al., 1989) with amino acid identity scores of 27.5%, 30.4% and 28%, respectively. The GABA permease also shows significant homology to the proline transport protein from *Aspergillus nidulans* (Sophianopoulou et al., 1989) with an amino

acid identity score of 31.2%. The highest degree of homology, however, was observed with the aromatic amino acid transport protein from *E. coli*, the product of the *aroP* gene (Honore et al., 1990). Furthermore, no sequence similarities were found between the GABA transporter and the two glutamate carriers from *E. coli* encoded by the *gltS* and *gltP* genes, respectively (Wallace et al., 1990; Kalman et al., 1991).

1.5 Pathways Involved in GABA utilization:

1.5.1 GABA transport in *E. coli*

Since GABA may serve as a source of either carbon or nitrogen, it is advantageous to the cell to be able to enhance its uptake in response to signals which relay a shortage in either one of them. To serve this purpose, there exists in *E. coli* a highly specific membrane transport carrier for GABA encoded for by the *gabP* gene. GABA permease, encoded by *gabP*, is responsible for the transport of γ-aminobutyrate (GABA) across the *E. coli* cell membrane. The permease is active in whole cells as well as in rightside-out vesicles (Kahane et al., 1978) and uptake of GABA is driven by membrane potential but abolished by proton ionophores (Niegemann et al., 1993). Previous studies (Kahane et al., 1978; Niegemann et al., 1993) show that the transporter is highly specific for γ-aminobutyrate transport, being unable to recognize alternative ligands such as the 20 common α-amino acids, ornithine and putrescine, all of which exhibit superficial structural similarity to GABA. The requirement of the GABA transport system has been demonstrated by the use of GABA transport mutants (Kahane et al., 1978) where mutants isolated were found to have lost the ability to accumulate GABA due to mutational lesions existing within *gabP*, but retained the parental high

levels of GABA transaminase and succinylsemialdehyde dehydrogenase, the degradative enzymes of the GABA catabolic pathway.

More recent studies (Brechtel et al.,1995a; King et al., 1995b), however, now indicate that the *gabP* transport channel can recognize and translocate a far more diverse range of chemicals than previously imagined. In fact, numerous molecules having different sizes and shapes are capable of interacting with the GABA permease either as inhibitory ligands and/or as transported substrates. Moreover, there is evidence which suggests that these inhibitory molecules exert their affect by acting within the *gabP* transport channel itself rather than at an allosteric inhibitory site (Brechtel et al., 1996).

1.5.2 GABA metabolism in *E. coli*

Once transported into the *E. coli* cell, γ-aminobutyrate catabolism occurs in two successive steps leading to its conversion to succinate which then gets utilized in the TCA cycle (Figure 2). The enzymes involved in this two step degradative reaction are 4-aminobutyrate transaminase, encoded by *gabT*, and succinic semialdehyde dehydrogenase, encoded by *gabD*, respectively. The pathway of GABA breakdown in *E. coli* K-12 seems to be the same as that in *Pseudomonas fluorescens* (Scott et al., 1959; Jakoby et al., 1960; Jakoby et al., 1959). The first step is transamination between GABA and α-ketogluterate to yield succinyl semialdehyde and glutamate. This reaction is catalyzed by γ-aminobutyrate-α-ketoglutarate transaminase. Succinyl semialdehyde is then oxidized to succinate by succinyl semialdehyde dehydrogenase for use in the tricarboxylic acid cycle.

Wild-type *E. coli* K-12 strains unable to utilize GABA as the sole source of nitrogen or carbon or both exhibit very low activity of these two enzymes involved in GABA breakdown, but mutants selected for their ability to utilize GABA as the sole source of nitrogen show a six to nine-fold increase in the activities of these two enzymes (Dover and Halpern, 1972a). This increase in enzymatic activity is not accompanied by any changes in the affinities of the mutant enzymes for their respective substrates (α-ketoglutarate for *gabT* and NADP for *gabD*) (Dover et al., 1972b) which strongly suggests that the mutations responsible which enable the cells to utilize GABA as the sole source of nitrogen occur at a control locus which regulates the synthesis of these enzymes, rather than affecting a structural gene. A similar situation has been described on the utilization of histidine by *Salmonella typhimurium* (Smith et al., 1971a; Smith et al., 1971b). The wild-type strain unable to utilize histidine as a single source of carbon or nitrogen or both, exhibits very low activities of histidase and urocanase, enzymes which catalyze the first two steps in the breakdown of histidine to glutamate. However, a mutant isolated for its ability to utilize histidine as a nitrogen source showed greatly increased levels of these two enzymes.

Further support for the hypothesis that the two enzymes of the pathway of GABA utilization belong to a single regulatory unit is evident by the fact that the synthesis of γ-aminobutyrate transaminase and succinic semialdehyde dehydrogenase is strictly coordinate under a great variety of growth conditions (Dover and Halpern, 1972b). That is, changes in conditions affect the rate of synthesis of these two enzymes to the same extent. These observations disprove earlier evidence which argued against the two

GABA enzymes constituting a single operon (Dover and Halpern, 1974) and support the contention that the two enzymes of the GABA utilization pathway constitute an operon (Jacob et al., 1961; Bartsch et al., 1990b; Niegemann et al., 1993).

1.6 Response of the *gab* operon to catabolite repression

There is significant evidence which suggests that the *gab* operon is sensitive to strong catabolite repression. Studies (Kahane et al., 1978) suggest that the synthesis of the GABA transport carrier is subject to strong catabolite repression. Evidence for this springs from the fact that the rate of GABA uptake by cells grown in glucose-NH_4^+ medium is only about one-ninth of the rate obtained with succinate-NH_4^+ grown cells which in turn is 50% less than the GABA transport activity of glucose-GABA grown cells (Kahane et al., 1978).

Likewise, the two enzymes involved in the GABA degradation pathway in *E. coli* K-12 are also subject to severe catabolite repression (Magasanik et al., 1961). In cultures grown in minimal media with ammonium salts as the sole source of nitrogen, both enzymes are severely repressed by glucose. Under these conditions, whether serving as the sole carbon source or when present in the medium together with succinate or GABA, glucose repressed the synthesis of γ-aminobutyrate transaminase and succinic semialdehyde dehydrogenase to less than 10% of that found in cells grown in succinate medium in the absence of glucose. Addition of GABA did not relieve glucose repression, nor did GABA affect the levels of the two enzymes in succinate-ammonia medium in the absence of glucose (Dover and Halpern, 1972b).

Interestingly, unlike other catabolite-sensitive systems, the GABA system resists catabolite repression under conditions of limited nitrogen supply (Dover and Halpern, 1972b). That is, escape from repression by glucose occurs only when the ammonia in the growth medium is substituted with GABA as the sole nitrogen source. However, this escape from repression is not a specific effect of GABA because this escape from repression of the enzymes involved in GABA metabolism may also be brought about by substitution of ammonia with glutamate or aspartate, although the extent of derepression is less marked than in the case of GABA as the sole nitrogen source (Dover and Halpern, 1972b). Nevertheless, in *E. coli*, GABA nitrogen utilizing mutants have been isolated in which the synthesis of γ-aminobutyrate succinic semialdehyde transaminase remains repressed in glucose media even under conditions of nitrogen limitation, while the synthesis of succinic semialdehyde dehydrogenase is derepressed under the same conditions (Dover and Halpern, 1974). This finding seemed to argue that the transcription of the two structural genes involved in GABA metabolism is initiated at two separate promoters and that there is more than one dehydrogenase. Metzer and Halpern (1990) cloned the *E. coli* K-12 *gab* region and postulated that the *gab* genes may be divergently transcribed by two different promoters. Nevertheless, more recent studies now confirm that the two structural genes of the GABA regulon do constitute one operon (Niegemann et al., 1993).

A similar escape from catabolite repression has been described for the histidine utilization enzymes in *Aerobacter aerogenes* following substitution of the ammonia in the growth medium with histidine (Neidhardt et al., 1957). Conversely, in *Salmonella*

typhimurium, in which histidine utilization is also subject to catabolite repression, no such escape was observed (Magasanik et al., 1961). It is important to mention that this escape from glucose repression upon substitution of ammonia with other nitrogen sources was found to be specific for the enzymes of the GABA utilization pathway and did not involve other enzymes sensitive to catabolite repression, such as β-galactosidase and aspartase. In fact, β-galactosidase and aspartase were as strongly repressed by glucose in the presence of GABA or aspartate as in the presence of ammonia as the source of nitrogen (Dover and Halpern, 1972b).

It has also been shown that the factor responsible for this relief from repression is glutamine synthetase which is derepressed under conditions of nitrogen limitation (Zaboura et al., 1978). Experiments with glutamine synthetase negative and glutamine synthetase constitutive mutants strongly indicate that glutamine synthetase is the effector which regulates GABA carrier synthesis as a function of nitrogen availability (Zaboura et al., 1978) and by doing so acts as a positive regulator in the *E. coli* GABA control. Glutamine synthetase probably acts as an activator of transcription in the *gab* system of *E. coli* (Magasanik et al., 1974). Activation by glutamine synthetase is not required in situations where no catabolite repression prevails, probably because of the availability of sufficient CRP-cyclic AMP complex to serve as an alternative activator. Another transport system that may be regulated by glutamine synthetase is that of glutamine in *Salmonella typhimurium* (Ayling et al., 1976) and possibly also in *E. coli* (Furlong et al., 1975).

1.7 Pathway of arginine catabolism

L-Arginine can serve as sole carbon and nitrogen source for *K. aerogenes*, as a poor nitrogen source for *S. typhimurium* and *E. coli*, and as a source of carbon for some *E. coli* and Proteus strains (Cunin et al., 1986; Friedrich et al., 1978; Kustu et al., 1979; Shaibe et al., 1985). In bacteria, two pathways exist for arginine catabolism (Cunin et al., 1986). The first pathway (Figure 3) is initiated by arginine decarboxylase, hence the name ADC pathway, which degrades arginine to agmatine and then eventually through a series of steps to succinate (Cunin et al., 1986). This pathway (Shaibe et al., 1985; Wilson et al., 1969) consists of six enzymatic steps and it converges with the GABA degradation pathway (Figure 2). Another pathway of arginine catabolism, called the arginine succinyltransferase (AST) pathway, exists in bacteria which is initiated as a response to succinylation of arginine. Degradation of arginine via this AST pathway eventually yields glutamate and succinate (Cunin et al., 1986).

1.8 Project Outlines and Objectives

This project was initiated by Dr. Rouha Kasra and Dr. Herb Schellhorn in the summer of 1999. Dr. Kasra began by isolating the three classes of GABA utilizing mutants from the wild type MC4100 background under Dr. Schellhorn's supervision. Due to problems in locating her strains after her departure, this work was repeated by myself in the fall of 1999, shortly after being assigned this project. Dr. Schellhorn carried out the transductions referred to in Materials and Methods.

The goal of the project was to characterize GABA mutants in order to elucidate the mutations responsible for GABA utilization in these mutant strains. Primers were designed to amplify the potential *gab* promoter (*csiD* promoter) and sequence analysis was performed to identify the possible mutation site. Furthermore, as a result of this study, we were able to show the existence of alternative GABA utilization pathway(s) that do not require the *gab* operon. Finally, we tested various putative inducers of *gab* expression and analyzed the protective functions of glutamate against salt and acid stress.

Table 1. Members of the RpoS Regulon in *E. coli*.[a]

Gene/ Operon	Function	RpoS-dependence[b]	Reference
aidB	involved in leucine metabolism; defends against DNA methylated damage	20	Landini *et al.*,1984; Volkert *et al.*, 1994
appY	controls expression of *hya* and *cyxAB- appA*	2	Atlung *et al.*,1994
bolA	confers round morphology, controls synthesis of PBP6	12	Aldea *et al.*,1989
cbpA	chaperone, curved DNA binding protein	5	Yamashino *et al.*,1994
cfa	?	6	Wang *et al.*,1994
csiE	?	15	Weichart *et al.*, 1993
fic	involved in folate synthesis	3	Utsumi *et al.*,1993
gabT	involved in GABA metabolism, encodes glutamate-succinic semialdehyde transaminase	18	Beca-DeLancey *et al.*,1999
gabD	involved in GABA metabolism, encodes succinic semialdehyde dehydrogenase	19	Schellhorn *et al.*,1998
gabP	involved in GABA transport, encodes GABA permease	31	Schellhorn *et al.*,1998
glgS	glycogen synthesis	3	Hengge-Aronis and Fischer, 1992
himA	gene regulation	2	Aviv *et al.*, 1994
hmp	anaerobic metabolism of nitrogen	2	Membrillo-Hernandez *et al.*, 1997
hyaABC DEF	oxidation of hydrogen	20	Brondsted and Atlung, 1994
katE	protection against H_2O_2	52	Sak *et al.*, 1989; Schellhorn and Stones, 1992
ldcC	maintains pH homeostasis	7	Kikuchi *et al.*, 1998; Van Dyk *et al.*,1998
osmY	?	68	Lange and Hengge-Aronis, 1993
otsBA	trehalose synthesis, osmoprotection, thermotolerance	32	Kaasen *et al.*, 1992; Hengge-Aronis *et al.*, 1991
poxB	synthesis of acetate	99	Chang *et al.*, 1994
proP	osmoprotection	4	Manna *et al.*, 1994
xthA	DNA repair, hydrogen peroxide resistance	3	Sak *et al.*, 1989
yciG	?	188	Van Dyk *et al.*, 1998
yohF	?	51	Fang *et al.*, 1996; Van Dyk *et al.*, 1998

[a]All genes/operons listed are positively regulated by RpoS.
[b]Values for RpoS dependence were calculated by comparing the maximum activity of each fusion in an *rpoS*$^+$ and *rpoS*$^-$ background.

Figure 1. Schematic representation of the *gab* region. Shown are the structural genes of the *gab* cluster, the nucleotide sequence and important structural features (transcriptional and translational start sites, -10 and -35 positions of the *csiD* promoter, and highly conserved nucleotides in the CRP binding site) of the *csiD* promoter and the primers designed to amplify the *csiD* promoter. Note that *gabC* is located upstream of the *gab* operon (Diab, K., Metzer, E. and Halpern, Y.S. unpublished, U68244).

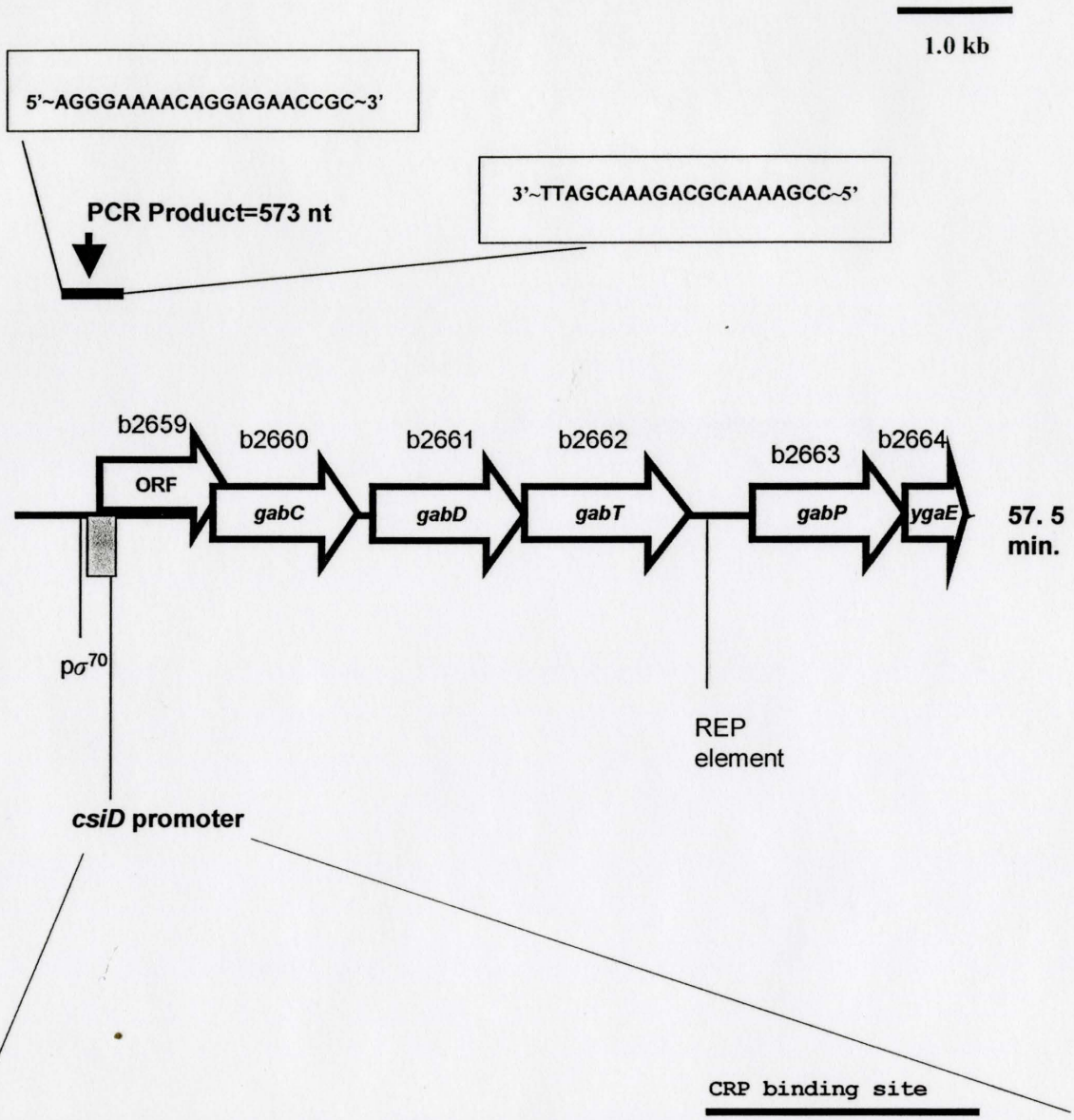

1.0 kb
5'~AGGGAAAACAGGAGAACCGC~3'
3'~TTAGCAAAGACGCAAAAGCC~5'
PCR Product=573 nt
b2659
ORF
b2660
gabC
b2661
gabD
b2662
gabT
b2663
gabP
b2664
ygaE
57. 5 min.
pσ70
REP element
csiD promoter
CRP binding site
gctttagttattcatttcctgtctcactttgccttaataccctacgttaaatgttactaatttgttgcttttgatcacaataagaaaacaa
tatgtcgcttttgtgcgcatttttcagaaatgtagatattttagattatggctacgaaatgagcatcgccatgtcaccctacatctca
-35
-10
+1
taagaggatcgcttctgatg

Figure 2. The pathway of GABA degradation in *E. coli*. Shown are the three *gab* genes and the enzymes that they encode which are involved in the metabolism of GABA. The first step in this process is the transport of GABA across the *E. coli* cell membrane. This step is catalyzed by the product of *gabP*. The next two steps are responsible for GABA catabolism: the first of these being a transamination reaction catalyzed by the product of *gabT*, and the last being an oxidation reaction catalyzed by the *gabD* gene product.

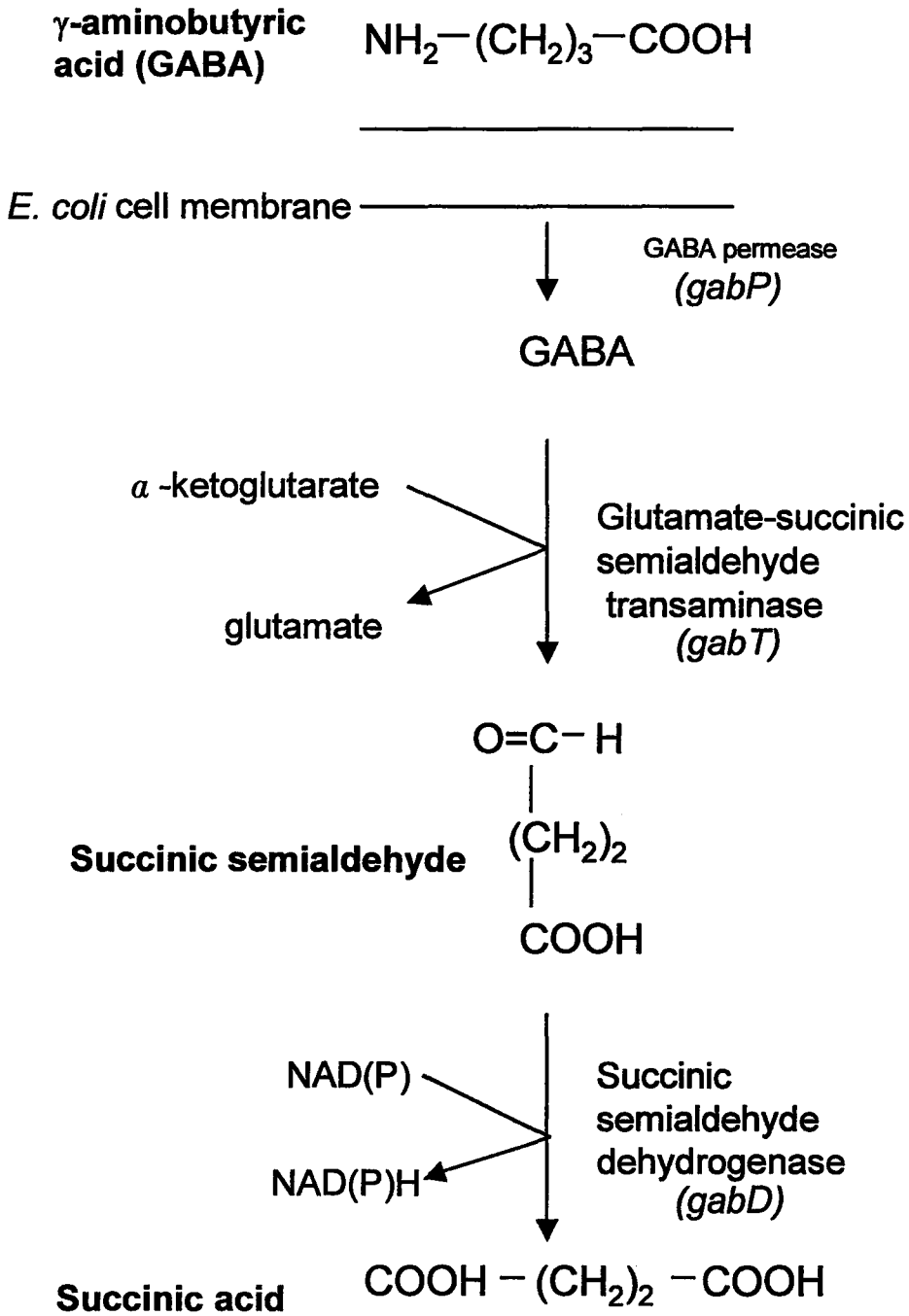
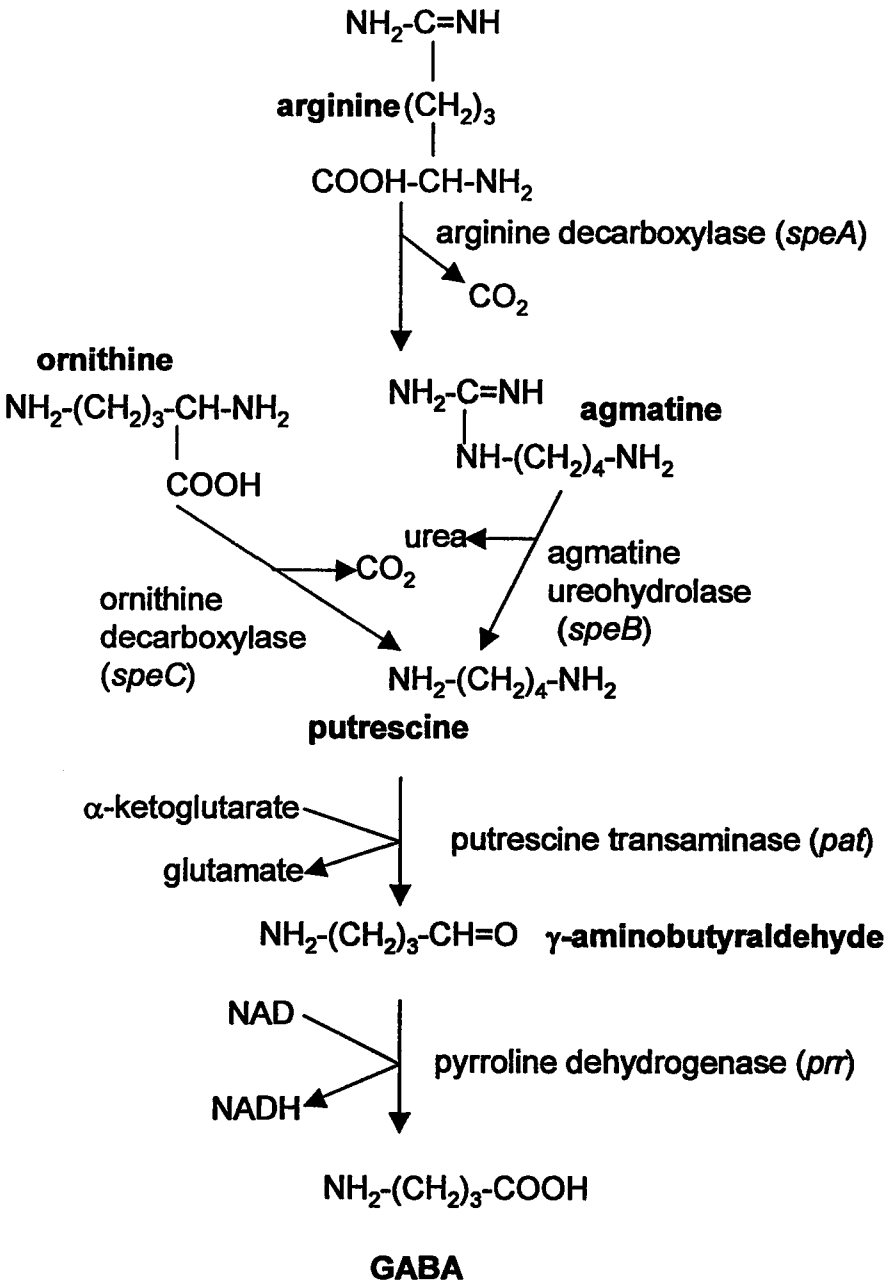

γ-aminobutyric acid (GABA)
NH2—(CH2)3—COOH
E. coli cell membrane
GABA permease
(gabP)
GABA
α -ketoglutarate
glutamate
Glutamate-succinic semialdehyde transaminase
(gabT)
Succinic semialdehyde
O=C—H
(CH2)2
COOH
NAD(P)
NAD(P)H
Succinic semialdehyde dehydrogenase
(gabD)
Succinic acid
COOH—(CH2)2—COOH

Figure 3. The arginine decarboxylase-initiated (ADC) pathway of arginine catabolism. Enzymes involved at every step are indicated and genes encoding for these respective enzymes are italicized in parenthesis (Shaibe et al., 1985).


NH2-C=NH
arginine (CH2)3
COOH-CH-NH2
arginine decarboxylase (speA)
CO2
ornithine
NH2-(CH2)3-CH-NH2
COOH
NH2-C=NH
NH-(CH2)4-NH2
agmatine
urea
CO2
agmatine ureohydrolase (speB)
ornithine decarboxylase (speC)
NH2-(CH2)4-NH2
putrescine
α-ketoglutarate
glutamate
putrescine transaminase (pat)
NH2-(CH2)3-CH=O
γ-aminobutyraldehyde
NAD
NADH
pyrroline dehydrogenase (prr)
NH2-(CH2)3-COOH
GABA

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacterial strains

The bacterial strains used in this study are listed in Tables 2a and 2b.

2.2 Chemicals and media

All chemicals were supplied by either Fisher Scientific, Ltd. (Toronto, Ontario, Canada), Sigma Chemical Co. (St. Louis, Mo.), Gibco BRL (Burlington, Ontario, Canada), or BDH Inc. (Toronto, Ontario, Canada). Antibiotics and other nonautoclavable stock solutions were filter sterilized with Gelman Sciences (Ann Arbor, Michigan) acrodisc sterile filters (pore size, 0.45 um). Liquid media was prepared as described by Miller (1992). The concentrations of antibiotics used were as follows: kanamycin (50 ug/ml), streptomycin (100 ug/ml), and tetracycline (15 ug/ml). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 50 ug/ml.

2.3 Growth Conditions

All strains used were MC4100 derivatives (see Table 2a). Cultures were grown routinely in Luria-Bertani (LB) rich media at 37°C containing the appropriate antibiotics. Cell growth was monitored spectrophotometrically (UV-VIS spectrophotometer, model UV-1201, Shimadzu Corporation, Kyoto, Japan) at optical density of 600 nm (OD_{600}).

2.4 Assay for β-galactosidase

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

Cultures were grown overnight in LB media containing the appropriate antibiotics. For expression studies, the bacterial cultures were maintained in early exponential phase ($OD_{600} < 0.2$) for at least eight generations prior to the start of each growth experiment. This ensured that measured β-galactosidase activity in early exponential phase cultures was due to *de novo* enzyme synthesis rather than being due to carry over from overnight stationary phase cultures. To ensure that the bacteria were in exponential growth, overnight cultures were diluted 1:1000 in fresh LB medium and incubated at 37°C with vigorous shaking (250 rpm). After 3 hours, the cultures were diluted again 1:100 (OD_{600}=0.02). For induction studies, conditioned medium and LB media supplemented with GABA (0.2%) were used as the growth medium. For the latter, the assay was done in duplicate. Subsequently, the cells were sampled and assayed for β-galactosidase activity at the times indicated. Cultures were grown in flasks at a culture/flask volume ratio of 1 to 5 at 37°C and 250 rpm to maintain proper aeration. β-galactosidase activity was assayed as described by Miller (1992). Units of activity were calculated as [1000 X OD_{420}]/[time of incubation (min.) X volume (ml) X OD_{600}] and were expressed as Miller units.

2.5 PCR amplification and sequence analysis

Primers used to amplify the *E. coli csiD* (carbon starvation-inducible gene) promoter (Marschall et al., 1998) were as follows: forward primer (AB19298) 5'-AGGGAAAACAGGAGAACCGC-3' and reverse primer (AB19299) 5'-CCGAAAACGCAGAAACGATT-3'. These primers were specifically designed using Primer Design Program (http://www.williamstone.com/) on the GenBank database

sequence (AE000351) consisting of the *csiD* promoter sequence as listed in Marschall et al. (1998). Each PCR tube contained 1 X PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 50 pmoles of each of the forward and reverse primers, 0.4 mM of each of the 4 nucleotide triphosphates, 4 mM $MgCl_2$, ~ 50 ng of *E. coli* MC4100 template DNA and ~ 5 U of Taq polymerase in a final volume of 50 ul. Amplification was performed under the following conditions: (1) 95°C for 2 minutes (pre-denaturation step); (2) (denaturation: 95°C for 15 seconds, annealing: 62°C for 30 seconds, elongation: 72°C for 1 minute, 25 cycles); (3) 72°C for 7 minutes (final extension step). We obtained a DNA fragment of 573 bp in length consisting of the *csiD* promoter region. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc. Valencia, CA). All PCR-amplified products were sent for DNA sequencing at the Mobix facility (McMaster University) and sequence comparison of amplified sequences to the wild type (MC4100) *csiD* promoter sequence was done using Clustal W (1.8) multiple sequence alignment program (http://www2.ebi.ac.uk/).

2.6 Isolation of GABA utilizing mutants

Many strains of *E. coli* K-12 (such as MC4100) cannot utilize GABA, except as a nitrogen source in the presence of a poor carbon source such as succinate (Donnelly and Cooper, 1981). The reason for inability to grow on GABA for these strains is not clear. However, it is possible to isolate GABA utilizing mutant strains which can use GABA as sole carbon and/or nitrogen source (Dover and Halpern, 1972a). In this study, three classes of GABA utilizing mutants, eight mutants of each class, were isolated directly from the wild type MC4100 background by selection on minimal media supplemented

with γ-aminobutyrate (Figure 4). Obtaining these GABA utilizing mutants was to be the first step towards characterizing the factors enabling these strains to metabolize GABA.

Group I mutants (HS1700) were selected to use GABA as both a carbon and nitrogen source. Group II mutants (HS1701) were selected to use GABA as a nitrogen source only and Group III mutants (HS1702) could use GABA as a carbon source only. Group I mutants were selected with minimal salts media (without NH_4Cl salts)+GABA (0.7% GABA as nitrogen and carbon source), while Group II mutants were selected with minimal salts media (without NH_4Cl salts)+glucose+GABA (0.2% glucose as carbon source, 0.2% GABA as nitrogen source). Group III mutants were selected with M9+NH_4Cl+GABA (0.5% GABA as carbon source). Proline, thiamine and $MgSO_4$ were added giving final concentrations of 0.002%, 0.001% and 1.0 mM, respectively.

The mutant isolation scheme was carried out in the following manner. Wild-type MC4100 *E. coli* cells were streaked out onto a LB plate and incubated overnight at 37°C. Independent colonies were used to inoculate eight different LB broth tubes which were grown overnight at 37°C to stationary phase. Each of these tubes was then used to streak onto an LB-X-gal(5 bromo 4-chloro-3-indolyl-B-D-galactopyronoside)-Streptomycin (LXS) plate containing streptomycin (50 ug/ml). These eight LXS plates were incubated overnight at 37°C. The purpose of this step was to consolidate phenotypic similarities between Group I and Group III mutants at the genotypic level. Independent colonies from each of the eight LXS plates were then spread plated onto the eight different plates for each of the three types of selective media. These spread plates were incubated at 37°C for 3-4 days. At this point, spontaneous mutants, easily distinguishable from wild-

type cells due to the significantly larger colony size of the GABA utilizing strains, were streaked onto fresh selective media to purify the GABA utilizing mutants. After three rounds of purification, these mutant cells were stored (in duplicate) in glycerol at -80°C. Finally, the phenotype of these GABA utilizing mutants was confirmed by replica plating onto selective media supplemented with GABA. See Table 2a for complete list of all strains and their genotypes.

2.7 Genetic methods

After selecting the three classes of GABA utilizing mutants (Table 2a), transductants were made with these mutants as recipients. The genetic procedures for transduction were done by Dr. Herb Schellhorn as described by Silhavy et al. (1984). *LacZ* fusions to genes in the *gab* operon were introduced into the GABA utilizing mutant strains by P1-mediated transduction. P1 (HS1010) was known to contain *lacZ* fusion to the *gabP* promoter and P1 (HS1057) was known to contain *lacZ* fusion to the *gabD* promoter.

2.8 Isolation of GABA utilizing mutants from fusion strains

Results obtained from this study gave reason for postulating an alternate pathway of GABA utilization that may work independently of *gab*-encoded functions. Therefore, we wanted to test the notion whether the *gab* operon was dispensable (i.e., non-essential) for GABA utilization. For this purpose, we attempted to isolate GABA utilizing mutants directly from strains carrying *lacZ* fusions to genes in the *gab* operon (HS1010^{o}, HS1010p^{o}, HS1057^{o}, HS1057p^{o}) (from Table 2b). MC4100 and HS1600 (from Table 2a) were used as control (i.e., non-fusion) background strains for isolation of GABA utilizing

mutants. The procedure used is outlined in Figure 5. Cells from HS1010° (*gabP-lacZ*), its *rpoS*⁻ derivative HS1010p°, HS1057° (*gabD-lacZ*), its *rpoS*⁻ derivative HS1057p°, and the control non-fusion background strains (MC4100, HS1600) were washed in 10 mM K_2HPO_4 and 200 ul of each suspension was spread plated onto the three types of selective media plates for the selection of the three classes of GABA utilizing mutants (composition of the selective plates same as mentioned previously). The spread plates were incubated at 37°C for 2-3 days. Five mutant colonies of each class generated from the fusion strains were streaked onto LB-X-gal plates and only those mutants that appeared to be over-expressing the functions of the *gab* operon (by comparing expression levels with the parental fusion strains) were stocked (in duplicate) in glycerol at –80°C. See Table 5 for list of over-expressing GABA mutants obtained.

2.9 Calculation of mutational frequency

Mutational frequencies for the GABA utilizing mutants obtained from the two *rpoS*⁺ fusion backgrounds were calculated as follows: cells from the two parental fusion strains were resuspended in 10 mM K_2HPO_4 to an $OD_{600} \sim 0.2$. This corresponds to ~ 1 X 10^8 cells/ml. Since 200 ul of cell suspension was spread plated, the total number of cells per plate amounts to ~ 2 X 10^7 cells. Therefore, to calculate per cell mutational frequency, the total number of mutant colonies obtained was divided by 2 X 10^7 cells.

2.10 Preparation of Conditioned Medium

Conditioned medium was prepared as described (Baca-DeLancey et al., 1999). 800 ml of LB broth was inoculated with 1:6000 dilution of an overnight culture of *E. coli* MC4100 followed by shaking (250 rpm) at 37°C. Cells were harvested 3-4 hours after

entering stationary phase at an OD_{600} of 1.6-2, and cells were pelleted by centrifugation for 10 minutes. The resulting supernatant was supplemented with the addition of 20 X TY (tryptone, yeast extract) to a final concentration of 0.5X, and the pH was brought to 7.5. Conditioned medium was then filter-sterilized through a 0.2-um-pore filter and stored at 4°C.

2.11 GABA induction of *gab* expression in Minimal Medium

MC4100, HS1600 (Table 2a), and all strains listed in Table 2b were grown overnight in LB media with the appropriate antibiotics. Overnight cultures were diluted 1:1000 in fresh LB medium and grown to stationary phase (OD_{600} ~ 1.0) at 37°C with shaking (250 rpm). Cells were then pelleted by centrifugation for 10 minutes and washed twice in M9 media (without NH_4Cl salts). This suspension was then replica plated onto minimal salts medium with ammonium as the nitrogen source and either glucose (0.2%) or succinate (0.5%) as the carbon source. Proline, thiamine and $MgSO_4$ were added as indicated previously. The test compound (γ-aminobutyrate) was added at a concentration of 0.2% (Figure 12). The plates were incubated overnight at 37°C.

2.12 Acid Adaptation Test

Glutamate, an amino acid, has been documented to serve a protective function against acidic shock (De Biase et al., 1999). This amino acid gets converted to GABA by means of a decarboxylation reaction catalyzed by glutamic acid decarboxylase under conditions of low pH (Dover and Halpern, 1972a). Glutamic acid decarboxylase deficient mutants are sensitive to acid shock (De Biase et al., 1999). Therefore, it is likely that GABA confers acid resistance to bacteria. To check for the possible role of GABA

in acid resistance, we tested the sensitivity of our GABA utilizing mutant strains (from Table 2b) along with appropriate controls (from Tables 2a & 2b) to various weak acids. Sensitivity was tested against sodium acetate (5-50 mM), benzoate (2-10 mM) and salicylate (1-5 mM). These weak acids were filter sterilized and the pH was adjusted to 7.0. Minimal salts medium was used with ammonium salts as the nitrogen source and glucose (0.2%) as the carbon source. Proline, thiamine and $MgSO_4$ were added as indicated previously. Plates were made with and without GABA (0.2%) to test for GABA's contribution towards acid adaptation. Cells were replica plated onto plates and the plates were incubated overnight at 37°C.

2.13 Osmoprotection test

Glutamate is also postulated to play a protective role against salt stress (De Biase et al., 1999). Since the pathway of GABA breakdown in *E. coli* converges with that of glutamate catabolism by means of a decarboxylation reaction catalyzed by glutamic acid decarboxylase (Dover and Halpern, 1972a), investigating glutamate's role as an osmoprotectant had relevance towards this project. Hence, to test for glutamate's role as an osmoprotectant, we tested the sensitivity of our GABA utilizing mutant strains (from Table 2b) along with appropriate controls (from Tables 2a & 2b) to varying concentrations of salt (0 mM NaCl, 250 mM NaCl, 500 mM NaCl). Minimal salts medium was used with ammonium salts as the nitrogen source and glucose (0.2%) as the carbon source. Proline, thiamine and $MgSO_4$ were added as indicated previously. The test compound (glutamate) was filter sterilized and the pH was adjusted to 7.0 with KOH. Plates were made with and without glutamate which was added at a concentration of 50

mM. Cells were replica plated onto plates and the plates were incubated overnight at 37°C.

Figure 4. Strategy employed to isolate three different groups of GABA utilizing mutant strains directly from the wild type (MC4100) background.

MC4100 streaked on LB, incubate O/N at 37°C

↓

8 different colonies to inoculate 8 LB broth tubes, let grow O/N at 37° C to Stationary Phase

↓

Each LB broth tube to streak out a LXS plate, incubate O/N at 37° C

↓

Independent colonies from each of the 8 LXS plates spread plated onto the 8 different plates for each of the 3 types of selective media, incubate at 37° C for 3-4 days

↓

Spontaneous mutants (big colonies) appearing
Group I (CN)--M9 (without NH_4Cl) + GABA (0.7%)
Group II (N)--M9 (without NH_4Cl) + Glucose+ GABA (0.2%)
Group III (C)--M9 (with NH_4Cl) + GABA (0.5%)

↓

3 rounds of purification, store cells in glycerol at -80° C

Figure 5. Schematic representation of the strategy used to isolate GABA utilizing mutants directly from strains carrying *lacZ* fusions to members of the *gab* operon. Mutational frequency (MF) values represent per cell mutational frequency.

- Spread plating cells from HS1010°, HS1010p°, HS1057° and HS1057p° onto the 3 types of selective media

↓

3 classes of GABA mutants from HS1010°

↓

CN-- MF=2.8×10^{-6} per cell
N -- MF=1.3×10^{-5} per cell
C -- MF=2.8×10^{-6} per cell

2 classes of GABA mutants from HS1057° (no N utilizing mutants obtained)

↓

CN-- MF=1.2×10^{-6} per cell
C -- MF=1.6×10^{-6} per cell

(No GABA mutants obtained from the *rpoS*$^-$ strains)

↓

Selected for over-expressing GABA mutants by streaking 5 mutant colonies of each class onto LXS plates along with appropriate controls

CHAPTER 3. RESULTS

3.1 GABA utilizing mutants obtained

Many strains of *E. coli* K-12 cannot utilize GABA as the sole source of carbon or nitrogen (Dover and Halpern, 1972a). Attempts were made to grow MC4100, a wild type strain of *E. coli* K-12, in media containing γ-aminobutyrate (GABA). This strain tested was unable to grow on most types of selective media supplemented with GABA. The only exception to this rule was found on minimal salts media (without NH_4Cl salts) with GABA (0.2%) and succinate (0.5%). Therefore, it seems that some *E. coli* K-12 strains, as well as *E. coli B* strains, can use GABA as a nitrogen source in the presence of a less preferred carbon source, such as succinate, but they cannot degrade GABA as a carbon source (Donnelly and Cooper, 1981).

Dover and Halpern (1972a) were unable to isolate GABA carbon utilizing mutants from wild-type strains. However, using appropriate selective media, we were able to obtain GABA utilizing mutants of all three classes directly from the wild-type strain (MC4100). We were able to isolate mutants which could use GABA as a carbon and nitrogen source or solely as a carbon or nitrogen source alone. The phenotype of these GABA utilizing mutants was confirmed by replica plating the strains on selective media (Table 3).

3.2 β-galactosidase assay using transduced GABA mutants

It is documented in the literature that GABA utilizing mutants exhibit a six- to nine-fold increase in the activities of the *gab* encoded functions compared to wild-type *E. coli* K-12 strains which exhibit very low activity of these *gab* genes (Dover and Halpern, 1972a). It is therefore postulated that this over-expression may play an important role for these mutants to be able to utilize GABA. Hence, in order to characterize the GABA utilizing mutants that we isolated and to identify the mutation(s) responsible for over-expression of the *gab* operon, these mutants were transduced with *lacZ* fusions to the two different *gab* genes by P1-mediated transduction as described in Materials and Methods (see Table 2a for strain list).

These transductants were then utilized in *lac* fusion studies. β-galactosidase assays were performed as described in Materials and Methods on isogenic wild type and *rpoS*$^-$ strains along with the three groups of GABA utilizing mutant strains carrying a *gabP::lacZ* reporter gene fusion (Figure 6). Expression studies revealed that these transduced GABA utilizing mutant strains were not over-expressing the functions of the *gab* operon. In fact, expression levels were similar to wild type expression. In rich media, β-galactosidase activity for *rpoS*$^+$ strains was very low in exponential phase and was induced approximately two to three fold upon entry into stationary phase. In *rpoS*$^-$ strains, total activity was greatly reduced in both phases. This supports previous findings by Schellhorn et al. (1998) which argue in favor of growth-phase and RpoS-dependent regulation of the *gab* genes.

Since the addition of GABA has been shown to be an inducer of *gab* expression in *E. coli* (Dover and Halpern, 1972b) on minimal salts media, we also tested whether the expression of the *gab* operon was specifically induced by this amino acid on rich media (Figure 6). However, the results indicate that GABA is not an inducer of *gab* operon expression when cells are growing in LB media.

3.3 Sequence analysis of the *gab* promoter in the GABA mutants

We wanted to explore the reasons for suppression of over-expression of the *gab* operon from our transduced GABA mutants (Figure 6). To determine if this could be due to the replacement of the mutation site with the wild-type site during the transduction event, we proposed that the mutation may lie somewhere within the linkage distance of the *gab* operon. Figure 1 shows a detailed schematic of the *gab* region. The *gab* operon has been previously identified as a member of the RpoS regulon (Schellhorn et al., 1998). It lies two ORFs downstream of the *csiD* gene, a carbon starvation-inducible gene in *E. coli*, that has been shown to be highly RpoS dependent (Marschall et al., 1998) and whose expression is driven from a single upstream promoter. This RpoS regulated promoter is the first promoter upstream of the *gab* operon (see fig. 1) and is therefore likely to drive expression of the *gab* genes. As a result, we designed primers as described in Materials and Methods to amplify this *gab* promoter in our GABA utilizing mutant strains in order to identify potential mutation(s) that could be responsible for over-expression of the *gab* genes thereby allowing GABA utilization. Wild-type sequence of the *csiD* promoter region was independently verified in our lab by sequencing this region in MC4100 wild-type strains and was found to be identical to the sequence provided by

Marschall et al. (1998). Comparison of the amplified products to wild type sequence was done using Clustal W sequence alignment program. The results (Figure 7) show that there was 100 % sequence identity between the wild-type and GABA utilizing mutant strains. Hence, no mutation was found in the *csiD* promoter region in the GABA utilizing mutants.

3.4 Dispensability of the *gab* operon for GABA utilization

To determine if the *gab* operon is a requirement for GABA transport and metabolism, we attempted to isolate GABA utilizing mutants from wild type fusion strains containing *lacZ* fusions to the two different *gab* genes (HS1010^{o} and HS1057^{o}) and their *rpoS*$^{-}$ derivatives (HS1010p^{o} and HS1057p^{o}). Hence, if the *gab* genes are necessary for GABA utilization, then we do not expect to obtain any GABA utilizing mutants from strains carrying mutations in the *gab* genes. Surprisingly, we were able to obtain GABA mutants from both these fusion strains in the *rpoS*$^{+}$ background, but not from the *rpoS*$^{-}$ background (Table 4). We were able to obtain all three classes of GABA mutants when *gabP* was disrupted and two classes of GABA mutants despite the *gabD* mutation. Interestingly, we did not obtain any nitrogen utilizing GABA mutants from strains carrying a mutation in *gabD*. This observation directly contradicts earlier findings by Metzer and Halpern (1979) where GABA nitrogen utilizing mutants obtained sustained normal transport and transamination but were completely devoid of succinic semialdehyde dehydrogenase activity. Furthermore, no GABA utilizing mutant strains were obtained from any of the *rpoS*$^{-}$ fusion strains used or the *rpoS*$^{-}$ non-fusion control strain (HS1600). Taken together, these results suggest that the *gab* operon is dispensable

for GABA utilization and that other RpoS regulated functions are possibly important in GABA uptake and metabolism.

Of the GABA mutants obtained, five mutant colonies from each class were tested on LB-X-gal plates to select for those mutants that appeared to be over-expressing the *gab* genes. Table 5 shows the percentage of GABA mutants obtained within each class that are potentially over-expressers. Table 2b is a list of these mutant strains that were stocked. For reasons that are not clear, I had difficulty replicating the over-expression that was evident on LB-X-gal plates (Figure 8) in liquid LB media (Figure 9). In fact, the liquid assay results indicate that *gab* expression levels of the GABA mutants are lower than the wild type fusion strain (HS1057^{o}) which cannot utilize GABA. Furthermore, the expression levels of these mutants are similar to those of the *rpoS*$^{-}$ fusion strain (HS1057p^{o}), except in stationary phase where activity is about five fold higher for the GABA utilizing mutants.

Induction Studies

We wanted to test putative inducers of *gab* operon expression to obtain some regulatory information about this RpoS dependent regulon. Both conditioned media (Baca-DeLancey et al., 1999) and γ-aminobutyrate (Dover and Halpern, 1972b) have been implicated as inducers of *gab* expression. β-galactosidase assays were done in LB media as described in Materials and Methods with the potential inducing substance added to the growth medium.

3.5 Putative inducers of *gab* expression:

3.5.1 Conditioned Medium

Baca-DeLancey et al. (1999) showed that one of the *gab* genes, *gabT*, which codes for γ-aminobutyrate transaminase, is induced by conditioned medium at mid-logarithmic phase. According to that study, the induction of *gabT* in the presence of conditioned medium vs. LB was 19-fold at mid-logarithmic phase, but this induction became less pronounced as cells reached high density. To determine if the other two members of this cluster elicited a similar response in the presence of conditioned medium, we used the two wild type fusion strains carrying *lacZ* fusions to the two remaining *gab* genes (HS1010° and HS1057°) along with their *rpoS*⁻ derivatives (HS1010p° and HS1057p°) in this induction experiment.

Induction studies revealed that neither *gabD* nor *gabP* is induced by conditioned medium (Figure 10). In fact, induction in conditioned medium happens at the same time as in 1 X LB. Nevertheless, our results suggest that expression of these *gab* genes was higher in conditioned medium vs. LB (Table 6). Expression levels for *gabP* were similar at mid-logarithmic phase, but 2-fold higher in conditioned medium vs. LB at stationary phase, and for *gabD*, expression levels were 25-fold higher in conditioned medium vs. LB at mid-exponential phase, but only 2-fold higher in conditioned medium at stationary phase. In *rpoS*⁻ strains, activity was greatly reduced in both types of media. Thus, there seems to be a factor present in conditioned medium which causes *gab* expression to be higher compared to LB media. Furthermore, this factor seems to exert its effect through RpoS.

3.5.2 LB media supplemented with GABA (0.2%)

Previous studies seem to indicate that γ-aminobutyrate may be an inducer of *gab* expression. For instance, it has been shown that when various strains of *E. coli* are grown on γ-aminobutyrate as the sole nitrogen source, an NAD(P)-dependent succinate semialdehyde dehydrogenase is induced coordinately with 4-aminobutyrate:2-oxogluterate transaminase (Dover and Halpern, 1972b). Furthermore, Donnelly and Cooper (1981) showed that when mutants are grown with γ-aminobutyrate as the sole nitrogen source, an NADP-dependent enzyme is induced. These observations were made when strains were growing in minimal salts media. To determine if the inducing effect of GABA was detectable in LB media, we used the wild type fusion strain carrying the *lacZ* fusion to *gabD* (HS1057°), along with its *rpoS*⁻ derivative (HS1057p°), in this induction experiment. The assays were done in duplicate and the results are plotted in Figure 11.

Our data (Figure 11) suggests that GABA does not induce expression of the *gab* genes when cells are growing in LB media. Expression studies revealed that induction in media containing GABA (0.2%) happens at the same time as in unsupplemented rich media. Furthermore, unlike the response to conditioned medium, in rich media GABA does not increase *gab* expression levels (Table 7). As a result, expression levels of the *gab* gene were similar in LB media in the presence or absence of GABA. For the *rpoS*⁺ strain, β-galactosidase activity in the absence of GABA increased 9-13 fold as cells transitioned from mid-exponential phase to stationary phase. In the presence of GABA, activity increased from 6-11 fold as cells went from mid-exponential phase to stationary phase. In the *rpoS*⁻ strain, activity was greatly reduced in both phases in both types of

media. These results support our findings using transduced GABA mutants in a previous experiment (Figure 6).

3.5.3 Minimal Media supplemented with GABA (0.2%)

To confirm the inducing effects of GABA in minimal salts media as suggested by previous findings (Dover and Halpern, 1972b; Donnelly and Cooper, 1981), we replica plated GABA mutants (from Table 2b) along with appropriate controls (MC4100, HS1600, HS1010°, HS1010p°, HS1057° and HS1057p°) on minimal plates as described in Materials and Methods. The results are shown in Figure 12. The replica plating results fail to show any indications of GABA being an inducer of *gab* expression when cells are grown in minimal salts media. In fact, minimal plates in the absence of GABA with ammonium as the nitrogen source and either glucose (0.2%) or succinate (0.5%) as the carbon source appear darker than plates supplemented with GABA to a final concentration of 0.2%. These findings are in conflict with earlier work done by other researchers (Dover and Halpern, 1972b; Donnelly and Cooper, 1981).

3.6 Role of glutamate in acid adaptation and osmotolerance

Glutamate, an amino acid, is converted to GABA by means of a decarboxylation reaction catalyzed by glutamic acid decarboxylase. This enzyme is encoded by the *gad* genes, *gadA* and *gadB*. It has been proposed that expression of these *gad* genes is positively regulated by acidic shock and salt stress (De Biase et al., 1999). Hence, to confirm the role of glutamate in acid resistance and osmotolerance, we tested the sensitivity of our GABA utilizing mutant strains obtained (from Table 2b) along with

appropriate wild type controls (from Tables 2a and 2b) to various weak acids and varying concentrations of salt.

For the acid adaptation test, sensitivity was tested against various weak acids. These included sodium acetate (5-50mM), benzoate (2-10 mM) and salicylate (1-5 mM). Sensitivity was tested on minimal plates in the presence and absence of GABA (0.2%) as described in Materials and Methods. The results are summarized in Table 8. For sodium acetate, maximal growth was observed up to 45 mM of acid. However, surprisingly at 50 mM of acid, cells grew better in the absence of GABA. Likewise, at 10 mM benzoate, cells exhibited enhanced growth in the absence of GABA. A similar phenomenon was seen in the case of 1 mM salicylate where having GABA in the media proved to be detrimental for growth. Interestingly, at this concentration of salicylate, no growth was observed for GABA utilizing mutant strains carrying a *gabD-lacZ* mutation (HS1903a, HS1903b, HS1903c), regardless of whether GABA was present or absent from the media. Furthermore, none of the strains were able to grow at higher concentrations of salicylate.

For the osmoprotection test, sensitivity was tested against varying concentrations of salt (0-500 mM NaCl) on minimal plates in the presence and absence of glutamate (50 mM) as described in Materials and Methods. The results are summarized in Table 9. Maximal growth was observed for wild type and mutant cells at 0 mM NaCl in the presence or absence of glutamate. However, as suspected, at 250 mM NaCl growth was markedly enhanced in media containing glutamate. Furthermore, at 500 mM NaCl no growth was observed in the absence of glutamate. Interestingly, in the presence of

glutamate at this highest concentration of salt, all strains except the GABA utilizing mutants carrying *gabD-lacZ* fusions were able to grow.

Table 2a. *E. coli* strains used in this study.

Strains	Genotype	Source/Reference
A) Strains		
MC4100	*Δ(argF-lacZ)205 araD139 flbB5301 relA1 rpsL150 thi ptsF25*	G. Weinstock
HS1600	as MC4100 but *rpoS13::Tn10*	H. Schellhorn
HS1700	as MC4100 but selected to use GABA as C and N source	R. Kasra
HS1701	as MC4100 but selected to use GABA as N source	R. Kasra
HS1702	as MC4100 but selected to use GABA as C source	R. Kasra
HS1710	as HS1700 but *gabP*::λ*plac*Mu53 [ϕ(*gabP-lacZ*)1]	H. Schellhorn
HS1711	as HS1701 but *gabP*::λ*plac*Mu53 [ϕ(*gabP-lacZ*)1]	H.Schellhorn
HS1712	as HS1702 but *gabP*::λ*plac*Mu53 [ϕ(*gabP-lacZ*)1]	H. Schellhorn
HS1713	as MC4100 but *gabP*::λ*plac*Mu53 [ϕ(*gabP-lacZ*)1] and *rpoS13::Tn10*	H. Schellhorn
HS1714	as MC4100 but *gabP*::λ*plac*Mu53 [ϕ(*gabP-lacZ*)1]	H. Schellhorn
HS1720	as HS1700 but *gabD*::λ*plac*Mu53 [ϕ(*gabD-lacZ*)1]	H. Schellhorn
HS1721	as HS1701 but *gabD*::λ*plac*Mu53 [ϕ(*gabD-lacZ*)1]	H. Schellhorn
HS1722	as HS1702 but *gabD*::λ*plac*Mu53 [ϕ(*gabD-lacZ*)1]	H. Schellhorn
HS1723	as MC4100 but *gabD*::λ*plac*Mu53 [ϕ(*gabD-lacZ*)1] and *rpoS13::Tn10*	H. Schellhorn
HS1724	as MC4100 but *gabD*::λ*plac*Mu53 [ϕ(*gabD-lacZ*)1]	H. Schellhorn
HS1700P	as HS1700 but *rpoS13::Tn10*	R. Kasra

Table 2a (continued). *E. coli* strains used in this study.

Strains	Genotype	Source/Reference
HS1701P	as HS1701 but *rpoS13::Tn10*	R. Kasra
HS1702P	as HS1702 but *rpoS13::Tn10*	R. Kasra
HS1710P	as HS1710 but *rpoS::Tn10*	H. Schellhorn
HS1711P	as HS1711 but *rpoS::Tn10*	H. Schellhorn
HS1712P	as HS1712 but *rpoS::Tn10*	H. Schellhorn
HS1720P	as HS1720 but *rpoS::Tn10*	H. Schellhorn
HS1721P	as HS1721 but *rpoS::Tn10*	H. Schellhorn
HS1722P	as HS1722 but *rpoS::Tn10*	H. Schellhorn
B) Transducing Phage		
P1*vir* (HS1010)	contains *lacZ* fusion to *gabP* promoter	laboratory stock
P1*vir* (HS1057)	contains *lacZ* fusion to *gabD* promoter	laboratory stock

Table 2b. *E. coli* strains used in this study.

Strains	Genotype	Source/Reference
A) Strains		
HS1010°	as MC4100 but λϕ(*gabP-lacZ*)1	transduced from MC4100 by L.C.Wei
HS1010p°	as HS1010° but *rpoS13::Tn10*	L.C.Wei
HS1057°	as MC4100 but λϕ(*gabD-lacZ*)1	L.C.Wei
HS1057p°	as HS1057° but *rpoS13::Tn10*	L.C.Wei
HS1900b	as HS1010° but selected to use GABA as C and N source	this study
HS1900c	as HS1010° but selected to use GABA as C and N source	this study
HS1900e	as HS1010° but selected to use GABA as C and N source	this study
HS1900g	as HS1010° but selected to use GABA as C and N source	this study
HS1901a	as HS1010° but selected to use GABA as N source	this study
HS1903a	as HS1057° but selected to use GABA as C and N source	this study
HS1903b	as HS1057° but selected to use GABA as C and N source	this study
HS1903c	as HS1057° but selected to use GABA as C and N source	this study
B) Transducing Phage		
P1*vir* (HS1010)	contains *lacZ* fusion to *gabP*	laboratory stock
P1*vir* (HS1057)	contains *lacZ* fusion to *gabD*	laboratory stock

Table 3. Analysis of GABA mutants obtained for utilization of GABA as a Carbon and/or Nitrogen source.[a]

Group	Selected to use GABA as:		Ability to use GABA as:		
	N source	C source	N	C	CN
I (CN)	+	+	+	+	+
II (N)	+	-	+	-	-
III (C)	-	+	+	+	+

[a] Results from replica plating experiment.
Poor or no growth (-); good growth (+)

Table 4. GABA mutants obtained from wild type fusion backgrounds.[a]

Parental Strain	GABA mutants[b]	mutant colonies	Mutational frequency[c]
HS1010[o]	CN	55	2.8×10^{-6}
	N	200	1.3×10^{-5}
	C	55	2.8×10^{-6}
HS1057[o]	CN	23	1.2×10^{-6}
	N	0	0
	C	31	1.6×10^{-6}

[a]Results from spread plating experiment as described in Materials and Methods.
[b]No GABA mutants were obtained from the *rpoS*⁻ fusion strains.
[c]Values for per cell mutational frequency were calculated as described in Materials and Methods.

Table 5. Over-expressing GABA mutants obtained.[a,b]

Strain	Genotype	percent of the total population[c]
HS1900	as HS1010^{o} but CN GABA mutant	80%
HS1901	as HS1010^{o} but N GABA mutant	20%
HS1903	as HS1057^{o} but CN GABA mutant	60%

[a]Over-expressers were selected by comparing *lac* expression to the appropriate wild type fusion strain on LB-X-gal plates as described in Materials and Methods.
[b]No over-expressing C utilizing GABA mutants were obtained from HS1010^{o}; also, no over-expressing C or N utilizing GABA mutants were obtained from HS1057^{o}.
[c]Percent values for over-expressers are calculated from a total of five colonies of each group.

Table 6. Expression of *gab* genes in response to conditioned medium.

		β-galactosidase accumulation[a]			
		- Conditioned Medium		+ Conditioned Medium	
Strain	**Class**	**Mid-log[b]**	**Stationary[c]**	**Mid-log**	**Stationary**
HS1010°	WT (*gabP-lacZ*)	30.4	23.3	30.5	67.1
HS1010p°	*rpoS*⁻ (*gabP-lacZ*)	0.7	0.7	1.2	1.1
HS1057°	WT (*gabD-lacZ*)	3.0	51.1	75.3	149.5
HS1057p°	*rpoS*⁻ (*gabD-lacZ*)	1.6	1.9	2.6	2.5

[a]Specific activity was determined in Miller Units.
[b]Mid-logarithmic phase was determined in cells to be at OD_{600} of 0.4-0.6.
[c]Stationary phase was determined in cells to be at OD_{600} of 1.0-1.6.

Table 7. Expression of *gab* genes in response to GABA (0.2%).[a]

		β-galactosidase[b]			
		- GABA		+ GABA	
Strain	**Class**	**Mid-log[c]**	**Stationary[d]**	**Mid-log**	**Stationary**
HS1057[o]	WT *(gabD-lacZ)*	12.30	135.10	15.05	130.10
HS1057p[o]	*rpoS*⁻ *(gabD-lacZ)*	3.15	4.75	3.6	6.45

[a]β-galactosidase assay was done in LB media supplemented with GABA (0.2%) as described in Materials and Methods.
[b]Specific Activity was expressed in Miller Units.
[c]Mid-logarithmic phase was determined in cells OD_{600} of 0.4-0.6.
[d]Stationary phase was determined in cells OD_{600} of 2.0-2.5.
Values represent the average determined from two independent samples.

Table 8. Growth of wild-type and GABA mutant strains on various weak acids in the presence and absence of γ-aminobutyrate.[a]

Strain	Class	Sodium Acetate[b,c]						Benzoate						Salicylate					
		+GABA			-GABA			+GABA			-GABA			+GABA			-GABA		
		5	45	50	5	45	50	2	6	10	2	6	10	1	3	5	1	3	5
MC4100	WT	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1600	*rpoS*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1010°	*gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1010p°	*rpoS*⁻*gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1057°	*gabD*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1057p°	*rpoS*⁻*gabD*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1900b	CN GABA mutant *gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1900c	CN GABA mutant *gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1900e	CN GABA mutant *gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1900g	CN GABA mutant *gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1901a	N GABA mutant *gabP*⁻	++	++	+	++	++	++	++	++	+	++	++	++	+	-	-	++	-	-
HS1903a	CN GABA mutant *gabD*⁻	++	++	+	++	++	++	++	++	+	++	++	++	-	-	-	-	-	-
HS1903b	CN GABA mutant *gabD*⁻	++	++	+	++	++	++	++	++	+	++	++	++	-	-	-	-	-	-
HS1903c	CN GABA mutant *gabD*⁻	++	++	+	++	++	++	++	++	+	++	++	++	-	-	-	-	-	-

[a]Results from replica plating experiment.
[b]no growth (-); weak growth (+); strong growth (++)
[c]Concentrations of acids used are in mM.

Table 9. Growth of wild-type and GABA mutant strains on varying concentrations of salt in the presence and absence of glutamate.[a]

Strain	Class	+Glutamate[b] 0 mM	+Glutamate[b] 250 mM	+Glutamate[b] 500 mM	-Glutamate 0 mM	-Glutamate 250 mM	-Glutamate 500 mM
MC4100	WT	++	++	+	++	+	-
HS1600	*rpoS*⁻	++	++	+	++	+	-
HS1010°	*gabP*⁻	++	++	+	++	+	-
HS1010p°	*rpoS*⁻*gabP*⁻	++	++	+	++	+	-
HS1057°	*gabD*⁻	++	++	+	++	+	-
HS1057p°	*rpoS*⁻*gabD*⁻	++	++	+	++	+	-
HS1900b	CN GABA mutant *gabP*⁻	++	++	+	++	+	-
HS1900c	CN GABA mutant *gabP*⁻	++	++	+	++	+	-
HS1900e	CN GABA mutant *gabP*⁻	++	++	+	++	+	-
HS1900g	CN GABA mutant *gabP*⁻	++	++	+	++	+	-
HS1901a	N GABA mutant *gabP*⁻	++	++	+	++	+	-
HS1903a	CN GABA mutant *gabD*⁻	++	++	-	++	+	-
HS1903b	CN GABA mutant *gabD*⁻	++	++	-	++	+	-
HS1903c	CN GABA mutant *gabD*⁻	++	++	-	++	+	-

[a]Results from replica plating experiment.
[b]no growth (-); weak growth (+); strong growth (++)

Figure 6a. Expression of *gabP* in wild type strains. Flasks containing either LB broth (control treatment) or LB + GABA (0.2%) were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. GABA was tested as a potential inducer of *gab* expression when cells are growing in LB media.
(A) HS1714 (*rpoS*$^+$, *gabP-lacZ*) grown in LB,
(B) HS1714 (*rpoS*$^+$, *gabP-lacZ*) grown in LB + GABA,
(C) HS1713 (*rpoS*$^-$, *gabP-lacZ*) grown in LB,
(D) HS1713 (*rpoS*$^-$, *gabP-lacZ*) grown in LB + GABA.
Symbols: o, Growth (OD_{600}); ■, β-galactosidase activity; O/N, overnight.

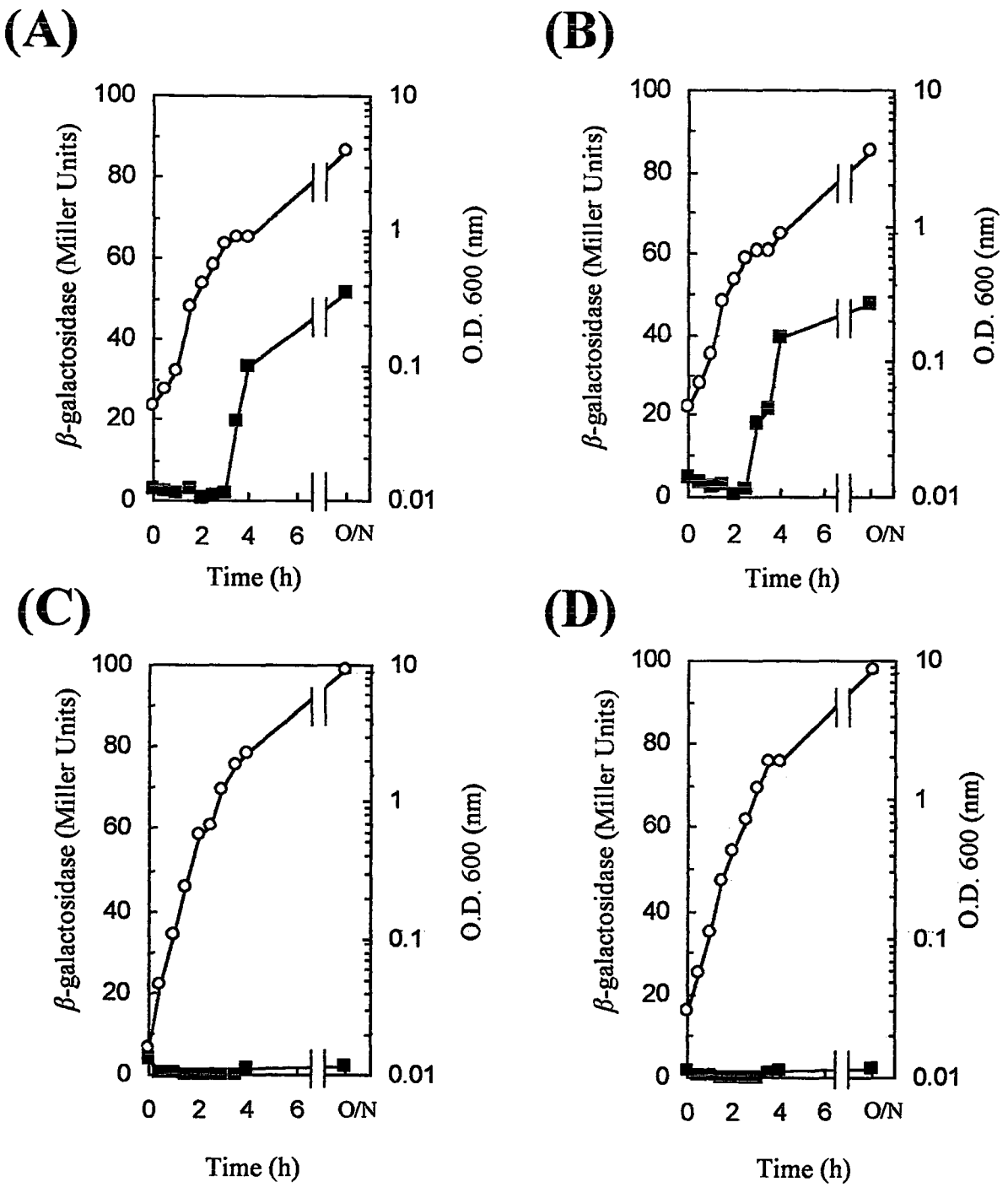

(A)
(B)
(C)
(D)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
2
4
6
O/N
100
80
60
40
20
10
1
0.1
0.01

Figure 6b. Expression of *gabP* in CN GABA utilizing mutant strains. Flasks containing either LB broth (control treatment) or LB + GABA (0.2%) were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and *β*-galactosidase activity. GABA was tested as a potential inducer of *gab* expression when cells are growing in LB media.

(A) HS1710 (*rpoS*$^+$, *gabP-lacZ*, CN GABA utilizing mutant) grown in LB,
(B) HS1710 (*rpoS*$^+$, *gabP-lacZ*, CN GABA utilizing mutant) grown in LB + GABA,
(C) HS1710p (*rpoS*$^-$, *gabP-lacZ*, CN GABA utilizing mutant) grown in LB,
(C) HS1710p (*rpoS*$^-$, *gabP-lacZ*, CN GABA utilizing mutant) grown in LB + GABA.

Symbols: o, Growth (OD_{600}); ■, β -galactosidase activity; O/N, overnight.

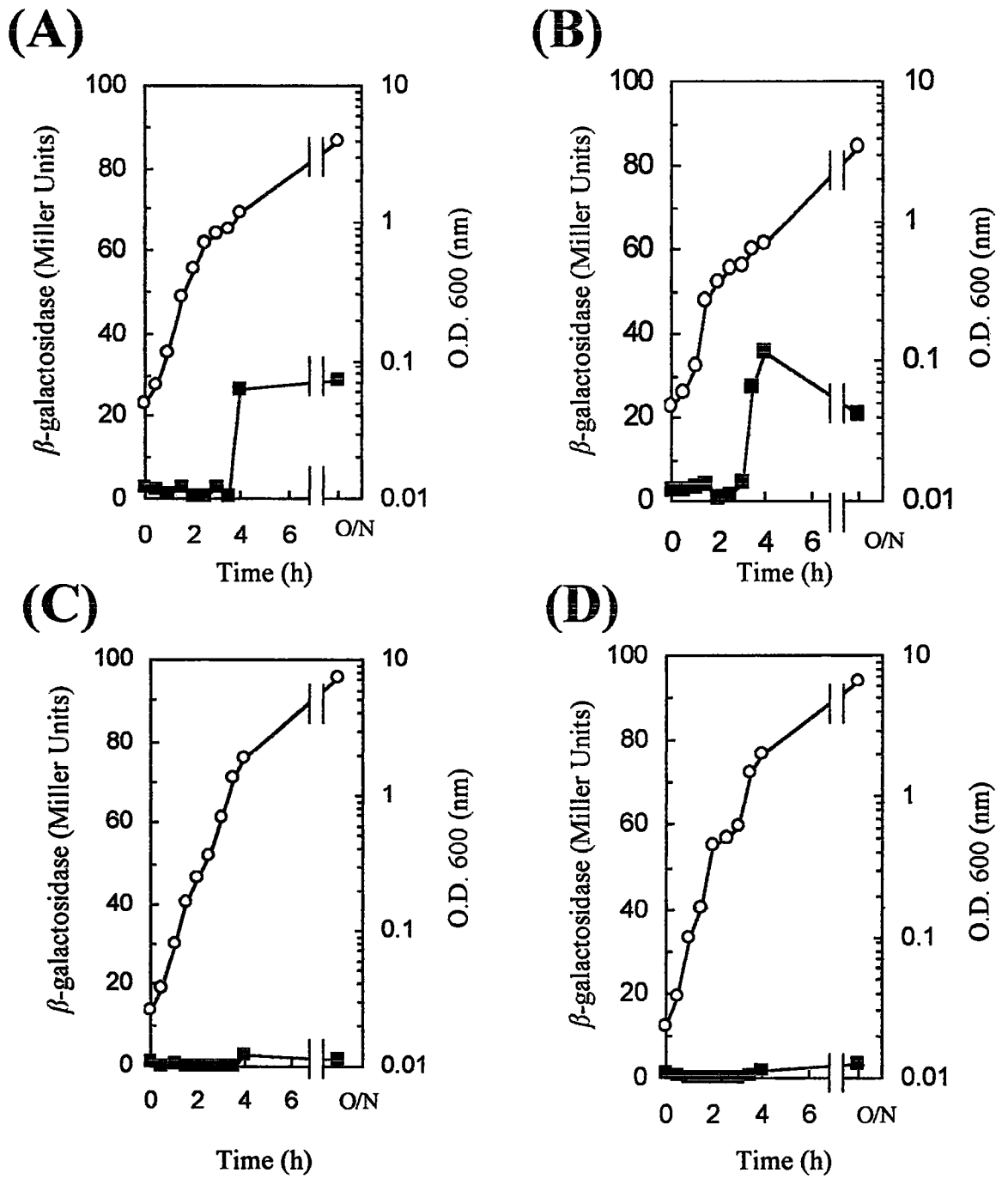

(A)
(B)
(C)
(D)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
2
4
6
O/N
100
80
60
40
20
10
1
0.1
0.01

Figure 6c. Expression of *gabP* in N GABA utilizing mutant strains.
Flasks containing either LB broth (control treatment) or LB + GABA (0.2%) were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. GABA was tested as a potential inducer of *gab* expression when cells are growing in LB media.
(A) HS1711 (*rpoS*$^+$, *gabP-lacZ*, N GABA utilizing mutant strain) grown in LB,
(B) HS1711 (*rpoS*$^+$, *gabP-lacZ*, N GABA utilizing mutant strain) grown in LB + GABA,
(C) HS1711p (*rpoS*$^-$, *gabP-lacZ*, N GABA utilizing mutant strain) grown in LB,
(D) HS1711p (*rpoS*$^-$, *gabP-lacZ*, N GABA utilizing mutant strain) grown in LB + GABA.
Symbols: o, Growth (OD_{600}); ■, β -galactosidase activity; O/N, overnight.

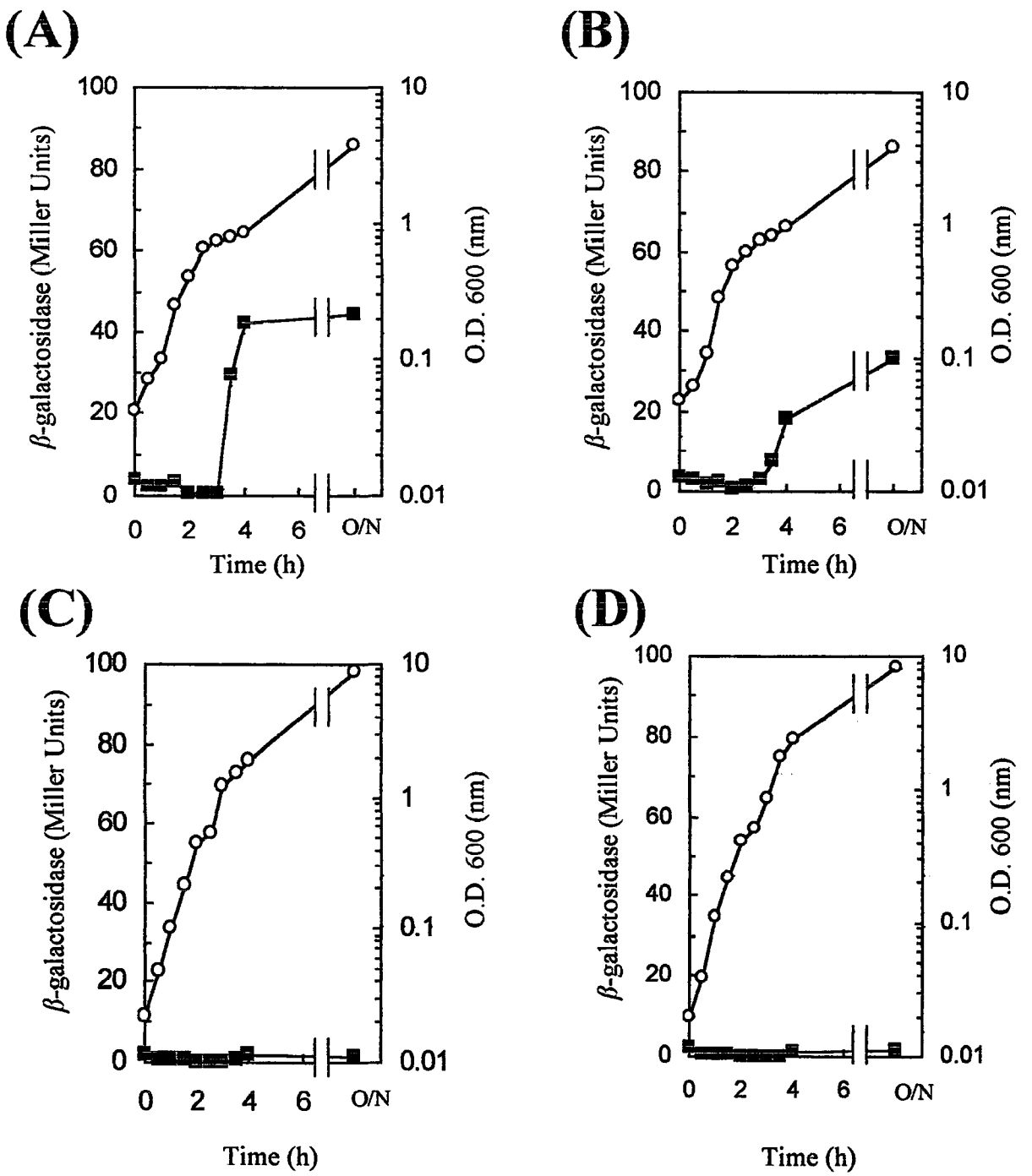

(A)
(B)
(C)
(D)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
2
4
6
O/N
100
80
60
40
20
10
1
0.1
0.01

Figure 6d. Expression of *gabP* in C GABA utilizing mutant strains.
Flasks containing either LB broth (control treatment) or LB + GABA (0.2%) were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. GABA was tested as a potential inducer of *gab* expression when cells are growing in LB media.
(A) HS1712 (*rpoS*$^+$, *gabP-lacZ*, C GABA utilizing mutant) grown in LB,
(B) HS1712 (*rpoS*$^+$, *gabP-lacZ*, C GABA utilizing mutant) grown in LB + GABA,
(C) HS1712p (*rpoS*$^-$, *gabP-lacZ*, C GABA utilizing mutant) grown in LB,
(D) HS1712p (*rpoS*$^-$, *gabP-lacZ*, C GABA utilizing mutant) grown in LB + GABA.
Symbols: o, Growth (OD_{600}); ■, β -galactosidase activity; O/N, overnight.

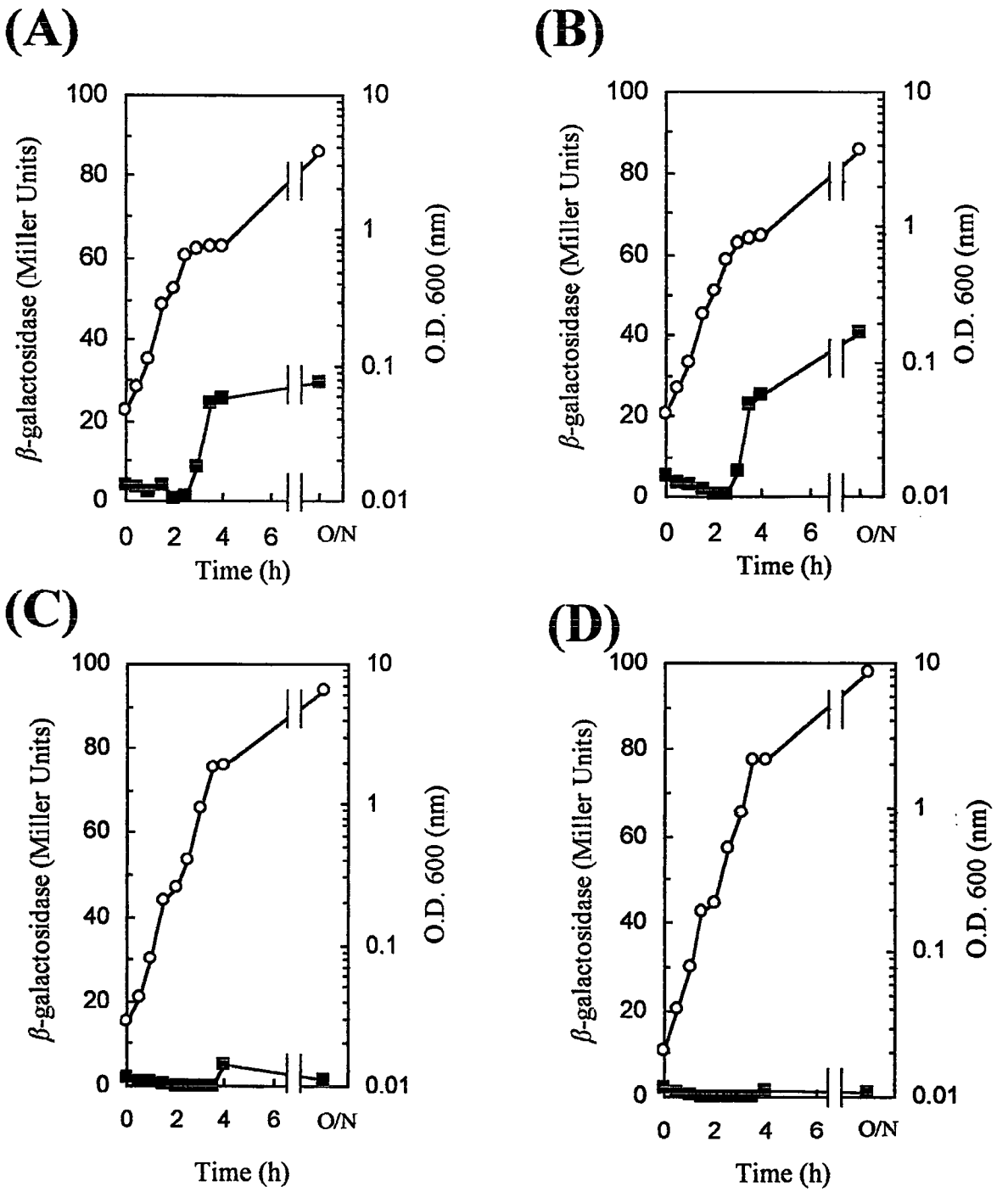

(A)
(B)
(C)
(D)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
2
4
6
O/N
100
80
60
40
20
10
1
0.1
0.01

Figure 7. Clustal W sequence alignment results of PCR amplified *csiD* promoter region in wild type (MC4100) strain and the three classes of GABA utilizing mutant strains generated from MC4100. HS1700 strains can use GABA as a carbon and nitrogen source, HS1701 strains can use it as a nitrogen source only, and HS1702 strains can use it as a carbon source only. One GABA utilizing mutant from each of the three classes was sequenced. The nucleotide sequence of the *csiD* promoter lies between nucleotides 160 and 360 in the amplified sequence. Important structural features of the *csiD* promoter are indicated as follows: highly conserved nucleotides in the CRP binding site are in bold, -10 and -35 positions of the *csiD* promoter and transcriptional and translational start sites are underlined. See figure 1 for complete nucleotide sequence and important structural features of the *csiD* promoter.

```
MC4100          -CGAACTA-----TCTCTATTTATAATTTAATGTTATATCTGCCCCGATAAAACGGGGCA 54
HS1700          -CGA-CTA-----TCTCTATTTATAATTTAATGTTATATCTGCCCCGATAAAACGGGGCA 53
HS1701          -TNNNTTCCGACTTCTCTNTTTATAATTTAATGTTATATCTGCCCCGATAAAACGGGGCA 59
HS1702          CCGCANTN-----TCTGTANCGANNNGTTANTGTTATATCTGCCCCGATAAAACGGGGCA 55
                      *      *** *    *    *** ****************************

MC4100          G-ATAATATGTTTAGTTTACTAACGGTCATTTTGCAGTGAAGCCATTTACTGTTTTTTAT 113
HS1700          G-ATAATATGTTTAGTTTACTAACGGTCATTTTGCAGTGAAGCCATTTACTGTTTTTTAT 112
HS1701          G-ATAATATGTTTAGTTTACTAACGGTCATTTTGCAGTGAAGCCATTTACTGTTTTTTAT 118
HS1702          GGATAATATGTTTAGTTTACTAACGGTCATTTTGCAGTGAAGCCATTTACTGTTTTTTAT 115
                * ************************************************************

MC4100          CGACCAGATAATCTGTTCTCTAATGTTAACTCCCCCTAACCTGTTGCTTTAGTTATTCAT 173
HS1700          CGACCAGATAATCTGTTCTCTAATGTTAACTCCCCCTAACCTGTTGCTTTAGTTATTCAT 172
HS1701          CGACCAGATAATCTGTTCTCTAATGTTAACTCCCCCTAACCTGTTGCTTTAGTTATTCAT 178
HS1702          CGACCAGATAATCTGTTCTCTAATGTTAACTCCCCCTAACCTGTTGCTTTAGTTATTCAT 175
                ************************************************************

MC4100          TTCCTGTCTCACTTTGCCTTAATACCCTACGTTAAATGTTACTAATTTGTTGCTTTTGAT 233
HS1700          TTCCTGTCTCACTTTGCCTTAATACCCTACGTTAAATGTTACTAATTTGTTGCTTTTGAT 232
HS1701          TTCCTGTCTCACTTTGCCTTAATACCCTACGTTAAATGTTACTAATTTGTTGCTTTTGAT 238
HS1702          TTCCTGTCTCACTTTGCCTTAATACCCTACGTTAAATGTTACTAATTTGTTGCTTTTGAT 235
                ************************************************************

MC4100          CACAATAAGAAAACAATATGTCGCTTTTGTGCGCATTTTTCAGAAATGTAGATATTTTTA 293
HS1700          CACAATAAGAAAACAATATGTCGCTTTTGTGCGCATTTTTCAGAAATGTAGATATTTTTA 292
HS1701          CACAATAAGAAAACAATATGTCGCTTTTGTGCGCATTTTTCAGAAATGTAGATATTTTTA 298
HS1702          CACAATAAGAAAACAATATGTCGCTTTTGTGCGCATTTTTCAGAAATGTAGATATTTTTA 295
                ************************************************************

MC4100          GATTATGGCTACGAAATGAGCATCGCCATGTCACCCTACATCTCATAAGAGGATCGCTTC 353
HS1700          GATTATGGCTACGAAATGAGCATCGCCATGTCACCCTACATCTCATAAGAGGATCGCTTC 352
HS1701          GATTATGGCTACGAAATGAGCATCGCCATGTCACCCTACATCTCATAAGAGGATCGCTTC 358
HS1702          GATTATGGCTACGAAATGAGCATCGCCATGTCACCCTACATCTCATAAGAGGATCGCTTC 355
                ************************************************************

MC4100          TGATGAATGCACTGACCGCCGTACAAAATAACGCTGTCGATTCAGGCCAGGACTATAGCG 413
HS1700          TGATGAATGCACTGACCGCCGTACAAAATAACGCTGTCGATTCAGGCCAGGACTATAGCG 412
HS1701          TGATGAATGCACTGACCGCCGTACAAAATAACGCTGTCGATTCAGGCCAGGACTATAGCG 418
HS1702          TGATGAATGCACTGACCGCCGTACAAAATAACGCTGTCGATTCAGGCCAGGACTATAGCG 415
                ************************************************************

MC4100          GATTCACCCTCACCCCGTCGGCGCAATCCCCGCGTCTGCTGGAACTCACCTTCACCGAAC 473
HS1700          GATTCACCCTCACCCCGTCGGCGCAATCCCCGCGTCTGCTGGAACTCACCTTCACCGAAC 472
HS1701          GATTCACCCTCACCCCGTCGGCGCAATCCCCGCGTCTGCTGGAACTCACCTTCACCGAAC 478
HS1702          GATTCACCCTCACCCCGTCGGCGCAATCCCCGCGTCTGCTGGAACTCACCTTCACCGAAC 475
                ************************************************************

MC4100          AGACGACCAAACAGTTTCTTGAGCAGGTTGCCGAGTGGCCCGTGCAGGCGCTGGAGTACA 533
HS1700          AGACGACCAAACAGTTTCTTGAGCAGGTTGCCGAGTGGCCCGTGCAGGCGCTGGAGTACA 532
HS1701          AGACGACCAAACAGTTTCTTGAGCAGGTTGCCGAGTGGCCCGTGCAGGCGCTGGAGTACA 538
HS1702          AGACGACCAAACAGTTTCTTGAGCAGGTTGCCGAGTGGCCCGTGCAGGCGCTGGAGTACA 535
                ************************************************************

MC4100          AATCGTTTCTGCGTTT----TTGG------------------------------------ 553
HS1700          AATCGTTTCTGCGTTT----TCGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 588
HS1701          AATCGTTTCTGCGTTTCGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 598
HS1702          AATCGTTTCTGCGTTTCGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 595
                ***************
```

Figure 8. Replica plate pattern of wild type strain HS1057° (*rpoS*$^+$, *gabD-lacZ*), its *rpoS*$^-$ derivative HS1057p°, and Group I (CN) GABA utilizing mutants generated from the wild type *rpoS*$^+$ fusion background only (HS1903a, HS1903b, HS1903c). All cells were grown in flasks containing LB broth to an OD_{600} ~ 1.0. The cells were then pelleted by centrifugation, washed in 1 X M9 salts (without NH_4Cl) and replica plated onto a LB-X-Gal plate. The plate was incubated overnight at 37°C.

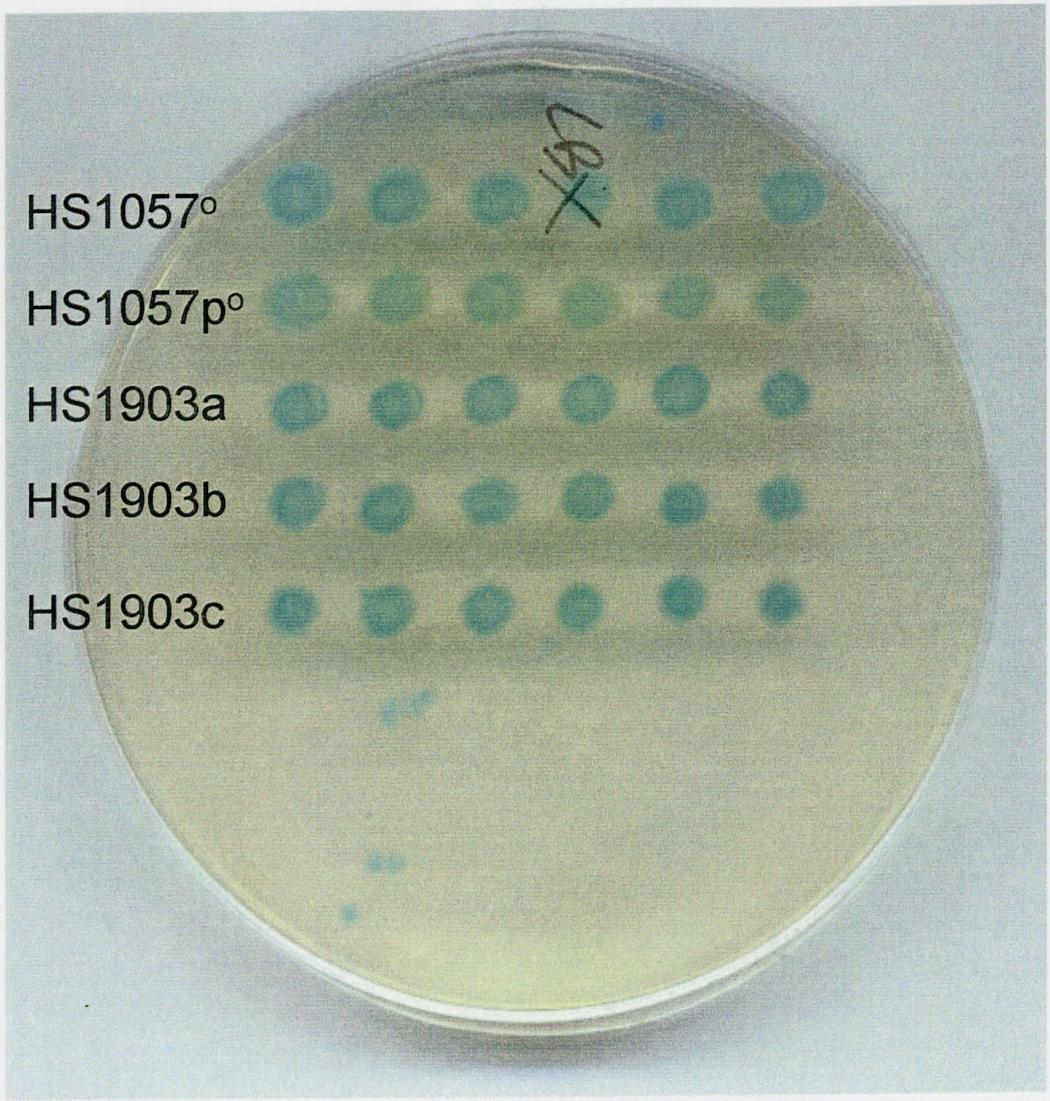
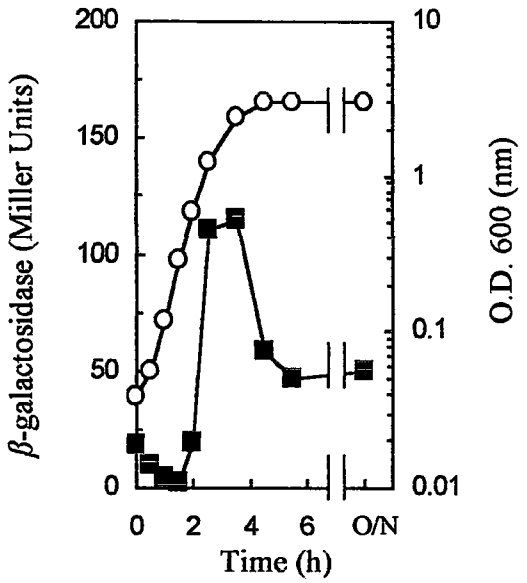
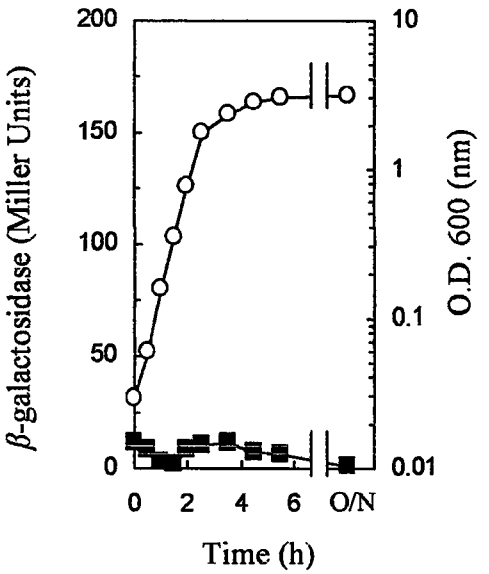

HS1057°
HS1057p°
HS1903a
HS1903b
HS1903c

Figure 9. Expression of *gabD* in wild type and "over-expressing" GABA utilizing mutant strains. This assay was done to duplicate the over-expression evident on LB-X-Gal plates (fig. 8) in liquid LB media. Flasks containing LB broth were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity.
(A) HS1057^{o} (*rpoS*$^{+}$, *gabD-lacZ*),
(B) HS1057p^{o} (*rpoS*$^{-}$, *gabD-lacZ*),
(C) HS1903a (*rpoS*$^{+}$, *gabD-lacZ*, CN GABA utilizing mutant),
(D) HS1903c (*rpoS*$^{+}$, *gabD-lacZ*, CN GABA utilizing mutant).
Symbols: o, Growth (OD_{600}); ■, β-galactosidase activity; O/N, overnight.

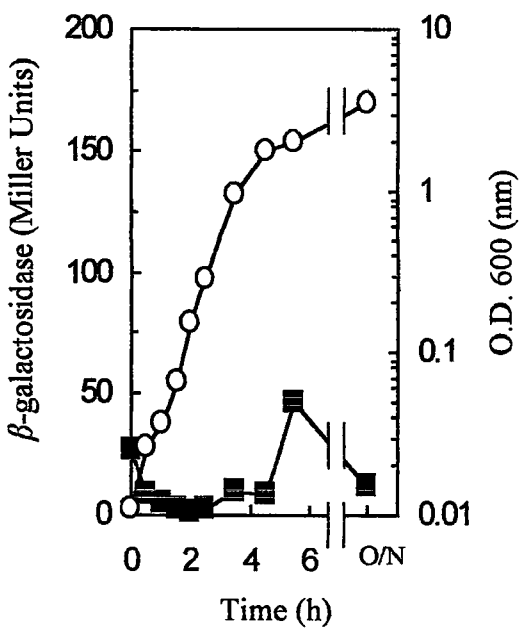
(A)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
50
100
150
200
0.01
0.1
1
10
0
2
4
6
O/N

(B)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
50
100
150
200
0.01
0.1
1
10
0
2
4
6
O/N

(C)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
50
100
150
200
0.01
0.1
1
10
0
2
4
6
O/N

(D)

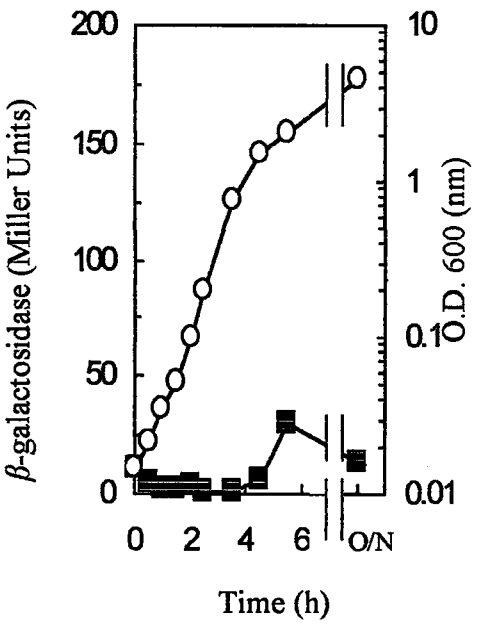
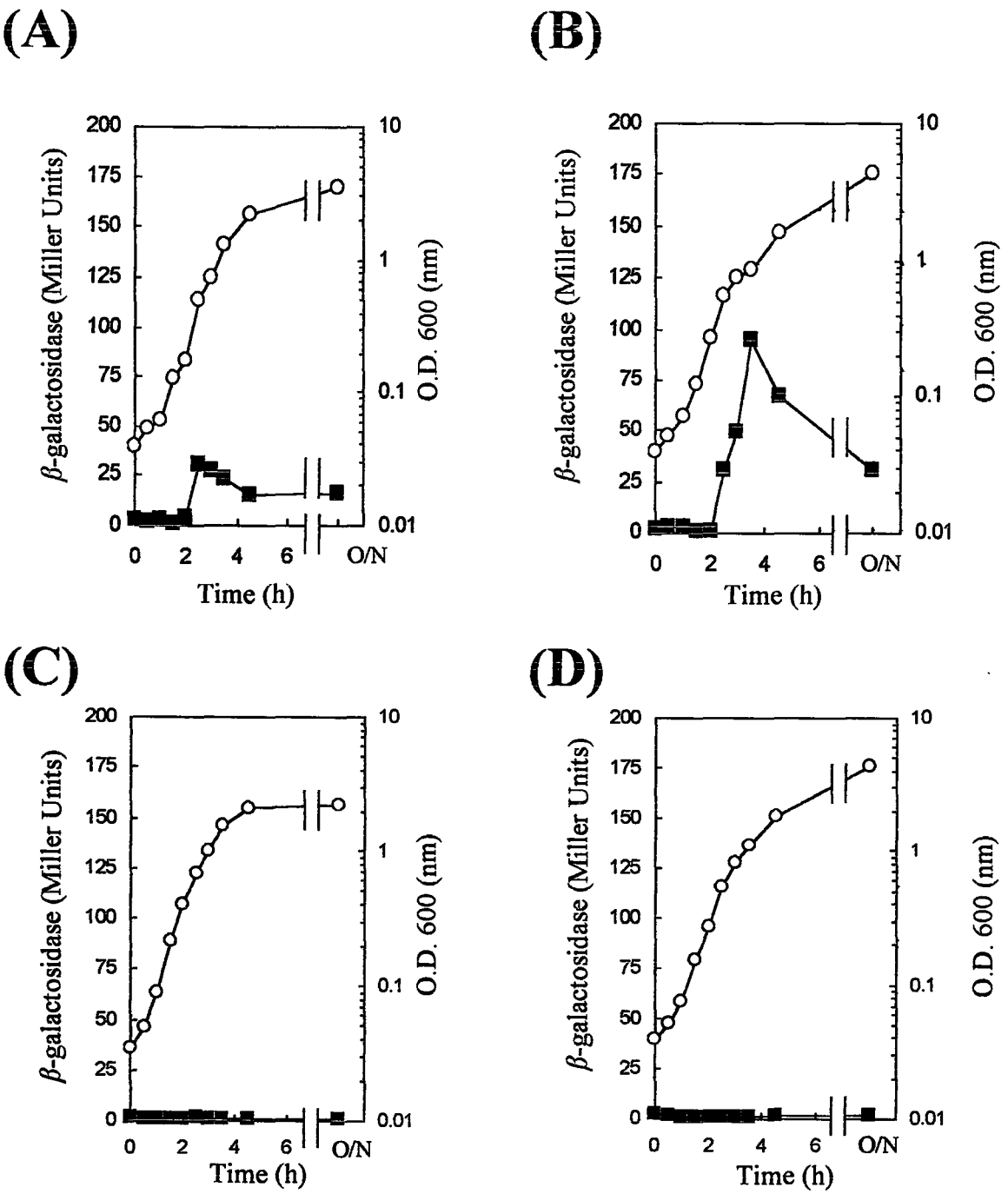
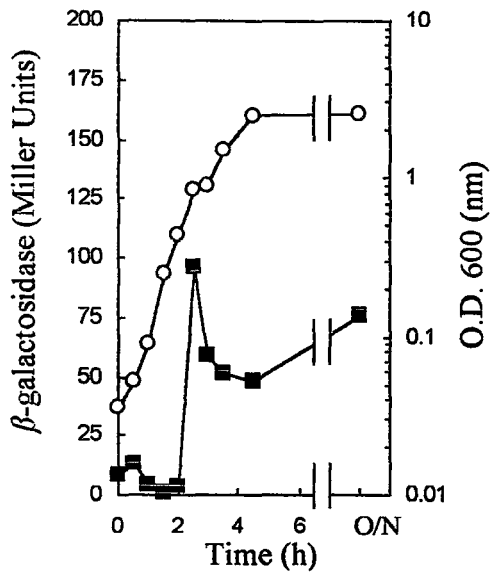

β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
0
50
100
150
200
0.01
0.1
1
10
0
2
4
6
O/N

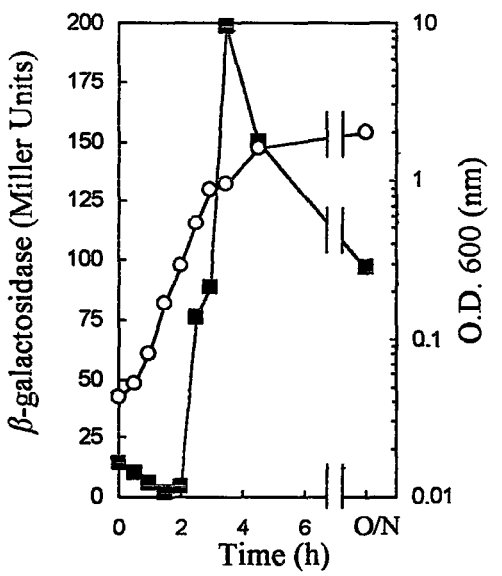
Figure 10. Conditioned medium as an inducer of *gab* expression. Flasks containing either LB broth (control treatment) or conditioned medium were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity.
(A) HS1010° (*rpoS*$^+$, *gabP-lacZ*) grown in LB,
(B) HS1010° (*rpoS*$^+$,*gabP-lacZ*) grown in conditioned medium,
(C) HS1010p° (*rpoS*$^-$, *gabP-lacZ*) grown in LB,
(D) HS1010p° (*rpoS-*, *gabP-lacZ*) grown in conditioned medium,
(E) HS1057° (*rpoS*$^+$, *gabD-lacZ*) grown in LB,
(F) HS1057° (*rpoS*$^+$, *gabD-lacZ*) grown in conditioned medium,
(G) HS1057p° (*rpoS*$^-$, *gabD-lacZ*) grown in LB,
(H) HS1057p° (*rpoS*$^-$, *gabD-lacZ*) grown in conditioned medium.
Symbols: o, Growth (OD_{600}); ■, β-galactosidase activity; O/N, overnight.


(A)
(B)
(C)
(D)
β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N
0
2
4
6
25
50
75
100
125
150
175
200
0.01
0.1
1
10

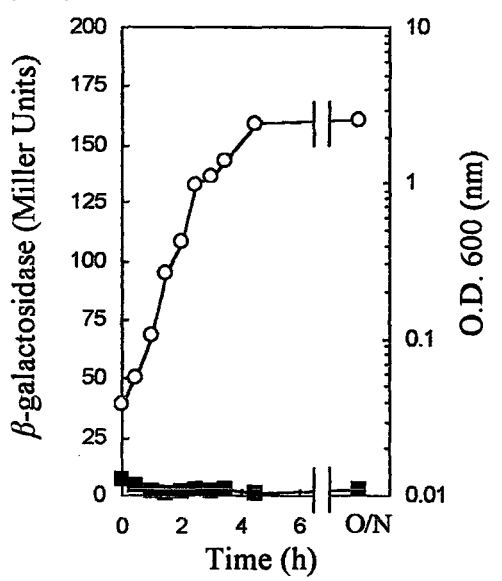
(E)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N
200
175
150
125
100
75
50
25
0
10
1
0.1
0.01
0
2
4
6

(F)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N
200
175
150
125
100
75
50
25
0
10
1
0.1
0.01
0
2
4
6

(G)


β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N
200
175
150
125
100
75
50
25
0
10
1
0.1
0.01
0
2
4
6

(H)

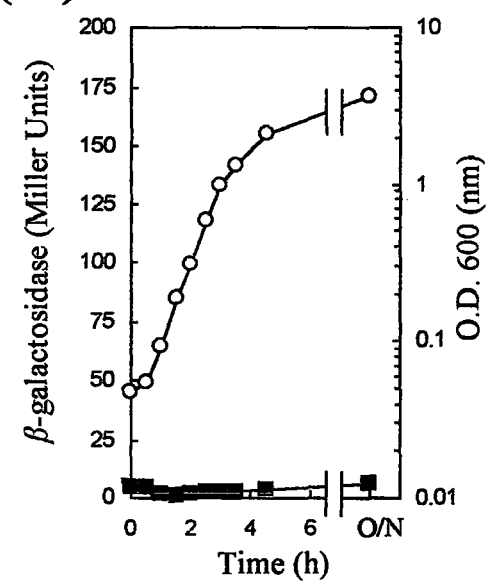
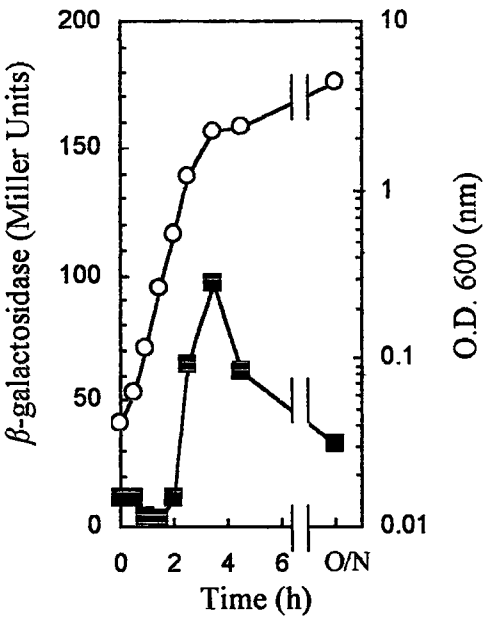
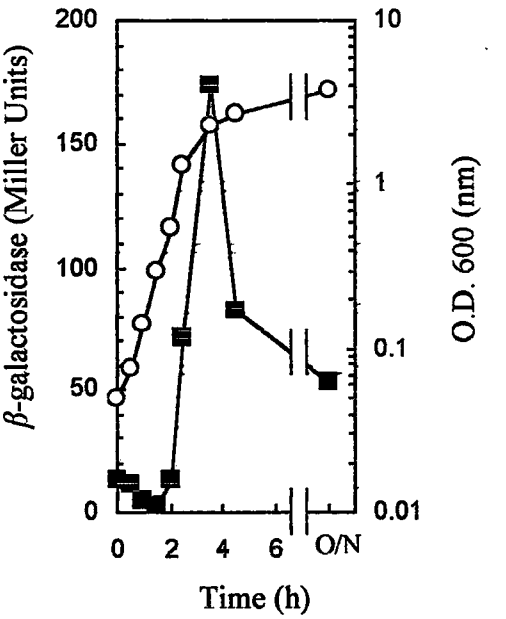

β-galactosidase (Miller Units)
O.D. 600 (nm)
Time (h)
O/N
200
175
150
125
100
75
50
25
0
10
1
0.1
0.01
0
2
4
6

Figure 11. GABA as an inducer of *gab* expression in LB media. Flasks containing either LB broth (control treatment) or LB broth + GABA (0.2%) were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. Also, the assays were done in duplicate.
(A) HS1057^{o} (*rpoS*$^{+}$, *gabD-lacZ*) grown in LB,
(B) HS1057^{o} (*rpoS*$^{+}$, *gabD-lacZ*) grown in LB,
(C) HS1057^{o} (*rpoS*$^{+}$, *gabD-lacZ*) grown in LB + GABA,
(D) HS1057^{o} (*rpoS*$^{+}$, *gabD-lacZ*) grown in LB + GABA,
(E) HS1057p^{o} (*rpoS*$^{-}$, *gabD-lacZ*) grown in LB,
(F) HS1057p^{o} (*rpoS*$^{-}$, *gabD-lacZ*) grown in LB,
(G) HS1057p^{o} (*rpoS*$^{-}$, *gabD-lacZ*) grown in LB + GABA,
(H) HS1057p^{o} (*rpoS*$^{-}$, *gabD-lacZ*) grown in LB + GABA.
Symbols: o, Growth (OD_{600}); ■, *β*-galactosidase activity; O/N, overnight.

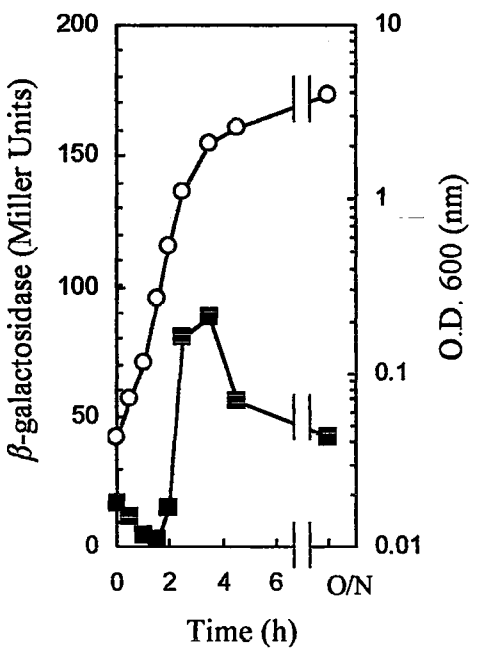
(A)


β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0
2
4
6
O/N
Time (h)

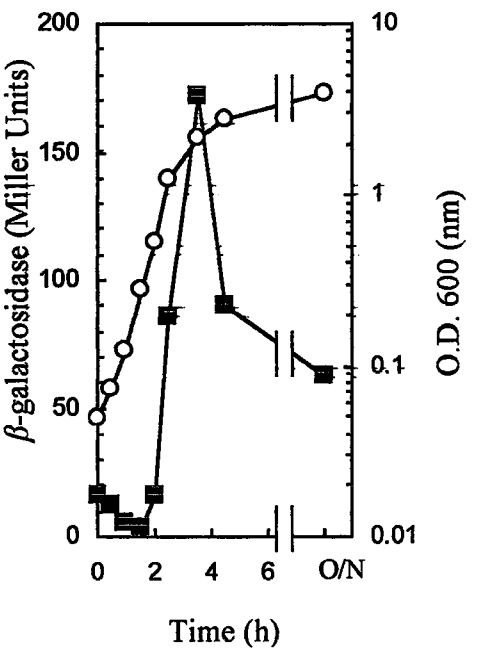
(B)

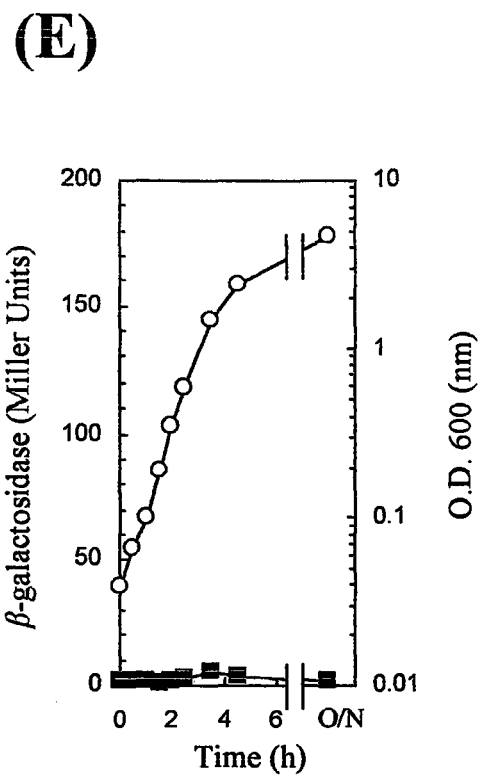

β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0
2
4
6
O/N
Time (h)

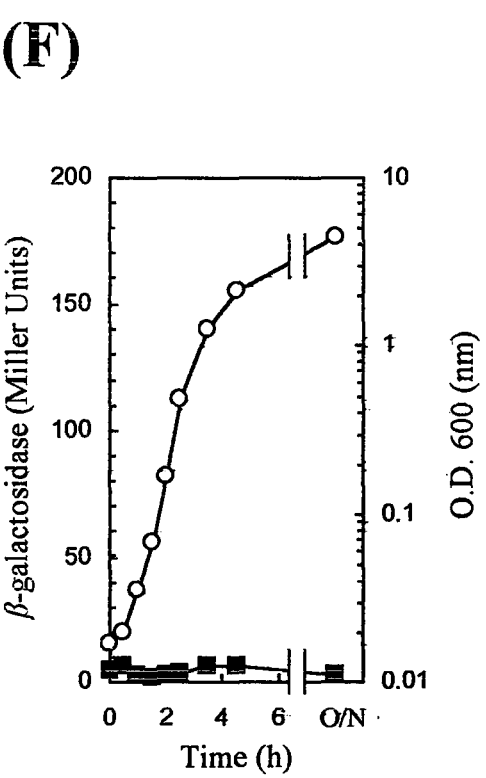
(C)

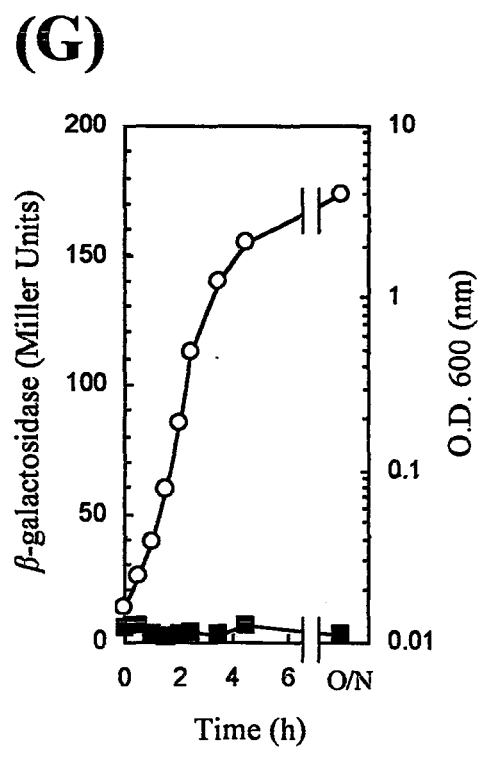

β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0
2
4
6
O/N
Time (h)

(D)


β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0
2
4
6
O/N
Time (h)


(E)
β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0 2 4 6 O/N
Time (h)


(F)
β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0 2 4 6 O/N
Time (h)


(G)
β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0 2 4 6 O/N
Time (h)

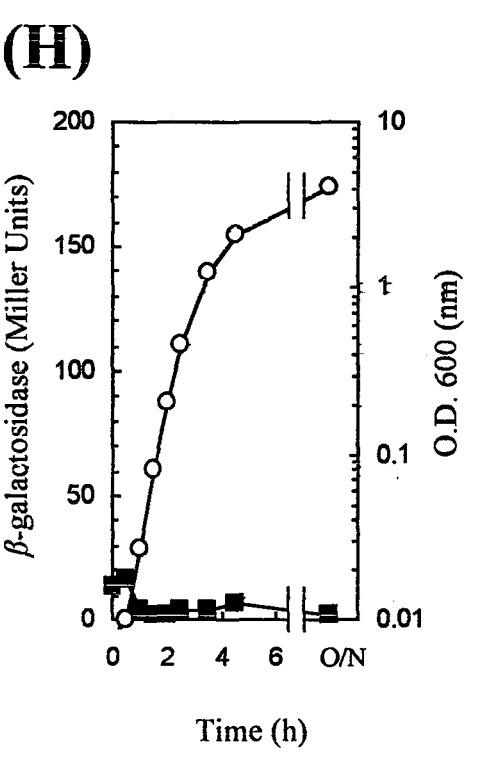
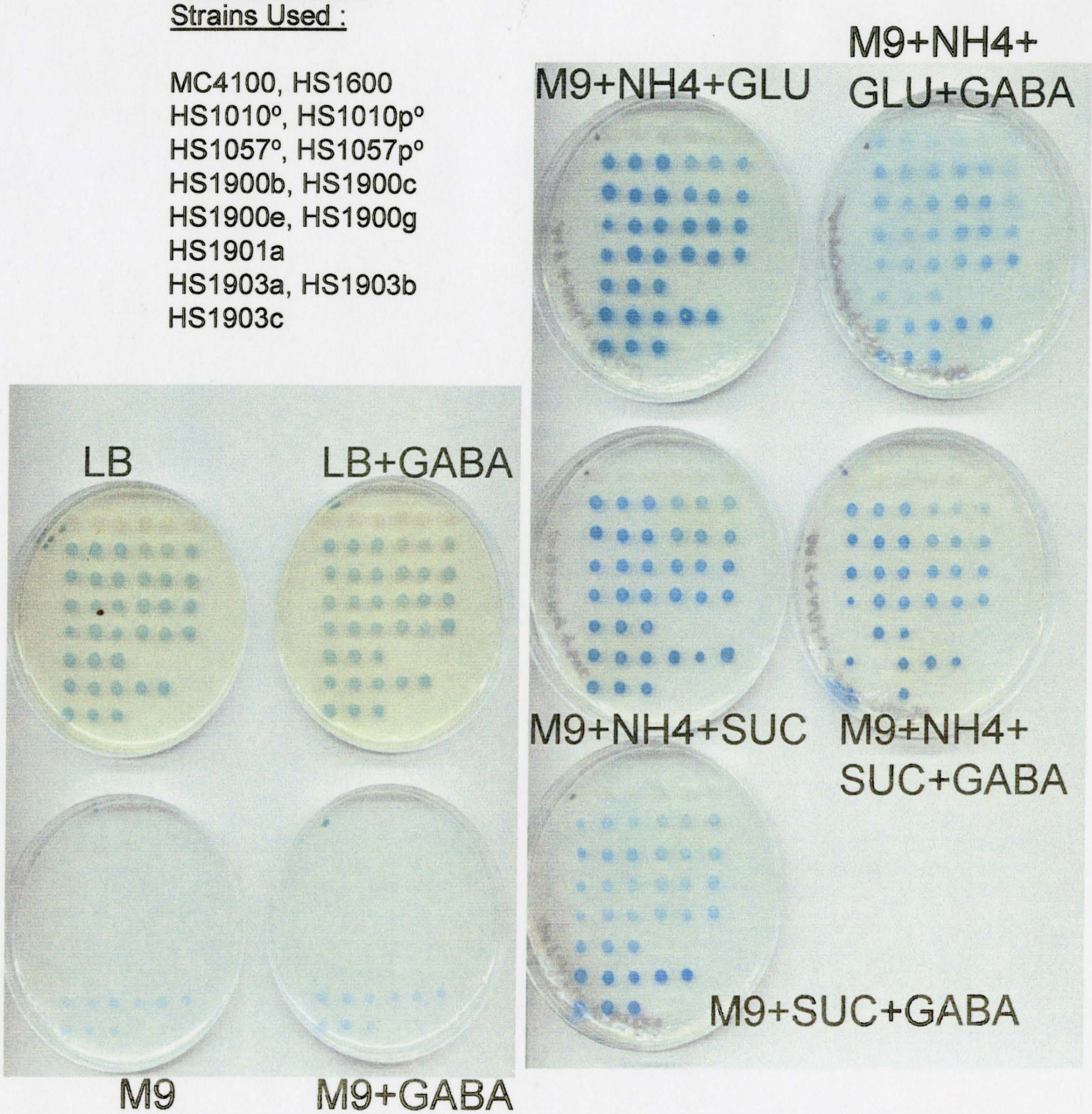

(H)
β-galactosidase (Miller Units)
200
150
100
50
0
10
1
0.1
0.01
O.D. 600 (nm)
0 2 4 6 O/N
Time (h)

Figure 12. GABA as an inducer of *gab* expression in minimal medium. Flasks containing LB broth were inoculated with overnight cultures as described in Materials and Methods. Cells were grown to early stationary phase (OD_{600} ~ 1.0), pelleted by centrifugation, washed in 1 X M9 salts (without NH_4Cl) and replica plated onto various minimal plates as described in Materials and Methods. The test compound (GABA) was added at a final concentration of 0.2%. Replica plating was done in triplicate using the following strains in the following order: MC4100 (WT), HS1600 (*rpoS*$^-$), HS1010° (*rpoS*$^+$, *gabP-lacZ*), HS1010p$^\circ$ (*rpoS*$^-$, *gabP-lacZ*), HS1057° (*rpoS*$^+$, *gabD-lacZ*), HS1057p$^\circ$ (*rpoS*$^-$, *gabD-lacZ*), HS1900b-g (*rpoS*$^+$, *gabP-lacZ*, CN GABA utilizing mutants), HS1901a (*rpoS*$^+$, *gabP-lacZ*, N GABA utilizing mutant), HS1903a-c (*rpoS*$^+$, *gabD-lacZ*, CN GABA utilizing mutants).

Replica Plate Pattern Order of Strains Used :

MC4100, HS1600
HS1010°, HS1010p°
HS1057°, HS1057p°
HS1900b, HS1900c
HS1900e, HS1900g
HS1901a
HS1903a, HS1903b
HS1903c



CHAPTER 4. DISCUSSION

About 25 different compounds, consisting mainly of amino acids but also including purines, pyrimidines, and nitrate, can serve as sole nitrogen sources for *E. coli* (Tyler, 1978) and *Salmonella typhimurium* (Gutnick et al., 1969). Most of the 20 natural L-amino acids and several D-amino acids serve as potential carbon and energy sources for members of the *Enterobacteriaceae*. γ-aminobutyrate (GABA), a four carbon amino acid, serves as an intermediate in the pathways of L-glutamate degradation in *E. coli* (Marcus et al., 1969) and L-arginine and putrescine catabolism in *E. coli* and *K. aerogenes* (Friedrich and Magasanik, 1979; Shaibe et al., 1985). GABA has been shown to serve as the sole carbon and nitrogen source in *K. aerogenes* (Friedrich and Magasanik, 1979) and in *E. coli* (Dover and Halpern, 1972b). The GABA degradation pathway is similar in *K. aerogenes* (Friedrich and Magasanik, 1979) and in *E. coli* (Dover et al., 1972b) in that it involves the *gab* operon, a highly RpoS-dependent regulon (Schellhorn et al., 1998). However, the *gab* system has been examined in much more detail in *E. coli*.

It has been well established that wild type *E. coli* cannot degrade GABA as the sole carbon source (Dover and Halpern, 1972a) and can utilize it as a nitrogen source only in the presence of succinate (Donnelly and Cooper, 1981). This inability of wild type strains to grow in the presence of GABA may be dependent on the expression levels

of the *gab* genes in these strains. In fact, the enzymes involved in GABA catabolism are expressed at low constitutive levels in wild type strains compared to GABA mutants which exhibit eightfold higher levels of these enzymes (Dover and Halpern, 1972a). Therefore, the reduced level of *gab* expression in wild type strains is postulated to be insufficient for the utilization of GABA as sole carbon or nitrogen source in these strains (Dover and Halpern, 1972a). This seems interesting as it forces one to consider the possible reasons for the presence of the *gab* operon in these wild type *E. coli* strains, something which is not clear at this point.

Even though wild type *E. coli* K-12 strains cannot utilize GABA, it is possible to isolate GABA utilizing mutant strains which can use GABA as sole carbon or nitrogen source. Dover and Halpern (1972a) were able to obtain GABA nitrogen utilizing strains but all attempts to obtain GABA carbon utilizing mutants directly from the wild type strains failed. Subsequently, secondary mutants derived from these GABA nitrogen utilizing strains were obtained which could use GABA as the sole source of carbon. Interestingly, unlike Dover and Halpern (1972a), we were able to isolate all three classes of GABA utilizing mutant strains directly from the wild type background (MC4100) by selection on appropriate minimal media supplemented with GABA (Table 2a). We were able to isolate mutants which could use GABA as a carbon and nitrogen source or solely as a carbon or nitrogen source alone. The reason for why we didn't encounter any difficulty in obtaining GABA carbon utilizing mutants directly from the wild type strain, as did Dover and Halpern (1972a), may be due to differences in wild type strains used between our study and theirs and differences in strategies used for isolating GABA

mutants. Their isolation criteria relied upon the use of ultraviolet mutagenesis (Dover and Halpern, 1972a), whereas we used selective minimal media for isolating GABA utilizing mutant strains.

To confirm the phenotype of our GABA utilizing mutants, these strains were replica plated on selective minimal media supplemented with GABA. The results (Table 3) show some interesting observations. We noticed that mutants selected to use GABA as the sole carbon source could also use GABA as a nitrogen source, but mutants selected to use GABA as the sole nitrogen source could not use GABA as a carbon source. Based on this data, we can make two predictions. First, it seems that GABA carbon and nitrogen utilizing strains are phenotypically similar to GABA carbon utilizing mutants. Secondly, GABA nitrogen utilizing mutants most likely outnumber other groups of GABA mutant strains.

Next, these GABA mutants obtained were transduced with *lacZ* fusions to members of the *gab* operon in order to expedite characterization of the factors responsible for GABA utilization. We found that the transductants lost the capacity to utilize GABA. The only transduced mutants that still retained the ability to utilize GABA could only use it as a nitrogen source. This observation strongly suggested that a *gab* operon dependent GABA utilization pathway was was altered in the mutants. Given that the *gab* pathway relies on over-expression of the *gab* operon for GABA metabolism (Dover and Halpern, 1972a), we assayed our transductants for β-galactosidase activity (Figure 6) to measure *gab* activity in order to characterize the mutations accomodating this over-expression sufficient for GABA uptake and catabolism. Our working model

was that if the transduced mutants were over-expressing the *gab* genes, then the mutation lay somewhere outside the linkage distance of the transducing phage. Conversely, if the transductants showed expression levels similar to wild type expression, then that would suggest one of two possibilities. One, the suppression of *gab* over-expression could be a result of the replacement of the mutation site with the wild type site during the transduction event, suggesting that the mutation lay somewhere within the linkage distance of the *gab* operon. Finally, the second possibility pointed towards some other alternative pathway of GABA utilization in these mutants. The results of β-galactosidase studies (Figure 6) clearly indicated that *gab* expression levels of the transduced GABA mutants were similar to wild type expression. In an attempt to uncover potential mutations in close vicinity to the *gab* operon, we sequenced the *csiD* promoter in wild type (MC4100) and the three classes of GABA mutant strains (HS1700, HS1701, HS1702) (Figure 7). Both *csiD* (Marschall et al., 1998) and the *gab* operon (Schellhorn et al., 1998) have been identified as members of the RpoS regulon and are transcribed in the same direction (Figure 1). As a result, these two adjacent and highly RpoS dependent transcriptional units may be driven from a single upstream RpoS dependent promoter classified as the *csiD* promoter, or the *gab* promoter. However, we didn't find a mutation in the *csiD* promoter region (Figure 7) suggesting that either the mutation responsible for *gab* over-expression lies in close proximity to the *gab* operon outside of the *csiD* promoter region or that some other pathway of GABA utilization is required in these mutants. Future work should include Northern blot analysis of *gab* expression in these wild type and GABA mutant strains to determine which factor is most likely responsible.

The literature pertaining to GABA comments on the role of the *gab* operon in GABA utilization (Dover and Halpern, 1972a, 1972b, 1974). GABA utilizing mutants exhibit a severalfold increase in *gab*-encoded functions which is postulated to be sufficient to allow for GABA utilization in these strains (Dover and Halpern, 1972a). Interestingly, this study speculates the presence of an alternate GABA utilization pathway that may be independent of *gab* expression. Hence, to validate the possibility that a second mechanism of GABA utilization exists that doesn't involve the *gab* operon, it was important to show that the *gab* operon was dispensable (i.e., non-essential) for GABA utilization. To accomplish this purpose, we attempted to isolate GABA mutants from wild type fusion strains (Figure 5) carrying *lacZ* fusions to members of the *gab* operon (HS1010^{o} and HS1057^{o}) and their *rpoS*$^{-}$ derivatives (HS1010p^{o} and HS1057p^{o}). The fact that we were able to obtain GABA utilizing mutants from both fusion strains in the *rpoS*$^{+}$ background only (Table 4), suggests two additional features of GABA metabolism in *E. coli*. One, that the *gab* operon indeed is dispensable for GABA utilization. In addition, other RpoS regulated functions, independent of *gab*-encoded functions, may allow the cell to metabolize GABA. These may include the *ast* operon, a five gene operon, involved in degradation of arginine and ornithine to generate succinate and glutamate and the *gad* genes involved in decarboxylation of glutamate to GABA by means of glutamic acid decarboxylase (Baca-DeLancey et al., 1999; De Biase et al., 1999). Surprisingly, from amongst these GABA mutants that seemed to be making use of some other GABA utilization pathway, we were able to identify those mutants that were over-expressing the functions of the *gab* operon . Over-expressers were identified by comparing *lac*

expression to the appropriate wild type fusion strain on LB-X-gal plates as described in Materials and Methods. Table 5 lists the number of over-expressing GABA mutants obtained. Figure 8 is an illustration of this over-expression. This raises an interesting question because if the *gab* operon is not being utilized for GABA degradation, then why is it being over-expressed in these GABA utilizing mutant strains? Perhaps, the *gab* operon serves another purpose, besides GABA utilization, in some strains of *Enterobacteriaceae*. Furthermore, in an attempt to elucidate the mutation responsible for this *gab* over-expression in these bacterial strains, we sequenced the *csiD* promoter in these GABA mutants also (data not shown). No mutation was found in this region. Future work entailing primer extension studies might prove useful in pinpointing the exact location of the mutation responsible for *gab* over-expression.

The next portion of my thesis involved testing putative inducers of *gab* expression to obtain some regulatory information about this operon. Conditioned media and γ-aminobutyrate were tested for their inducing effects. Conditioned media has been shown to an inducer of *gabT* expression (Baca-DeLancey et al., 1999) but failed to elicit a similar response for the other two structural genes in this operon (our study, Figure 10). This conflict in data may be a result of differences in strains used between our study and those of other researchers (Baca-DeLancey et al., 1999). Likewise, synthesis of the two γ-aminobutyrate –degrading enzymes has been shown to be induced by γ-aminobutyrate itself when cells are growing in minimal salts media (Dover and Halpern, 1972b; Donnelly and Cooper, 1981). However, we did not find GABA to be an inducer of *gab* operon expression, regardless of whether the cells were grown in minimal salts media

(Figure 12) or rich media (Figure 11). Once again, factors such as differences in strains used could be an issue responsible for this conflict in findings observed between our study and others. However, other reasons, which are not clear at this point, may also be at work.

Finally, we wanted to investigate the role of glutamate in acid adaptation and osmotolerance. Glutamate has been documented to serve a protective function against acidic shock and salt stress (De Biase et al., 1999). To confirm this effect, we tested the sensitivity of our GABA utilizing mutants to various weak acids and varying concentrations of salt on minimal plates as described in Materials and Methods. For acid adaptation, sensitivity was tested in the presence and absence of GABA. This is because the pathway of GABA breakdown in *E. coli* converges with that of glutamate catabolism by means of a decarboxylation reaction catalyzed by glutamic acid decarboxylase (Dover and Halpern, 1972a). Glutamic acid decarboxylase deficient mutants are sensitive to acid shock (De Biase et al., 1999). Therefore, our hypothesis was that perhaps the ability to generate GABA or have access to it confers acid resistance to bacteria. As a result, we expected adding GABA to the media to be beneficial for cell growth. Interestingly though, GABA proved to be detrimental for growth because we found that the cells exhibited enhanced growth in the absence of GABA (Table 8). An explanation for this observation is not clear at this point. As for the role of glutamate in osmotolerance, sensitivity was tested in the presence and absence of glutamate. We hypothesized optimal growth in the presence of glutamate and indeed this is what we found (Table 9).

Based on our findings, therefore, we can conclude that glutamate serves as an osmoprotectant, but its role in acid tolerance is not clear.

Acknowledgements

I would like to thank Dr. Herb Schellhorn for doing the transductions and Dr. Rouha Kasra for providing GABA mutant strains. This work was funded from an operating grant to H.E.S. from the Natural Sciences and Engineering Council (NSERC) of Canada.

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