Mutations in \textit{deoB} and \textit{deoC} alter an extracellular signaling pathway required for activation of the \textit{gab} operon in \textit{Escherichia coli}

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Abstract

In \textit{Escherichia coli}, a \textit{lacZ} fusion to the \textit{gabT} gene is activated by the accumulation of two self-produced extracellular signals, indole and a second unidentified signal (signal-2). Extracellular indole contributes approximately 25\% of this activation and signal-2 is responsible for the majority of activation. Using an \textit{E. coli} strain unable to produce indole and containing a \textit{gabT::lacZ} fusion, a genetic approach was used to search for genes involved in the production of signal-2. A spontaneous \textit{E. coli} mutant, MJ1, exhibited significantly less signal-2 activity based on the ability of spent culture supernatants from this mutant to activate the \textit{gabT::lacZ} fusion. Genetic analysis of MJ1 revealed that it contained two mutations, one in \textit{thyA} and a second unknown mutation, designated \textit{spl1} (signal production locus) that led to loss of signal-2 production. The \textit{spl1} second-site mutation arises at high frequency in a \textit{thyA} background because it suppresses the loss of viability. This study demonstrates that mutations in \textit{deoB} and \textit{deoC} were capable of suppressing the loss of viability in \textit{thyA} mutants and concomitantly resulted in loss of signal-2 activity in conditioned medium. Interestingly, both \textit{deoB} and \textit{deoC} mutations in an otherwise wild-type background resulted in higher levels of \textit{gabT::lacZ} expression in cells at low density. It is hypothesized that \textit{deoB} and \textit{deoC} mutations result in an enhanced rate of signal-2 uptake and thus deplete signal-2 from the external medium.

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Keywords: Cell-cell signaling; \textit{Escherichia coli}; \textit{deo} operon

1. Introduction

Many bacterial genes are expressed in response to self-produced extracellular signals in their environment [1]. These signals accumulate with an increase in cell density and elicit changes in gene expression when a threshold concentration is reached. This phenomenon has been demonstrated in both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, commonly used signals include the \textit{N}-acyl homoserine lactones and the LuxS-dependent AI-2 signal, recently characterized as a furanone derivative [2]. However, unlike many other Gram-negative bacteria, an \textit{N}-acyl homoserine lactone signal has not been identified in \textit{Escherichia coli} [2,3]. In \textit{E. coli} cell-to-cell signaling pathways are involved in the regulation of flagella and motility, expression of type III secretion systems, transcription of \textit{ftsQAZ}, inhibition of DNA replication, and activation of degradative pathways [4–11]. Previous work in our laboratory has determined that \textit{cysK}, \textit{astD}, \textit{tnaB} and \textit{gabT} genes in \textit{E. coli} are activated by the accumulation of extracellular signals [3]. One of these signals has been identified as indole and is the primary signal for activation of \textit{tnaB-lacZ} fusion [11]. However, for the \textit{astD} and \textit{gabT} genes, indole accounted for only 25–30\% of the activity in conditioned medium (CM). This suggested that a second signal, designated signal-2, was responsible for the majority of activation [11]. In this study, a genetic approach was used to search for genes involved in the production of signal-2. Using a \textit{tnaA} (tryptophanase) mutant (PR1), deficient in indole production and containing a \textit{gabT-lacZ} fusion as a biosensor, we have isolated a mutant (MJ1) with significantly reduced production of...
Unexpectedly, MJ1 was found to contain two mutations, a thyA mutation that enhanced production of signal-2 and a second-site suppressor (designated spl1 for signal-2 production locus) that restored viability to thyA mutants, but resulted in loss of signal-2 activity. Loss of function mutations in deoB and deoC all gave rise to the spl phenotype in both thyA and wild-type backgrounds.

2. Materials and methods

2.1. Bacterial strains, phages, plasmids, and growth conditions

*E. coli* strains used in this study are shown in Table 1. *E. coli* strains DH5α (Gibco BRL) and XL1 were used for plasmid propagation. Bacteriophage P1 was used for transduction. Plasmids pET21a, pSK and pBCKS were used as cloning vectors [12–14]. Luria–Bertani (LB) medium consisting of 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter was used as growth medium. Antibiotics were used at the following concentrations: kanamycin 20 μg ml⁻¹, trimethoprim 40 μg ml⁻¹, ampicillin 100 μg ml⁻¹, chloramphenicol 25 μg ml⁻¹, and streptomycin 25 μg ml⁻¹. All β-galactosidase assays were conducted by the method of Miller [15] using cells harvested at early exponential phase (OD₆₀₀ = 0.35)

### Table 1

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1</td>
<td>gabT::mini-Tn5SucZ, tnaA2, SmalT, SulZ</td>
<td>This study</td>
</tr>
<tr>
<td>MJ1</td>
<td>PR1 splI</td>
<td>This study</td>
</tr>
<tr>
<td>MJ6</td>
<td>PR1 thyA::cm</td>
<td>This study</td>
</tr>
<tr>
<td>MJ7</td>
<td>MJ6 splI isolate 1</td>
<td>This study</td>
</tr>
<tr>
<td>MJ8</td>
<td>MJ6 splI isolate 2</td>
<td>This study</td>
</tr>
<tr>
<td>MJ22</td>
<td>PR1 thyA</td>
<td>This study</td>
</tr>
<tr>
<td>MJ23</td>
<td>MJ22 deoB::cat</td>
<td>This study</td>
</tr>
<tr>
<td>MJ24</td>
<td>MJ22 deoC::cat</td>
<td>This study</td>
</tr>
<tr>
<td>MJ25</td>
<td>PR1 deoB::cat</td>
<td>This study</td>
</tr>
<tr>
<td>MJ26</td>
<td>PR1 deoC::cat</td>
<td>This study</td>
</tr>
</tbody>
</table>

2.2. Preparation of CM

To determine the ability of MJ1 to produce activating extracellular signals, its cell-free supernatants were prepared. Briefly, 30 ml of LB broth was inoculated with a single colony of MJ1 mutant and incubated with shaking (250 rpm) at 37°C. Cells were harvested after entering stationary phase (OD₆₀₀ of 1.5), and pelleted at 4300 × g for 10 min. The resulting supernatants were supplemented with 20 μg ml⁻¹ (tryptone, yeast extract) to a final concentration of 0.5×, and the pH was adjusted to 7.5; this is referred to as CM. The CM was then filter-sterilized with a 0.22-μm-pore filter attached to a disposable syringe and stored at −80°C until use.

2.3. Allelic replacement

The one-step replacement method described by Datsenko and Wanner [16] was used to inactivate selected genes. Polymerase chain reaction (PCR) products were generated from pKD3 or pACYC184 using the primers listed in Table 2. PCR products were electroporated into the appropriate strain carrying pKD46, which contains the phage λ red recombinase under AraC control and transformants were selected on the appropriate antibiotic. The replacement of a gene by the antibiotic marker was verified by PCR.

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer*</th>
<th>Sequence (5'-3')b</th>
<th>Position in the gene</th>
</tr>
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<td>thyA</td>
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<td>GCTTTAATCTACACGGAAAACATGTCACCATTCTGGGACGAATGCGATATGAATATCCTCCTCTACG</td>
<td>208–249</td>
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<tr>
<td></td>
<td>R</td>
<td>GATAATAAACGGCGACGGCAACGGGTCCGGCTGGTTATATATGATGGAGCTGAGCTCTCTC</td>
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<tr>
<td>deoB</td>
<td>F</td>
<td>CGAAGACACACAGGAAGTTCTACCGGCTGGTTATATATGATGGAGCTGAGCTCTCTC</td>
<td>185–226</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTTGTTGATACCGCAGTGTTGGATGCTGCGGACATATGGAATATGCTGCTCTAG</td>
<td>812–770</td>
</tr>
<tr>
<td>deoC</td>
<td>F</td>
<td>AACTCCGCGGGGCAATACCCCGCTGGCTTATGTACATATCCCTCATATGGAATATCCTCCTCTC</td>
<td>111–151</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTTTACCGGATAGGTGGTTATGATGATCCGACCACCCTTAGTGTGGAGCGCGCTGAGCTCTCCTC</td>
<td>517–477</td>
</tr>
<tr>
<td>tss</td>
<td>F</td>
<td>CTGAAACACAAACCGAGATATTTCTTGGCAGCTGCGGACGTTTCTTTCGTTACAG</td>
<td>68–108</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TAAATCGGAACCCCAGTCGAAGTTGGTGAAGCCGATGTAGC</td>
<td>651–611</td>
</tr>
</tbody>
</table>

*aF and R represent the forward and reverse primers respectively.

bThe underlined sequences are the primer sequences that amplify the antibiotic resistance cassette.
3. Results

3.1. Identification of a mutation that decreases signal-2 production or activity

The *gabT* gene encodes a glutamate:succinate semialdehyde aminotransferase and is part of the *gabDTPC* operon which functions in the generation of succinate from γ-aminobutyrate [17]. Previously, extracellular indole was shown to activate a *gabT::lacZ* fusion [11]. However, the use of CM from wild-type and an isogenic *tnaA::cat* mutant indicated that indole contributed only 25–30% of the total activity in *E. coli* CM [11]. We hypothesized that a second signal, designated signal-2, was responsible for the majority of *gabT-lacZ* activation. To identify genes required for signal-2 activity, the *E. coli* strain PR1 *gabT::lacZ, tna2, ΔmutT* was used. PR1 is unable to produce indole and the activation of *gabT::lacZ* by CM relies on signal-2. Transposon mutagenesis of PR1 was performed using the Tn5Tmp transposon. A mutant, designated MJ1, was obtained from PR1 by identifying colonies with altered expression of *gabT::lacZ* on LB plates containing trimethoprim and X-gal. MJ1 produced colonies that appeared darker blue than wild-type and exhibited a blue ring in the agar surrounding the colonies. This resulted in a characteristic ‘halo’ phenotype. To determine whether the apparent increase in blue color was a reflection of β-galactosidase expression from *gabT::lacZ*, cells of PR1 and MJ1 were assayed at early exponential phase in LB broth. The levels of β-galactosidase were slightly higher in MJ1 (120 ± 1 Miller units) relative to PR1 (81 ± 3 units). Next, the production of extracellular signal-2 was tested in CM from MJ1 and wild-type PR1 at OD<sub>600</sub> of 1.5 (Fig. 1). CM from PR1 activated the *gabT-lacZ* fusion 6.4-fold. In contrast, CM from MJ1 activated only 1.2-fold (Fig. 1). MJ1 colonies were darker blue than PR1; therefore, the reduced expression of signal-2 by MJ1 was surprising. The darker blue color of the MJ1 colonies and the characteristic blue halos may result from leakage of β-galactosidase or lysis of cells within the developing colony.

3.2. Characterization of MJ1

Unexpectedly, hybridization analysis revealed that MJ1 did not have a chromosomal Tn5Tmp insertion and arose as a spontaneous trimethoprim-resistant mutant during the selection for transposon insertions. Since mutations in *thyA* can result in trimethoprim resistance, MJ1 was tested for additional phenotypes associated with loss of *thyA* [18,19]. MJ1 was unable to grow in the absence of thymine and cells were three to four times longer than wild-type PR1 cells (data not shown). Both phenotypes suggested a *thyA* mutation was present in MJ1. A *thyA::cat* disruption was constructed in PR1 to determine whether loss of *thyA* resulted in the same phenotypes observed in MJ1. The resulting strain, MJ6 *thyA::cat*, formed long rods and was resistant to trimethoprim. However, MJ6 exhibited three phenotypes that were distinct from MJ1: (i) colonies of MJ6 did not form blue halos on X-gal plates, (ii) growth of MJ6 in LB medium stopped at an optical density of 1.2, and (iii) CM from MJ6 had an enhanced signal-2 activity with three-fold activation of *gabT::lacZ*, relative to the 1.7-fold activation observed with PR1 CM prepared at the same density (data not shown). It should be noted that the low-level activity from wild-type PR1 CM in this experiment resulted from the use of CM harvested from cells at a lower optical density (OD<sub>600</sub> = 1.0) than in the experiments reported in Fig. 1. This was necessary due to the fact that MJ6 *thyA::cat* did not grow well beyond an OD of 1.0.

3.3. A second-site suppressor, *spI*, accumulates in *thyA* mutants and results in loss of signal-2 activity

During the course of working with MJ6 *thyA::cat*, we noted that occasional colonies arose from this strain that exhibited the blue halo phenotype. In addition, the frequency of these blue, halo-forming colonies was increased when 2–3-day-old colonies were restreaked. These blue, halo-forming colonies appeared identical to the original MJ1 mutant and were hypothesized to contain a second-site suppressor in addition to the *thyA::cat* allele. The frequency of these second-site suppressors was examined in more detail. Duplicate cultures of MJ6 *thyA::cat* were grown to stationary phase and plated at daily intervals on LB plates containing X-gal. After initial growth to stationary phase, each culture gave rise to colonies with the halo phenotype at a frequency of 3.4% and 7.7%. By day 2, the frequency of colonies with blue halos in both cultures was greater than 70% of the total viable cells. An independent isolate with the blue halo phenotype was isolated from each culture and designated MJ7 and MJ8. To determine whether MJ7 and MJ8 were defective in signal-2 activity in a manner similar to the original MJ1 mutant,
CM from each strain was tested for activation of gabT::lacZ. CM from MJ7 and MJ8 failed to activate the gabT::lacZ fusion (Fig. 1). The second-site suppressor that accumulates in thyA mutants and results in the loss of signal-2 activity has been designated spl1. One explanation for the high frequency of second-site reversion to the halo phenotype is that the secondary spl mutations improve the survival of thyA::cat mutants in stationary phase. This was examined by conducting cell survival assays for PR1, MJ6 thyA::cat and MJ7 thyA::cat, spl1. In Fig. 2, the survival of MJ6 dropped off very rapidly with 10^6 viable cells at day 1. By day 4, virtually all the cells of MJ6 have either died or been replaced by cells with the halo phenotype. In contrast, the spl1 mutation in MJ7 significantly enhanced cell viability at all times tested. However, the restored level of viability did not approach the wild-type levels seen with PR1 (Fig. 2).

3.4. Inactivation of deoB or deoC suppresses the loss of viability in thyA mutants

Previous studies indicated that thyA mutants readily develop secondary mutations in deoB or deoC genes [20]. These mutations increase the pools of deoxyribose-1-phosphate and increase the efficiency of thymine salvage pathways allowing for growth at lower thymine concentrations. We hypothesized that the spl1 mutation may represent an allele of deoB or deoC. The thymine requirements were tested for MJ6 thyA::cat and MJ7 thyA::cat, spl1. We found that the minimal concentration of thymine required for growth of MJ7 was 10 μM, a concentration 10-fold less than that required by MJ6 (100 μM). Based on this result, the deoB and deoC genes were inactivated in the MJ22 ΔthyA background. P1 transduction was used to transfer the mutation into a fresh MJ22 ΔthyA background and the resulting mutants, MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat, were used for further studies. Both MJ23 and MJ24 now formed colonies with the blue halos, a phenotype that was not observed with the MJ22 ΔthyA parent, but was observed in MJ7 thyA::cat, spl1. The stationary phase survival of MJ22 ΔthyA, MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat was examined (Fig. 3). After an overnight culture was plated out (day 0), the viability of MJ22 ΔthyA was six orders of magnitude lower (1.7×10^3 cells ml^-1) than wild-type PR1 (3.3×10^5 cells ml^-1). Cells of MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat exhibited a viable cell count at day 0 that was five orders of magnitude above that seen with MJ22 ΔthyA. Upon prolonged incubation at stationary phase, MJ22 ΔthyA died off rapidly. However, at day 3–4, the viable cell count began to rise and the colonies that formed now all exhibited prominent blue halos. The survival of MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat remained at levels that were above 10^6 viable cells out to day 5. Therefore, mutations in either deoB or deoC significantly improve the survival of thyA mutants in stationary phase.

3.5. Loss of deoB or deoC reduces the amount or activity of an extracellular signal-2

The ability of CM prepared from MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat to activate the signal-2-dependent gabT::lacZ fusion was tested. CM prepared from MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat activated the gabT::lacZ fusion 2.7-fold and 1.7-fold, respectively, relative to the activity in LB alone (Fig. 4A). In contrast, CM from MJ22 ΔthyA activated the gabT::lacZ fusion 9.2-fold.

The loss of signal-2 activity observed in deoB and deoC mutants could be due to a reduced amount/activity, or could be due to the overproduction of a signal-2 antagonist. CM from each mutant and a LB control were mixed at various concentrations with wild-type CM and tested for activation of the gabT::lacZ fusion. These experiments demonstrated that MJ23 and MJ24 did not reduce signal-2 activity by the production of an antagonist (data not shown).

To investigate the role of the deoB and deoC mutations

Fig. 2. Cell viability in stationary phase. Individual cultures of PR1, MJ6 thyA::cat and MJ7 thyA::cat, spl1 were grown in 3 ml of LB broth and shaken at 250 rpm at 37°C. At daily intervals the viable cell count for each culture was determined. The values represent the mean of quadruplicate samples from two independent experiments, and the error bars represent S.D. The values for MJ22 reflect colonies with a non-halo phenotype. CFU represents log colony-forming units per ml.

Fig. 3. Role of deoB and deoC mutations in the survival of thyA mutants in stationary phase. Individual cultures of PR1, MJ22 ΔthyA, MJ23 ΔthyA, deoB::cat and MJ24 ΔthyA, deoC::cat were grown in 3 ml of LB broth and shaken at 250 rpm at 37°C. At daily intervals, the viable cell count for each culture was determined. The values represent the mean of quadruplicate samples from two independent experiments, and the error bars represent S.D. The values for MJ22 reflect colonies with a non-halo phenotype. CFU represents log colony-forming units per ml.
in signal-2 activity in a wild-type (PR1) background, P1 transduction was performed to move the deoB::cat or deoC::cat to a PR1 background. The corresponding transductants were designated MJ25 deoB::cat and MJ26 deoC::cat. CM prepared from MJ25 deoB::cat was capable of activating the gabT::lacZ fusion 2.6-fold, relative to the 6.4-fold activation observed when CM from PR1 wild-type was used (Fig. 4B). The deoB::cat mutation had a more severe effect on the production of signal-2 in CM, with a 1.9-fold activation of gabT::lacZ. The above experiment indicates that loss of deoB or deoC reduces signal-2 amount or activity in both thyA and wild-type backgrounds.

3.6. deoB and deoC mutations increase the basal levels of gabT::lacZ expression

The deoB and deoC null alleles increased on gabT::lacZ expression in cells at mid-exponential phase (OD_{600} = 0.35) in LB only medium. In MJ25 deoB::cat, the expression of gabT::lacZ was two-fold higher than wild-type PR1, and in MJ26 deoC::cat, gabT::lacZ expression was four-fold higher (data not shown). However, when cells were assayed at very early exponential phase (OD_{600} = 0.1), the deoB and deoC mutations did not alter the levels of gabT::lacZ expression when compared to wild-type (data not shown).

4. Discussion

In this study, an indole-negative strain of E. coli was used to identify loci responsible for the production of a second extracellular activating signal (signal-2) for a gabT::lacZ fusion. A spontaneous mutant, MJ1, which failed to produce signal-2 was isolated. Genetic analysis of MJ1 strongly supports the hypothesis that the phenotypes of MJ1 are due to two distinct mutations. MJ1 was fortuitously isolated as a spontaneous trimethoprim-resistant mutant. The trimethoprim resistance and the thymine auxotrophy are consistent with MJ1 containing a loss of function mutation in thyA. The second mutation, spl1, presumably arose early during the initial colony formation of MJ1 on trimethoprim plates. This hypothesis is supported by our ability to construct a thyA::cat null allele and demonstrate that cells with a second-site suppressor mutation (i.e., MJ7 and MJ8) arise at high frequency and have a phenotype identical to MJ1. These second-site suppressors presumably arise because they significantly increase the viability of thyA mutants at stationary phase where thymine is limited in the medium (Fig. 2).

One mechanism to compensate for the loss of thyA is to increase the efficiency of the salvage pathway for dTMP synthesis [20,21]. Mutations in deoB and deoC result in increased pools of deoxyribose-5-phosphate and deoxyribose-1-phosphate. In turn, this leads to a lower thymine requirement for cells because the salvage pathway is more efficient at generating dTMP [20]. This likely accounts for the ability of deoB and deoC mutations to greatly enhance the survival of thyA mutants in stationary phase. The spl1 allele in the thyA mutant background resulted in the same phenotypes as deoB and deoC mutations. These phenotypes include: (i) colonies with blue halos, (ii) loss of signal-2 activity, and (iii) improved survival in stationary phase. This suggests that spl1 may be an allele of deoB or deoC.

How does the loss of deoB or deoC lead to reduced signal-2 activity? Two possibilities are considered: (i) signal-2 is a product of the DeoB- or DeoC-catalyzed reac-
tion, or (ii) loss of deoB or deoC results in the enhanced uptake of signal-2 from the external medium. The products of DeoB- and DeoC-catalyzed reactions include: deoxyribose-5-phosphate, acetaldehyde and glyceralddehyde-3-phosphate [22]. However, these compounds did not alter expression of the gabT::lacZ fusion, even at millimolar amounts (data not shown). Glyceralddehyde-3-phosphate is further metabolized to dihydroxyacetone phosphate (DHAP) by the activity of triose phosphate isomerase, and DHAP is then converted to glyceraldehyde-3-phosphate (G3P) by the GpsA dehydrogenase [22,23]. Therefore, an additional consequence of deoB and deoC mutations is the reduced production of DHAP and G3P. Again, these compounds did not alter gabT::lacZ expression.

Loss of deoB or deoC results in the accumulation of deoxyribose-5-phosphate and deoxyribose-1-phosphate. In E. coli, deoxyribose-5-phosphate is reported to be the inducer for DeoR-repressed genes [22,24]. The DeoR repressor negatively regulates the expression of genes required for nucleoside uptake. These genes include tss, an outer membrane pore for nucleosides, and nupG, a specific nucleoside transporter [22,25,26]. Therefore, in a deoC mutant, accumulation of the inducer deoxyribose-5-phosphate is predicted to increase the transcription of tss and nupG. In turn, this is predicted to increase the uptake of nucleosides from the external medium. If signal-2 was a substrate for the tss pore and/or the nupG permease, then the increased uptake predicted to occur in deoB and deoC mutants would deplete this signal from the medium. However, a tss mutant exhibited normal gabT::lacZ activation in response to signal-2 in CM and produced similar amounts of signal-2 (data not shown). In addition, nucleosides that are known to use tss and nupG for import have been tested for signal-2 activity and none of these compounds exhibited activity at concentrations up to 10 mM (data not shown).

Based on the above information, we propose the following hypothesis to reconcile the paradoxical results that deoB and deoC mutations result in decreased extracellular signal-2, yet lead to increased expression of the gabT::lacZ fusion. Secreted signal-2 is depleted from the external medium in deoB and deoC mutants by the enhanced expression of an uptake system, possibly mediated by NupG. This would account for the reduced signal-2 activity in CM harvested from the deo mutants. The enhanced uptake of signal-2 would then lead to an increased intracellular concentration of signal-2. In turn, this activates a regulatory pathway for the gabT::lacZ fusion. Consistent with this hypothesis, the effects of deoB and deoC mutations on gabT::lacZ expression were not evident when cells were grown at very low density (OD600 = 0.1) were assayed. This suggests that the deoB and deoC mutations only increase the basal level of gabT::lacZ expression when signal-2 is present. Assuming that signal-2 is a nucleoside, the mechanism that exports it is unknown. The identification of mutations that alter signal-2 production as well as the identification of signal-2 is in progress to address these questions.

Acknowledgements

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References


