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Activation of the *gab* Operon in an RpoS-Dependent Manner by Mutations That Truncate the Inner Core of Lipopolysaccharide in *Escherichia coli*

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The *gab* operon (*gabDTPC*) in *Escherichia coli* functions in the conversion of γ-aminobutyrate to succinate. One component of *gab* operon regulation involves the RpoS sigma factor, which mediates activation at high cell density. Transposon mutagenesis was used to identify new genes that regulate *gab* operon expression in rich media. A Tn*5tmp* insertion in the *hldD* (formerly *rfaD*) gene increased *gabT*::*lacZ* expression 12-fold. The *hldD* gene product, an ADP-L-glycerol-D-mannoheptose-6-epimerase, catalyzes the conversion of ADP-D-glycerol-D-mannoheptose to ADP-L-glycerol-D-mannoheptose, a precursor for the synthesis of inner-core lipopolysaccharide (LPS). Defined mutations in *hldE*, required for heptose synthesis, and *waaF*, required for the addition of the second heptose to the inner core, also resulted in high-level *gabT*::*lacZ* expression. The *hldD*, *hldE*, and *waaF* mutants exhibited a mucoid colony phenotype due to production of a colanic acid capsule. However, in the *hldD*::*cat* background, the high-level expression of *gabT*::*lacZ* was independent of the regulatory components for colanic acid synthesis (*rcsA*, *rcsB*, and *rcsC*) and also independent of *manC* (*cpsB*), a structural gene for colanic acid synthesis. Activation of *gabT*::*lacZ* in the *hldD*::*cat* background was dependent on the RpoS sigma factor. The *hldD*::*cat* mutation resulted in a sixfold increase in the levels of a translational RpoS-LacZ fusion and had a marginal effect on a transcriptional fusion. This study reveals a stress-induced pathway, mediated by loss of the LPS inner core, that increases RpoS translation and *gab* operon expression in *E. coli*.

The *gabDTPC* operon in *Escherichia coli* functions in the conversion of γ-aminobutyrate (GABA) to succinate (3, 17). The *gabD* gene encodes a succinate:semialdehyde dehydrogenase that generates succinate from succinate-semialdehyde. The *gabT* gene encodes a succinate:semialdehyde aminotransferase that catalyzes the formation of succinate semialdehyde from GABA. The *gabP* gene encodes a GABA permease (3). In addition to the catabolism of γ-aminobutyrate, the *gab* operon has been proposed to contribute to polyamine homeostasis during nitrogen-limited growth (25) and to maintain high internal glutamate concentrations under stress conditions (14).

Regulation of the *gab* operon occurs at multiple levels. At least three promoters that transcribe the *gab* operon have been identified. The RpoS-encoded sigma factor σ^S or σ^{38} transcribes the *gab* operon, and recent studies indicate that two RpoS-dependent promoters are present upstream of the *gab* operon (2, 14, 23). In addition, a σ^{70}-dependent promoter, together with the Nac regulatory protein, contributes to expression under nitrogen-limiting conditions (25, 33). In nitrogen-rich environments, two accumulated extracellular signals, indole and a second unidentified signal, independently contribute to activation of the *gab* operon at high cell density in an RpoS-dependent manner (2, 31). Expression of the *gab* genes is also enhanced at high pH (28). Negative regulation of *gab* expression is mediated by the GabC repressor (23), also termed CsiR (14). It has been proposed that activation by extracellular signals and RpoS is dependent on loss of the GabC repressor (25).

In this study, a genetic approach was used to search for genes that regulate expression of a *gabT*::*lacZ* fusion under conditions of nitrogen excess. The parent strain for these studies was PR1, a strain unable to produce indole (Table 1). Therefore, PR1 relies on a second unknown signal for activation of *gabT*::*lacZ* by cell-cell signaling (31). We were originally interested in mutations that altered the production or response to the second signal. However, in this study, mutations were identified that altered *gabT*::*lacZ* expression by a previously unreported mechanism. Mutations in *hldD* or *hldE*, which truncate the inner core of lipopolysaccharide (LPS), resulted in high-level expression of the *gabT*::*lacZ* fusion along with the induction of mucoidy. However, the colanic acid capsule itself did not have a role in the high-level expression of *gabT*::*lacZ*. In addition, the overexpression of *gabT*::*lacZ* in the *hldD*::*cat* background was independent of RcsA and the RcsB/C two-component system. This pathway for activation of *gabT*::*lacZ* in the *hldD*::*cat* background was dependent on RpoS, and the loss of *hldD* increased RpoS translation.

Isolation of a mutant with high-level expression from a *gabT*::*lacZ* fusion. To search for genes that regulate a *gabT*::*lacZ* transcriptional fusion in *E. coli* strain PR1, a library of mini-Tn*5tmp* insertions was constructed using the EZ::TN

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TABLE 1. Strains used in this study

E. coli strain	Genotype	Source or reference
ZK126	*tna-2* Δ*lacU169*	31
VS20320	*rcsB*::mini-Tn*10tet*	V. Stout
PR1	ZK126 *gabT*::mini-Tn*5lacZ-tet/1*	This study
MJ2	PR1 *hldD*::Tn*5tmp*	This study
MJ11	PR1 *hldD*::*cat*	This study
MJ12	MJ11 *rcsB*::mini-Tn*10tet*	This study
MJ13	PR1 *hldE*::*cat*	This study
MJ18	MJ11 *cpsB*(*manC*)::*tet*	This study
MJ19	PR1 *waaL*::*cat*	This study
MJ20	PR1 *waaF*::*cat*	This study
MJ27	MJ11 *rcsA*::*tet*	This study
MJ31	MJ11 *rpoS*::Tn*10*	This study
MJ32	MJ2 *rcsC*::*cat*	This study
DDS1626	*rpoS-lacZ*	D. Sledjeski
PR1000	DDS1626 *hldD*::*cat*	This study

DHFR-1 Tnp Kit (Epicentre Technologies). The Tn*5tmp* transposome was electroporated into *E. coli* PR1, and cells were plated on Luria-Bertani (LB) agar containing trimethoprim (50 mg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). From approximately 14,600 trimethoprim-resistant colonies, a pale blue, mucoid colony was isolated and designated MJ2. To determine whether the pale blue phenotype was related to *gabT*::*lacZ* expression, PR1 and MJ2 cells were assayed for β-galactosidase in LB broth. Unexpectedly, despite the pale blue phenotype on X-Gal plates, the expression of *gabT*::*lacZ* was 12-fold higher in MJ2 (864 ± 188 U) than in PR1 (72 ± 4 U) (Fig. 1). The discrepancy between colony color and β-galactosidase expression was due to a masking of the colony color by the capsular material.

Loss of *hldD* leads to increased expression of *gabT*::*lacZ*. MJ2 was found to be resistant to P1 lysis, and repeated attempts to use transduction to confirm linkage of trimethoprim resistance to the MJ2 phenotypes were unsuccessful. The Tn*5tmp* insertion was cloned, and sequence analysis of the transposon junction indicated an insertion at position 1286 in the 2,037-bp *hldD* (formerly *rfaD*) gene. HldD is an ADP-L-glycerol-D-mannoheptose-6-epimerase that catalyzes the conversion of ADP-D-glycerol-D-mannoheptose to ADP-L-glycerol-D-mannoheptose, a precursor for the synthesis of inner lipopolysaccharide (5). The phenotypes of MJ2, such as the formation of mucoid colonies, resistance to P1 lysis, and sensitivity to detergents, were consistent with *hldD* mutants described in the literature (5, 6, 18, 20, 24). Since P1 transduction could not be used to move the *hldD*::Tn*5tmp* mutation into a fresh background, a *hldD*::*cat* mutation was constructed in strain PR1 by the one-step inactivation procedure of Datsenko and Wanner (7). PCR products were generated from pKD3 using the primers listed in Table 2. The *hldD*::*cat* mutation in PR1 was verified by PCR, and this strain was designated MJ11. Like MJ2, strain MJ11 was mucoid, resistant to P1 lysis, and sensitive to detergents and exhibited constitutive expression of the *gabT*::*lacZ* fusion (Fig. 1). Therefore, we conclude that an insertion in *hldD* was responsible for the increased expression of *gabT*::*lacZ*. In addition, the above phenotypes of the *hldD*::*cat* allele, i.e., the mucoid phenotype, detergent sensitivity, and increased *gabT*::*lacZ* expression, were restored to the wild type by a plasmid containing the *E. coli hldD* gene (data not shown).

Expression of the *gabT*::*lacZ* fusion used in this study was previously shown to be dependent on the RpoS sigma factor (2). To determine whether the high-level *gabT*::*lacZ* expression in the *hldD*::*cat* background was RpoS dependent, an *hldD*::*cat rpoS*::Tn*10* double mutant was constructed. The activation of *gabT*::*lacZ* was abolished in the double mutant, with levels of β-galactosidase (58 ± 2 Miller units) that were slightly lower than those in the wild type (72 ± 4 Miller units) (Fig. 1).

The loss of the inner-core domain of lipopolysaccharide results in constitutive *gabT*::*lacZ* expression. *hldD* is the first gene in the *hldDFCL* operon, and the HldD enzyme (ADP-L-glycerol-D-mannoheptose-6-epimerase) catalyzes the last step in the biosynthetic pathway of the heptose LPS precursor (1, 11, 22). The increased *gabT*::*lacZ* expression in the *hldD* mutant could directly result from the loss of heptose. However, an alternative possibility, given the unexpected role in regulation, is that the HldD enzyme carries out a second undefined activity that accounts for the changes in *gabT*::*lacZ* expression. To address the possibility that a general loss of heptose can lead to this phenotype, a heptoseless mutant was created by inactivation of the *hldE* (*rfaE*) gene using the PCR-mediated one-step procedure (7). HldE is a bifunctional enzyme with D-β-D heptose phosphate-7-kinase and D-β-D heptose-1-phosphate adenylyltransferase activities. This enzyme is required for the second and fourth step in the biosynthesis of ADP-L-β-D-heptose (11). Strain MJ13 *hldE*::*cat* was mucoid and exhibited a 5.7-fold increase in expression of the *gabT*::*lacZ* fusion. There-

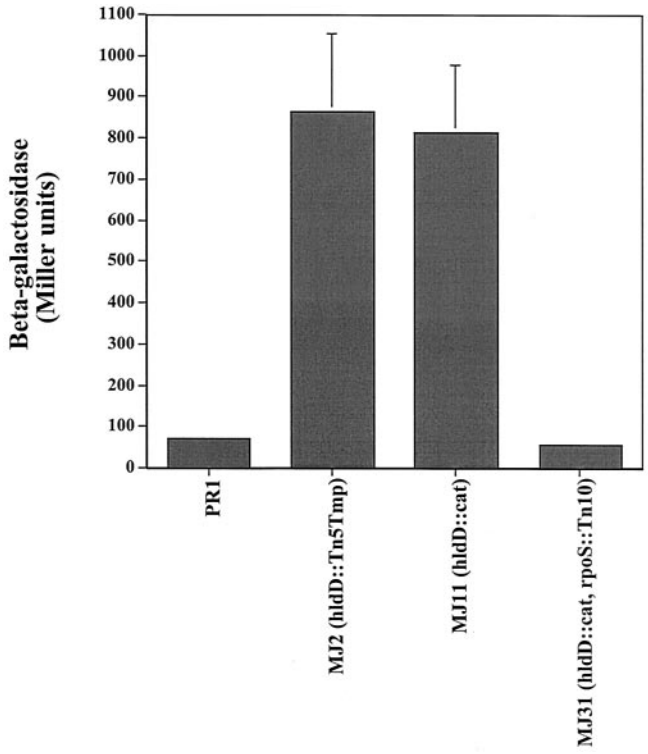

FIG. 1. Loss of *hldD* leads to activation and *gabT*::*lacZ*. The indicated strains were grown to early log phase (A_{600} = 0.35) in 0.5× LB medium at pH 7.5 and analyzed for β-galactosidase by the method of Miller (15). Data shown are means of duplicate samples from three independent experiments. Error bars, standard deviations.

TABLE 2. PCR primers used for one-step inactivation of genes

Gene	Primer[a]	Sequence (5′→3′)[b]
hldD	F	CCACGAAGGCGCGTGCTCTTCCACCACCGAGTGGGACGGCACATATGAATATCCTCCTTAG
	R	AACGGAAGCCAACAATCTGCGAGTTCGCTTCAGGCAGGATTTGTGTAGGCTGGAGCTGCTTC
hldE	F	ATGAAAGTAACGCTGCCAGAGTTTGAACGTGCAGGAGTGATGCATATGAATATCCTCCTTAG
	R	CTTCTTGATGATGTTGGTCGTCGAGCAACCGTCTTCAAAGTTGTGTAGGCTGGAGCTGCTTC
waaL	F	AATGACCGCATTTCTTTTGGTGTAGGAACGGCAACAGGAGCCATATGAATATCCTCCTTAG
	R	AATTCCCATCAGACCTTTCAGTGACCCTGCTTCAATTATCTTGTGTAGGCTGGAGCTGCTTC
rcsA	F	GTTGATGACCTTGCCATAGCTTGTGATTCACAGCGCCCTTCGGTTTTTTCGTTTTCAGAG
	R	CGGTCTTGGCTTTGATATTCATTTGGTCAGAGATTTGAATCGCTGCCCGAGATGCGCCGC
manC	F	AACCGAATCTGGAAACCGCTCAGGCCTATGTGGCAAGCGGCGGTTTTTTCGTTTTCAGAG
	R	CTGTACCGCGTTACGGTCGGCAATCAGCACCGCATCTTTGCGCTGCCCGAGATGCGCCGC
waaF	F	GCCGGAAGTTAACGAAGCTATTCCTATGCCTCTCGGTCACGCATATGAATATCCTCCTTAG
	R	TCATGATCTTTCGCCGAGCCAAACAGAACCACCTGATAACCTGTGTAGGCTGGAGCTGCTTC
rcsC	F	TGTTCAGAGCATTGGCGTTAGTGCTCTGGCTGTTGATTGCTTCATATGAATATCCTCCTTAG
	R	CGTCAGCGTCTGTTTTATCACATCCAGCGTTACCGGCTTCGACAGTGTGTAGGCTGGAGCTGCTTC

[a] F and R represent the forward and reverse primers, respectively.
[b] The underlined sequences are the primer sequences that amplify the antibiotic resistance cassette.

fore, loss of heptose is sufficient to confer constitutive activation of *gabT*::*lacZ*.

The insertional mutations in *hldD* are likely to be polar on the downstream *waaFCL* genes that are involved in the assembly of the heptose precursor into LPS. WaaC transfers the first heptose to the inner-core backbone, and WaaF transfers the second heptose to the core (26, 32). WaaL is required for the addition of O antigen to the core. To address the role of the downstream genes in *gabT*::*lacZ* expression, the *waaF* and *waaL* genes were individually mutated by the insertion of a chloramphenicol resistance cassette via PCR-mediated mutagenesis (7). The *waaF*::*cat* mutation in MJ20 resulted in a 5.6-fold increase in *gabT*::*lacZ* expression relative to that in the wild type. In contrast, the *waaL*::*cat* mutation in MJ19, which prevents the addition of O antigen to the core, had no effect on *gabT*::*lacZ* expression. Since *E. coli* K-12 strains are already devoid of O antigen, this result was expected. However, the results with the *waaF* and *hldD* mutations are consistent with truncation of the LPS inner core leading to *gabT*::*lacZ* activation.

The colanic acid capsule and the Rcs regulatory proteins are not required for *gabT* activation in *hldD* mutants. A prominent phenotype of the *hldD* mutant was the production of capsular polysaccharide. Synthesis of the colanic acid capsule is controlled by the regulators of colanic acid synthesis (*rcs*) genes composed of the activator RcsA, the sensor kinase RcsC, and the response regulator RcsB (4, 8, 9, 10, 19, 30). First, to determine whether the production of colanic acid in *hldD*::*cat* cells was responsible for the constitutive activity of *gabT*::*lacZ*, a double-mutant MJ18 strain (*hldD*::*cat manC*::*tet*) was constructed. The *manC* gene, formerly called *cpsB*, encodes a mannose-1-phosphate guanosyltransferase required for production of colanic acid (29). As expected, MJ18 exhibited a nonmucoid phenotype. The *gabT*::*lacZ* fusion in MJ18 *hldD*::*cat manC*::*tet* was activated 7.9-fold above the levels seen in PR1, indicating that colanic acid itself was not responsible for the high-level *gabT*::*lacZ* expression in the *hldD* background (Fig. 2). The role of the *rcs* genes in *gabT*::*lacZ* activation was also examined. The loss of *rcsA* or *rcsB* in the *hldD*::*cat* background did not reduce the high-level *gabT*::*lacZ* expression, and the loss of *rcsB* enhanced expression (Fig. 2). The loss of *rcsC* in the *hldD*::Tn*5tmp* background resulted in levels of *gabT*::*lacZ* expression that were slightly reduced, with 5.1-fold

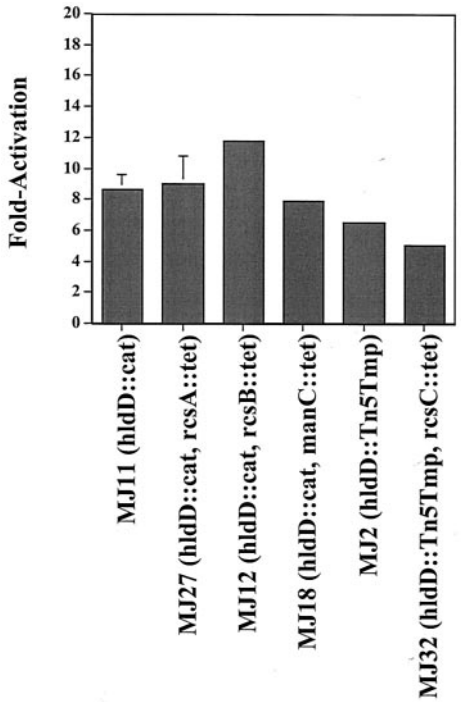

FIG. 2. The colanic acid capsule and the Rcs regulatory proteins are not required for *gabT*::*lacZ* activation in the *hldD*::*cat* background. Cells were grown in 0.5× LB medium at pH 7.5, harvested for β-galactosidase analysis at early log phase (A_{600} = 0.35), and assayed by the method of Miller (15). The reported values represent the fold activation of the *gabT*::*lacZ* fusion in the various strains relative to the expression in the isogenic parent PR1. The baseline value of β-galactosidase in PR1 *gabT*::*lacZ* was 68 ± 1 U.

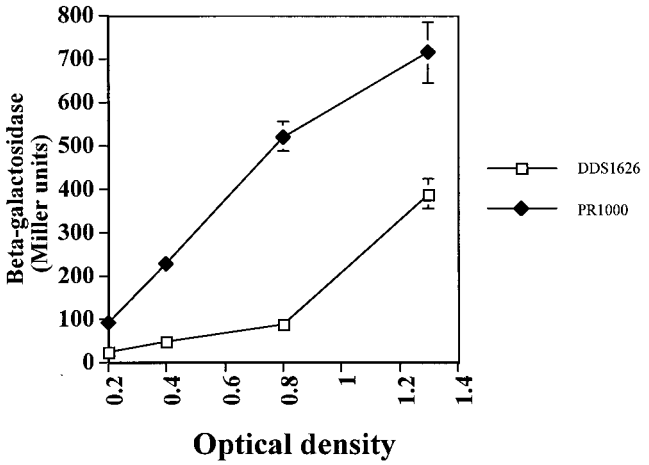

FIG. 3. Loss of *hldD* increases RpoS translation. Cultures of either DDS1626 (RpoS-LacZ) or PR1000 (RpoS-LacZ, *hldD*::*cat*) were grown in 0.5× LB medium, and β-galactosidase activities were assayed at the indicated optical densities. The reported values represent the average of quadruplicate samples from two independent experiments.

activation versus the 6.5-fold activation in MJ2 *hldD*::Tn*5tmp* (Fig. 2).

Loss of *hldD* increases RpoS translation. To further investigate the role of RpoS in the increased expression of *gabT* in the *hldD* background, we addressed whether RpoS itself was increased in *hldD* mutants. An RpoS-LacZ translational fusion was used to examine expression in wild-type and *hldD*::*cat* backgrounds. As shown in Fig. 3, the expression of RpoS-LacZ was markedly increased in the *hldD*::*cat* background relative to that in the wild type. The maximum difference was observed at an optical density at 600 nm of 0.8, where a sixfold increase was evident in the *hldD* mutant (Fig. 3). The effect of *hldD*::*cat* on *rpoS* transcription was also examined by reverse transcription-PCR analysis. The *hldD:cat* allele has less than a twofold effect on *rpoS* mRNA levels (data not shown).

These data combined with those in Fig. 1 suggest that RpoS overexpression in the *hldD*::*cat* background is a primary mechanism for the activation of *gabT*::*lacZ.* Two additional experiments were conducted to verify that increased RpoS expression results in overexpression of the *gabT*::*lacZ* fusion. First, RpoS expression is increased at low temperature (27). Expression of the *gabT*::*lacZ* fusion was increased fourfold at 25°C compared to that at 37°C (51 ± 0.35 versus 13 ± 1 Miller units). Second, the response regulator RssB (SprE) targets RpoS for proteolysis, and *rssB* mutants accumulate increased levels of RpoS (16, 21). The expression of *gabT*::*lacZ* was elevated fivefold in an *rssB*/*sprE* mutant compared to that in the wild type (data not shown).

Concluding remarks. In this study, a *gabT*::*lacZ* fusion was used as a biosensor to identify loci involved in its regulation. Mutations in *hldD*, *hldE*, and *waaF*, which result in loss of the heptose precursor for the inner core of lipopolysaccharide, resulted in activation of a *gabT*::*lacZ* fusion. This pathway for *gabT*::*lacZ* activation was independent of RcsA, RcsB, and RcsC. However, one possible caveat in the interpretation of a role for RcsA is the fact that the *hldD rcsA* double mutant formed small colonies that were genetically unstable and gave rise to larger colonies. Although great care was taken in assaying the *hldD rcsA* double mutant, including assays on primary transductants, we cannot rule out the possibility that second-site suppressor mutations restored some level of *gabT*::*lacZ* expression in this background.

In the *hldD*::*cat* background, the activation of *gabT*::*lacZ* required a functional RpoS (Fig. 1). Moreover, the expression of an RpoS-LacZ translational fusion was increased sixfold in the *hldD*::*cat* background (Fig. 3). These data indicate that the increased expression of *gabT*::*lacZ* was likely due to increased levels of RpoS. To our knowledge, the increase in RpoS translation in response to loss of the LPS inner core has not been previously described. Studies by Majdalani et al. have demonstrated that a small regulatory RNA, *rprA*, regulates RpoS translation and that cell envelope stress increased the expression of *rprA* in a manner that was dependent on the RcsC/YojN/RcsB phosphorelay (12, 13). Moreover, it has also been demonstrated that loss of the LPS inner core acts directly, or indirectly, to regulate activity of the RcsC/YojN/RcsB phosphorelay (19, 24). Therefore, the mechanism that increases RpoS translation in response to loss of the LPS inner core could involve the RcsC/YojN/RcsB phosphorelay. However, this phosphorelay is clearly not required for the increased expression of the *gab* operon in the *hldD*::*cat* background (Fig. 2). This information, together with the fact that the high-level *gab* expression in the *hldD*::*cat* background is strictly RpoS dependent (Fig. 1), suggests that a second pathway for increasing RpoS expression in the *hldD*::*cat* background exists. Osmotic shock, a condition that may mimic loss of the LPS inner core, has previously been shown to increase RpoS translation in a manner that required both *rprA* and a second small regulatory RNA, *dsrA* (12, 27). Our preliminary studies indicate that the individual loss of *rprA* or *dsrA* had only a minor effect on the increased RpoS translation in the *hldD*::*cat* background. However, both *rprA* and *dsrA* may be required for the increased RpoS translation in mutants defective in the LPS inner core. The identification of gene products that are required for the increased RpoS translation in the *hldD*::*cat* background will provide important information on how cells transmit alterations in LPS to regulation of RpoS and the *gab* operon.

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