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The *leuO* Gene Product Has a Latent Ability To Relieve *bgl* Silencing in *Escherichia coli*

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The *Escherichia coli bgl* operon is of interest, since its expression is silent (phenotypically Bgl$^-$), at least under standard laboratory conditions. Here we attempted to identify a *trans*-acting factor(s) that is presumably relevant to the regulation of *bgl* by a random insertion mutagenesis with mini-Tn*10*. These collected mutations, conferring the phenotype of Bgl$^+$, were localized in three loci on the genetic map, two of which appeared to be *hns* and *bglJ*, which were previously implicated as the factors affecting the Bgl phenotype. The other locus at 1 to 2 min on the genetic map appeared to be a new one. In this case, the insertion mutation was found to be just in front of the *leuO* gene encoding a putative LysR-like DNA-binding protein. Genetic analyses revealed that overproduction of LeuO in the wild-type cells causes the phenotype of Bgl$^+$. A *leuO* deletion mutant was also characterized in terms of expression of *bgl*. From these results, the possible function of LeuO in *bgl* expression will be discussed from an evolutionary and/or ecological point of view.

The *bgl* operon, located at about 84 min on the *Escherichia coli* K-12 genetic map, is involved in the utilization of certain β-glucosides, such as salicin and arbutin (9, 15). This operon is of interest in that, although it is intact in the genetic sense (*bgl*$^+$), its expression is normally silent in the wild-type background (i.e., phenotypically Bgl$^-$) but is enhanced upon the occurrence of various types of spontaneous mutations, resulting in a phenotype of Bgl$^+$. Such Bgl$^+$ mutations include insertion of IS*1* or IS*5* upstream of the *bgl* promoter, point mutations within a CRP (cyclic AMP receptor protein) binding site and deletions within a region upstream of the CRP binding site (10, 11, 13, 14). Interestingly, mutations outside the *bgl* operon can also result in Bgl$^+$. Specifically, certain mutations in the genes encoding the subunits of DNA gyrase (*gyrA* and *gyrB*) or the H-NS nucleoid protein (*hns* [formerly called *bglY*]) can activate the *bgl* operon (1, 2). Recently, another *trans*-acting gene, named *bglJ*, was reported to affect *bgl* silencing, although its significance is less clear (3). In this context, the molecular mechanism underlying *bgl* silencing has previously been characterized to some extent, and a plausible model of Schnetz (12) postulated that a region of the *bgl* operon including its promoter is organized into a nucleoprotein structure, within which the activity of the *bgl* promoter is repressed somehow. Nevertheless, no clear picture with regard to such a "silencing nucleoprotein structure" has emerged.

Based on a number of current studies, it is believed that H-NS is directly implicated in the formation of such a presumed silencing nucleoprotein structure (12, 16). From our recent results, however, it is also evident that the DNA-binding ability of H-NS is dispensable for *bgl* silencing (21). That is, a carboxyl-terminally truncated form of H-NS, lacking the entire DNA-binding domain, is still able to fully repress *bgl* expression. This fact may suggest that other *trans*-acting DNA-binding protein(s) must also be involved in *bgl* silencing. In this study, we thus attempted to search for such a new *trans*-acting factor(s) that may be relevant to the underlying mechanism of *bgl* silencing.

Isolation of Bgl$^+$ mutants. A method of transposon mutagenesis with mini-Tn*10cam* (*cam* gene confers chloramphenicol resistance [Cm^r]) (7) was adopted by employing a specialized *E. coli* strain (BGL1), which was designed to carry not only the intact *bgl* operon on the chromosome but also a *bgl-lacZ* fusion gene at the λ*att* site (21). Note that this particular strain is phenotypically Bgl$^-$ and LacZ$^-$ due to *bgl* silencing. Cells of CSH26 [Δ(*pro-lac*) *ara thi*], which is the parental strain of BGL1, were extensively mutagenized with mini-Tn*10cam*. Then, P1 phage lysates from the mutagenized cells were used for P1 transduction into strain BGL1, yielding 3.6×10^4 Cm^r cells. Among these cells, we searched for *trans*-acting mutants, which should exhibit simultaneously the phenotypes of Bgl$^+$ and LacZ$^+$. The former was screened on agar plates containing salicin and bromothymol blue, and the latter was screened on MacConkey-lactose plates containing salicin. After such an extensive double screening, we isolated 29 mutants that exhibited the desirable phenotypes of Cm^r, Bgl$^+$, and LacZ$^+$. They were assumed to carry a Tn*10* insertion mutation in a certain *trans*-acting gene, thereby resulting in a relief of *bgl* silencing. The positions of these 29 mutations on the *E. coli* chromosome were roughly mapped by P1 transduction with the help of a large set of Tn*10* insertions in the entire *E. coli* chromosome, which were previously constructed by Singer et al. (*zxx* series::Tn*10*) (20). This whole set of Tn*10* insertions provided us with appropriate markers (Tet^r [tetracycline resistant]), whose positions are known and located evenly on the entire *E. coli* chromosome. The results of such analyses showed that 12 mutations (class A) were linked to *zab-3051*::Tn*10* (1 to 2 min on the chromosome) (99% linkage), 11 mutations (class B) were linked to *zci-506* (27 to 28 min) (80% linkage), and the remainder (class C) were linked to *zji-202*::Tn*10* (98 to 99 min) (80% linkage). Together with the previous notions with regard to the *trans*-acting mutations affecting *bgl* silencing (see the introduction), we assumed that class A mutations most likely represent a novel mutation, while the other classes, B and C, correspond to each known gene, *hns* and *bglJ*, respectively. From these 29 mutants, we thus selected representative strains

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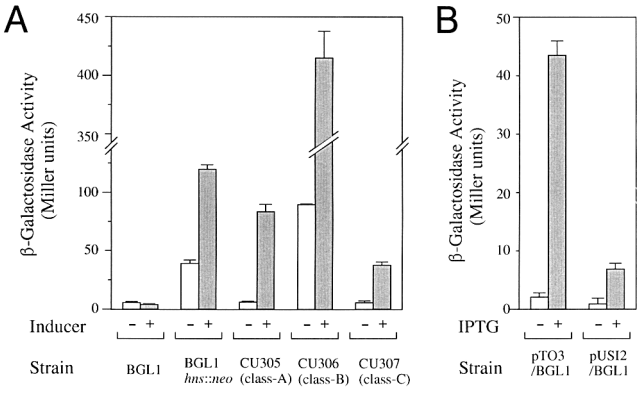

FIG. 1. (A) Expression of the *bgl* operon in the set of transposon insertion mutants. Cells of the indicated strains, all carrying a *bgl-lacZ* transcriptional fusion on the chromosome, were grown at 37°C to mid-logarithmic phase in TB medium (19) in the absence (−) and presence (+) of 5 mM β-methyl-D-glucoside, an inducer for the *bgl* operon. β-Galactosidase activities expressed by these cells were measured by the method of Miller (8). Each value is the mean ± standard deviation from four independent assays. (B) Expression of the *bgl* operon in the cells in which LeuO is overproduced. Transformants of BGL1 with pTO3 (see FIG. 2C) or pUSI2 (a control vector) were grown, and then β-galactosidase activities expressed by these cells were measured, as described above.

for each class (namely, CU305 for class A, CU306 for class B, and CU307 for class C).

With these representatives, we first demonstrated that each mutant does indeed exhibit the expected phenotype, Bgl^+, by monitoring expression of the *bgl-lacZ* fusion in these mutants (Fig. 1A). The levels of β-galactosidase activities, expressed in these mutants, increased in an inducer (β-methyl-D-glucoside)-dependent manner, as remarkably as in the case of the known *hns* deletion mutant. Thus, we succeeded in isolating at least one new class of Bgl^+ mutants, in which the mini-Tn*10cam* insertion results in a relief of *bgl* silencing. We then focused our attention on this particularly new mutant, CU305, while the presumed *hns* and *bglJ* mutants, CU306 and CU307, were discarded.

Derepression of *bgl* expression in CU305 is due to overproduction of LeuO. By employing standard techniques of recombinant DNA, we clarified the structure of the chromosomal region of CU305 where mini-Tn*10cam* had been inserted (Fig. 2A). The inserted element was found to be 19 bp upstream of the known open reading frame (ORF), named *leuO*, which is located at 1.8 min on the *E. coli* genetic map, and between the *leuABCD* and *ilvIH* operons. The *leuO* gene specifies a putative DNA-binding protein, which appears to be a member of the LysR family of regulatory proteins (6). Based on its location on the map (Fig. 2A), it has previously been speculated that LeuO might be implicated in the regulation of the *leuABCD* operon, but no evidence for such a role has been reported (e.g., in fact, the Δ*leuO* strain constructed in this study is not leucine auxotrophic). In CU305, this *leuO* ORF is not interrupted by the mini-Tn*10cam* insertion. Then, the question of how this particular insertion causes the Bgl^+ phenotype arose. Among several explanations envisaged, we first tested the idea that overproduction of LeuO, due to the mini-Tn*10cam* insertion upstream of the coding sequence, may result in the Bgl^+ phenotype. This was found to be the case, as demonstrated below. The *leuO* gene was cloned, and placed under the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter in a versatile expression vector (named pUSI2) (18), to yield pTO3 (Fig. 2C). When this plasmid was introduced into the wild-type background (BGL1), it was found that expression of *bgl-lacZ* was greatly enhanced in a manner dependent on the presence of IPTG (Fig. 1B). The level of *bgl* expression was more or less the same as in the case of CU305. These results collectively supported the view that overexpression of LeuO somehow results in the phenotype of Bgl^+. In this respect, it may be noted that, although it was previously reported that overproduction of LeuO negatively affects *cadBA* expression (17), the result described here demonstrated that overproduction of LeuO also has a somewhat positive effect on expression of this particular operon (*bgl*).

Overproduction of LeuO affects *bgl* expression in H-NS-independent manner. We then asked the question of what might be the possible mechanism underlying *bgl* derepression caused by LeuO overproduction. We focused our attention particularly on a possible linkage between the functions of H-NS and LeuO, since H-NS is a major *trans*-acting factor, so far known to be implicated in the *bgl* silencing (1, 16). Based on the fact that expression of the *bgl* operon is fully derepressed in a Δ*hns* background (Fig. 1A), one can assume that overproduction of LeuO might cause inactivation of H-NS either directly or indirectly, which in turn should result in *bgl* derepression. However, we found by an immunoblotting analysis with an anti-H-NS antiserum that the cellular content of H-NS in CU305 is essentially the same as that in its parental cells (data not shown). We also confirmed that H-NS in strain CU305 is fully functional, because the expression of the *proVWX* operon is not derepressed by the LeuO overproduction (data not shown). Note that whether H-NS is functional can be monitored sensitively by examining whether the *proVWX* operon is depressed, as demonstrated previously (21). Thus, the above assumption was dismissed.

The further question was what would happen if the *leuO* gene on the wild-type chromosome were knocked out. To see this consequence with special reference to *bgl* silencing, a region on the BGL1 chromosome was replaced by a chloramphenicol-resistant cassette, so as to yield a Δ*leuO*::*cam* mutant (Fig. 1B). The resultant strain, named TO2, was examined for *bgl* silencing by monitoring *bgl-lacZ* expression, showing that no effect was seen (Fig. 3). This result suggested that LeuO is not essential to keep the *bgl* operon to be silent. We then needed to see the phenotype of the *leuO* deletion mutation in

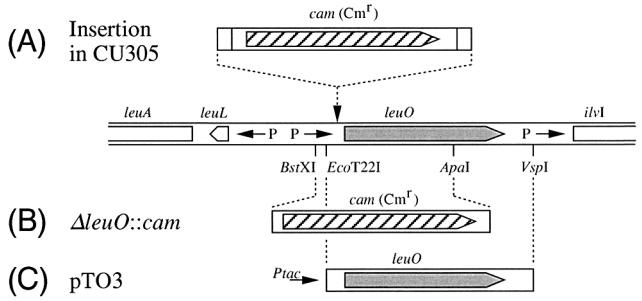

FIG. 2. Schematic representations of *leuO* mutations on the chromosome and of the cloned *leuO* gene on the plasmid. (A) Structure of the chromosomal region encompassing the *leuO* gene is shown schematically, in which the position of the mini-Tn*10cam* insertion in CU305 was indicated. Each polygonal symbol shows the indicated ORFs as well as their relative directions of transcription (the letter P shows each putative promoter). (B) Construction of the Δ*leuO*::*cam* deletion mutation used in this study. The *Bst*XI-*Apa*I DNA region encompassing the *leuO* coding sequence was replaced by the DNA fragment carrying the *cam* (Cm^r) gene on the chromosome of TO2. (C) Construction of the plasmid pTO3 that can overproduce LeuO. The *Eco*T22I-*Vsp*I fragment encompassing the entire region of the *leuO* gene was cloned under the *tac* promoter on pUSI2 (17), to yield pTO3.

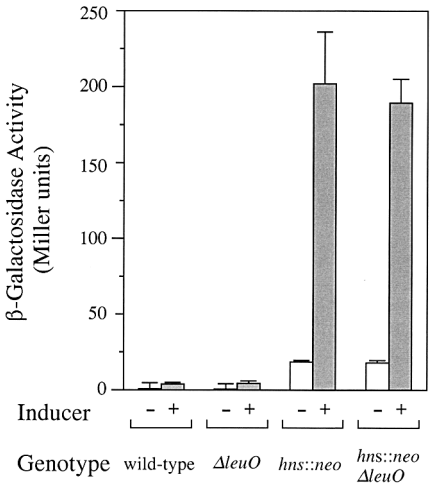

FIG. 3. Expression of the *bgl* operon in the *leuO* deletion mutant. A set of strains including BGL1 (wild type), TO2 (Δ*leuO*), BGL1 *hns*::*neo* (an *hns*::*neo* derivative of BGL1), and TO3 (an *hns*::*neo* derivative of TO2) were assayed for their expression of *bgl-lacZ*. The cells were grown at 37°C to the mid-logarithmic phase in TB medium (19) in the absence (−) and presence (+) of 5 mM β-methyl-D-glucoside, and then β-galactosidase activities expressed were measured by the method of Miller (8). Each value is the mean ± standard deviation from four independent assays.

appropriate genetic backgrounds, under which *bgl* silencing had been relieved. To do so, the Δ*hns* mutation was again concerned, since expression of the *bgl* operon is fully derepressed in this background (21). A strain with both the Δ*hns* and Δ*leuO* mutations was constructed and examined for *bgl* expression. Again, no effect was seen (Fig. 3), suggesting that LeuO is not essential for *bgl* transcription at least under certain conditions.

LeuO is a LysR-like protein, and thus, it is most likely a DNA-binding protein. In this study, we found that LeuO is able to lead the *bgl* operon to its derepressed state (i.e., Bgl^+), even in the presence of H-NS, provided that LeuO was overexpressed under certain conditions. It is not known at present how LeuO can do this task. However, we demonstrated that LeuO appears not to exert its function through H-NS, which is a major contributor for *bgl* silencing. It was also found that LeuO is not required for *bgl* activation caused by the Δ*hns* mutation. These results suggest that LeuO exhibits its effect on the *bgl* operon in an H-NS-independent manner, that is, LeuO can fully antagonize the negative effect of H-NS on the *bgl* operon. Thus, LeuO may function as an activator for the *bgl* operon through its direct binding to this target. Alternatively, LeuO may have the ability to disrupt the presumed silencing nucleoprotein structure on the *bgl* promoter. Of course, more complex explanations cannot be excluded at present. In any case, it will be of interest to see whether LeuO can bind directly to a region of the *bgl* operon.

Implications. It is not clear at present whether the single-copy *leuO* gene is relevant to the regulation of the *bgl* operon, because we have not so far succeeded in finding a phenotype for the *leuO* deletion mutant. However, it can be assumed that there may be certain natural conditions under which the expression of LeuO is enhanced. Under such a presumed circumstance, the *bgl* operon would be inducibly activated. In this context, it is also worth mentioning that these features of LeuO, described here, are very similar to those of BglJ (3). Overproduction of BglJ, caused by an IS insertion, was also reported to result in the phenotype of Bgl^+. This protein belongs to yet another type of DNA-binding protein, a NarL/RcsB-like protein with a potential helix-turn-helix motif. These facts suggest that *E. coli* has at least two potential activators, which can relieve the tight *bgl* silencing. If they can exert a redundant effect on the *bgl* operon, then the *leuO* deletion mutation alone would not show any noticeable phenotype with regard to the *bgl* operon. It will be thus of interest to construct a Δ*leuO* Δ*bglJ* double mutant in order to see the Bgl phenotype.

In connection with a physiological significance of our finding, the issue should finally be addressed of why *E. coli* must keep the *bgl* operon, which can be activated (or used) only upon fortuitous mutations, and is otherwise useless. Why should *E. coli* have such proteins, like LeuO, which possesses a powerful but latent ability to activate the *bgl* operon? As pointed out by Giel et al. (3), one might expect that the *bgl* operon in *E. coli* would be lost eventually. Instead, the silent *bgl* operon is present in a variety of *E. coli* strains isolated from different geographical locations (4). Hall et al. (5) have proposed that the presence of the *bgl* operon, which can be activated solely by mutation, may confer a selective advantage to those organisms that have maintained this cryptic system (i.e., the *bgl-leuO* system). If so, with the onset of an urgent situation in nature, potential *trans*-acting genes for the *bgl* operon, like *leuO* and *bglJ*, may serve as a secret weapon, which can be triggered by naturally occurring IS insertions. From an evolutionary and/or ecological point of view, this may be one of the positive physiological functions of *leuO.*

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