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Isolation and Characterization of the *lacA* Gene Encoding β-Galactosidase in *Bacillus subtilis* and a Regulator Gene, *lacR*

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We have isolated transposon insertions in the *lacA* gene encoding an endogenous β-galactosidase of *Bacillus subtilis*. Upstream of the putative operon containing *lacA* is a negative regulator, *lacR*, which encodes a product related to a family of regulators that includes the lactose repressor, *lacI*, of *Escherichia coli*. New strains with insertions in the *lacA* gene should be of use in studies using *lacZ* fusions in *B. subtilis*.

The *lacZ* gene from *Escherichia coli* has been used extensively as a reporter gene for studies of gene expression in *Bacillus subtilis*. These studies have sometimes been hindered by an endogenous β-galactosidase activity that is induced during sporulation (6, 8) and, in mutagenesis experiments, by regulatory mutations in a gene apparently encoding a repressor of the endogenous activity. We previously reported the isolation both of mutations in the regulatory gene, designated *lacR*, giving rise to increased levels of endogenous β-galactosidase and of secondary mutations in a linked gene which abolished the activity (9). The latter mutations, designated *lacA*, were thought to lie in the structural gene for the enzyme. The *lacRA* locus lies between *hisA* and *thr*, at about 290° (see reference 2) on the *B. subtilis* chromosome. A second, unlinked gene encoding a cryptic endogenous β-galactosidase activity was reported by Zagorec and Steinmetz (20). Here we report the isolation of transposon insertions in the *lacA* and *lacR* genes, the DNA sequence of the region, and the construction and characterization of strains with defined insertions in the two genes.

Transposon mutagenesis was done with the mini-Tn*10 cat* plasmid pHV1248 (see Table 1 for bacterial strains and plasmids). The plasmid was introduced into *B. subtilis* SG68, which carries a mutation in the *lacR* gene leading to constitutive levels of endogenous β-galactosidase and hence blue colonies on indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (9). The strain was incubated at the nonpermissive temperature for plasmid replication on plates maintaining selection for the mini-Tn*10*-borne chloramphenicol resistance determinant. Three white colonies were obtained among approximately 5,000 colonies.

Transformation of DNA from each of the chloramphenicol-resistant strains back into SG68 showed that the transposon insertion was completely linked to the mutation giving rise to the white colony phenotype. To recover DNA adjacent to the transposon insertion, one of the new strains, SG80, was transformed with plasmid pRD110. Selection for resistance to spectinomycin (50 μg/ml) led to insertion of the plasmid into the transposon by homologous recombination with the *cat* gene. Plasmids with inserts extending out from the transposon were

TABLE 1. *B. subtilis* strains and plasmids

Strain or plasmid	Relevant characteristics	Source, reference, or construction
Strains		
168	*trpC2*	C. Anagnostopoulos[a]
SG64	*trpC2 lacR1 lacA17*	9
SG68	*trpC2 lacR1*	9
SG77	*trpC2 lacR1* Ω(*lacA*::mini-Tn*10 cat*)*77*	Insertion of mini-Tn*10* (17) giving a Lac$^-$ phenotype
SG80	*trpC2 lacR1* Ω(*lacA*::mini-Tn*10 cat*)*77*	DNA from SG77 transformed into SG68 with selection for Cm^r
SG81	*trpC2* Ω(*lacA*::*neo*)*81*	This study
SG82	*trpC2* Ω(*lacA*::*tet*)*82*	This study
SG83	*trpC2* Ω(*lacR*::*spc*)*83*	This study
SG84	*trpC2* Ω(*lacA*::*neo*)*81* Ω(*lacR*::*spc*)*83*	This study
Plasmids		
pBEST309	*bla tet*	12
pBEST501	*bla neo*	13
pHV1248	*bla ori*C_{pE194}(Ts) ermC (mini-Tn*10 cat*)	17
pIC156	*bla spc*	18
pRD91	*bla spc lacA*$^+$	7-kb *Pst*I-*Sst*I DNA fragment cloned in pUS19
pRD110	*bla cat spc*	Chloramphenicol resistance cassette from pSG1 cloned into the *Bam*HI site of the pUS19 polylinker
pRD112	*bla cat lacA*$^+$	2.8-kbp PCR product cloned in *Xba*I-*Cla*I-digested pSG1301[b]
pRD116	*bla cat lacR*$^+$	1.0-kbp PCR product cloned in *Xba*I-*Xho*I-digested pSG1301[c]
pRD117	*bla cat lacR*::*spc*	*spc* cassette from pIC156 cloned in the *Cla*I site of pRD116
pSG1	*bla cat*	7
pSG1301	*bla cat*	19
pUS19	*bla spc*	3

[a] C. Anagnostopoulos, Laboratoire de Génétique Microbienne, INRA, 78352 Jouy en-Josas Cedex, France.

[b] PCR amplification was done with the synthetic oligonucleotides (5′-3′) CCT TCAATCTAGACAGTGGTATGTCAACTC and AAAATCGATTCCGCCCA TATCGAGCGGAGC.

[c] PCR amplification was done with the synthetic oligonucleotides AACAAC TCGAGCTGAATAATGGAGGCAG and CCTATCTAGACCTTAATTCAT ACTCTTCC.

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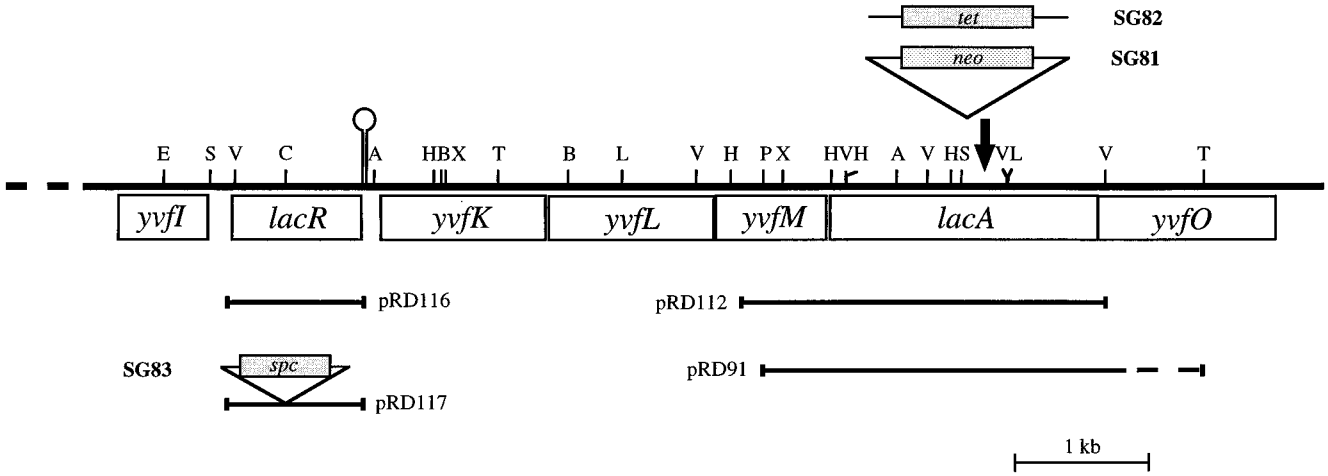

FIG. 1. Physical map of the *lacRA* region of the *B. subtilis* chromosome. The central thick solid line represents the cloned and sequenced segment of chromosomal DNA, together with some restriction endonuclease cleavage sites. The abbreviations for these are as follows: A, *Sph*I; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; L, *Sal*I; P, *Pst*I; S, *Sma*I; T, *Sst*I; V, *Eco*RV; X, *Bst*XII. Below, the positions of the major ORFs are indicated by boxes, all of which would be transcribed from left to right. The *lacA* and *lacR* genes are indicated. The stem and loop symbol indicates the position of a likely transcription termination signal. Below the boxes are represented the plasmids used to characterize the region and to make defined insertions in the chromosome. The *tet* and *neo* insertions in *lacA* were made by ligation of cassettes from pBEST309 or pBEST501 to *Sma*I-digested pRD91 and direct transformation into 168. The insertions were checked by PCR and for the absence of the *spc* marker on the plasmid. The thick black arrow indicates the position of the mini-Tn*10 cat* insertion in strain SG77. The names of strains derived by insertion of resistance markers are shown alongside the boxes representing the resistance cassettes.

recovered by digestion with various restriction endonucleases and religation, followed by transformation of *E. coli* DH5α (Gibco-BRL). Further rounds of plasmid insertion and recovery were used to clone a region of about 13 kb flanking the site of the original insertion, part of which is shown in Fig. 1.

Comparison of preliminary sequence information with the segment of DNA from the *his-thr* region being sequenced as part of the *B. subtilis* genome project (15) revealed the precise location of the transposon insertion. (Full details of the DNA sequencing methods are described elsewhere [10].) Analysis of the complete sequence of the region (accession number Z94043) revealed seven long open reading frames (ORFs) (Fig. 1), with almost no significant nontranslated spaces. The predicted product of each ORF was compared to protein sequence databases by using the BLAST network service and default parameters (1). All of the predicted products showed highly significant matches; the best match in each case is shown in Table 2. The plasmid insertion lay in a large ORF showing substantial sequence similarity with a *Bacillus circulans* gene encoding β-galactosidase. This result strongly supported the notion that the transposon had inserted into the structural gene (*lacA*) for the endogenous *B. subtilis* enzyme. This was confirmed by transformation of strain SG64 with plasmid pRD112 (Fig. 1), which resulted in segregation of Lac^+ and Lac^- transformant colonies. Three ORFs upstream of *lacA* (*yvfK*, *yvfL*, and *yvfM*) and one downstream ORF (*yvfO*) all had predicted products with significant sequence similarities to transmembrane and binding components of the ABC transporter family (11), especially those involved in maltose or maltodextrin transport (Table 2). This suggests that they are involved in sugar transport, though no ATPase component was present. The downstream ORF had a predicted product similar to arabinogalactan endo-1,4-β-galactosidases from various organisms. All five of these ORFs were especially closely spaced (<22 bp), with no obvious transcription terminators, suggesting that they comprise a single polycistronic operon. Upstream of *yvfK* was a space (139 bp) containing a likely transcriptional terminator sequence. This space seems likely to contain the promoter for the putative operon. Upstream of the transcription terminator was a gene encoding a predicted product significantly similar to a family of DNA-binding regulatory proteins that includes the lactose repressor, LacI, of *E. coli*.

TABLE 2. Similarities between the predicted products of the *B. subtilis lac* operon and proteins in sequence databases

B. subtilis lac operon gene[a]	Properties of predicted products with the most similar sequence				% Identity[b]
	Gene	Organism	Function or activity	Swiss-Prot accession number	
yvfI (219)	*pdhR*	*E. coli*	Pyruvate dehydrogenase repressor	P06957	31 (211)
lacR (330)	*ebgR*	*E. coli*	Regulatory gene for "evolved" β-galactosidase (*ebg*) operon	P06846	34 (326)
yvfK (421)	*malX*	*Streptococcus pneumoniae*	Part of maltose transport system, binding protein	P29850	33 (417)
yvfL (421)	*malC*	*S. pneumoniae*	Part of maltose transport system, permease	Q04698	48 (425)
yvfM (283)	*malD*	*S. pneumoniae*	Part of maltose transport system, permease	Q04699	46 (267)
lacA (687)	*bgaB*	*B. circulans*	β-Galactosidase	P48843	51 (674)
yvfO (454)	*ganA*	*Pseudomonas fluorescens*	Arabinogalactan endo-1,4-β-galactosidase	P48841	38 (322)

[a] Size of predicted protein, in amino acid residues, given in parentheses.
[b] Length of sequence alignment, in amino acid residues, given in parentheses.

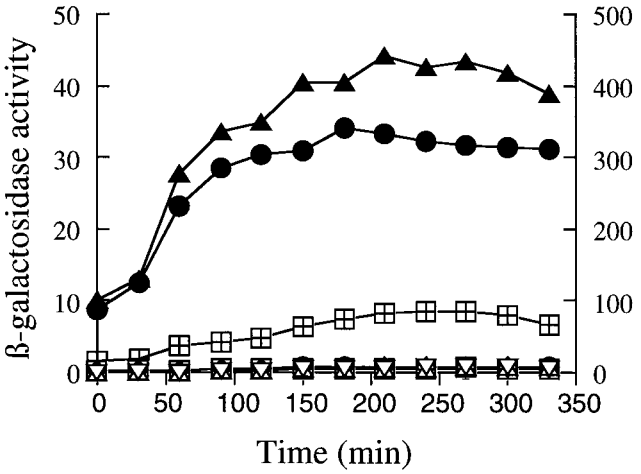

FIG. 2. Time courses of β-galactosidase synthesis by *B. subtilis* strains 168 (*lacA*$^+$*R*$^+$) (⊞), SG64 (*lacA17 lacR1*) (○), SG68 (*lacR1*) (●), SG80 (Ω*lacA lacR1*) (□), SG81 (Ω*lacA*) (▽), SG82 (Ω*lacA*) (△), SG83 (Ω*lacR*) (▲), and SG84 (Ω*lacA* Ω*lacR*) (◇). The strains were grown in CH medium and induced to sporulate as described previously (16). Time zero represents the time at which the cells were resuspended in the starvation medium. Samples were taken at intervals and assayed for β-galactosidase as specified by Daniel et al. (5). Note that strains SG68 and SG83 (solid symbols) produced much higher levels of β-galactosidase and are plotted on the 10-fold reduced scale to the right of the panel. All of the other plots (open symbols) refer to the left-hand scale. Some of the symbols for plots from strains producing negligible amounts of β-galactosidase are not visible because of overlap.

Intriguingly, the best match was to the EbgR repressor, which regulates the cryptic operon that mutates to give rise to lactose utilization in the absence of *lacZ* in *E. coli* (14). Two lines of evidence suggest that this gene is the previously identified negative regulator, *lacR*, of the operon containing *lacA* (9). First, transformation of plasmid pRD116 into a strain containing a *lacR* mutation (SG68) gave rise to segregation of blue and white colony types on plates containing X-Gal. Second, construction of an insertion in this ORF gave rise to a strain (SG83) with a greatly elevated level of endogenous β-galactosidase, similar to that of the original *lacR1* point mutant, SG68 (Fig. 2). Although these results confirm the location of the *lacR* gene, it is interesting to note that immediately upstream of *lacR* was another gene (*yvfI*) encoding a likely transcriptional regulator, this time of the GntR family. It is possible that the *yvfI* gene also plays a role in regulation of the operon containing *lacA* but we have not tested this.

To confirm the identity of the *lacA* gene we constructed derivatives of strain 168 in which it was insertionally disrupted with cassettes conferring resistance to either kanamycin (strain SG81) or tetracycline (strain SG82) (Fig. 1). Both strains showed negligible endogenous β-galactosidase activity during both growth and sporulation (Fig. 2), even in the presence of a *lacR* mutation (strain SG84). Strains SG80, SG81, and SG82 should provide useful tools for experiments in which the *lacZ* gene of *E. coli* is used as a reporter, especially if mutants showing increased or decreased activity are sought.

It is not yet clear precisely which substrates would be transported and degraded by this system. *B. subtilis* does not utilize lactose as a sole carbon and energy source, even when the operon is expressed constitutively in a *lacR* mutant (3). We have attempted to find substrates that can induce expression of the endogenous β-galactosidase, using a wide range of sugars without success (4). It is possible that they respond to and enable utilization of a relatively exotic carbon source, such as a plant exudate, that we have not tested.

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