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Isolation and Characterization of Mutations in the Gene Encoding an Endogenous *Bacillus subtilis* β-Galactosidase and Its Regulator

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We have isolated mutations that appear to inactivate the gene (*lacA*) encoding an endogenous β-galactosidase activity in *Bacillus subtilis* and in a closely linked negative regulatory element (*lacR*). Both genes map to the *hisA-thrA* region. The *lacA* mutations may help to avoid some of the problems arising from the use of the *Escherichia coli lacZ* gene as a reporter gene in *B. subtilis*.

Gene fusions have been used extensively as convenient means of measuring gene expression in many systems, for example, during sporulation in *Bacillus subtilis* (9–11). An important experimental approach that such gene fusions allow is looking for secondary mutations that alter the normal pattern of expression of the target gene, such mutations being detected by very convenient chromogenic or fluorogenic indicators that may be incorporated into the growth medium. The *lacZ* gene from *Escherichia coli* has been the most extensively used reporter gene in *B. subtilis* (6, 14). However, a common, though not often reported, problem in the use of this gene is a background β-galactosidase activity produced by the host (5, 7). Although the enzyme activity is produced at a relatively low level, it is specifically induced during sporulation and creates problems, particularly in dealing with sporulation genes that are also weakly expressed. Another problem arises in experiments in which second-site mutations increasing the expression of a target gene fused to *lacZ* are to be isolated. In such circumstances, the vast majority of mutations isolated affect the endogenous β-galactosidase activity rather than the reporter gene. Here we document the fact that mutations affecting the regulation of the endogenous *lacZ*-like gene occur frequently. The new mutations map to the *hisA* region of the chromosome and presumably define a negative regulatory element, designated *lacR*, of the endogenous β-galactosidase. Starting with *lacR* mutations that overexpress the β-galactosidase activity, we have isolated secondary mutations that lie in the closely linked *lacA* locus, which is probably the structural gene for the enzyme, and that eliminate β-galactosidase activity.

Isolation and characterization of *lacR* mutations. The strains of *B. subtilis* used in our study are listed in Table 1. Strain 168 (ϕ105J43) was grown at 37°C to an A_{600} of 1.0 in 10 ml of Penassay broth. The cells were mutagenized by adding 2.5 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma Chemical Co., St. Louis, Mo.), incubated with shaking for 15 min, washed twice in Penassay broth, and plated directly on nutrient agar containing the chromogenic β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma). After 3 days of incubation, blue colonies (16 from a total of about 10,000 colonies) were picked and purified by two rounds of single-colony isolation. Because the mutagenized cells were plated directly after mutagenesis and were not allowed to undergo vegetative growth, they were unlikely to be clonal. They were presumed to arise by mutations altering the regulation of the *spoIIIC-lacZ* gene carried on the ϕ105J43 prophage (13). Several of the mutants were induced to sporulate by the resuspension method of Sterlini and Mandelstam (12) and were assayed at intervals for β-galactosidase with *o*-nitrophenylphosphate as described previously (7). All of the mutants still induced β-galactosidase only during sporulation, but the overall activities were always higher than those observed for the parental strain. A representative time course (for strain SG61) is shown in Fig. 1 together with the responses produced by the parental strain. Note that the levels of expression in the mutant are so great that on the scale shown in the figure, induction of *spoIIIC-lacZ* is almost insignificant. All of the mutants sporulated normally, as determined by measuring the incidence of heat-resistant spores (85°C, 15 min) at 9 h after resuspension in the sporulation medium.

The mutations giving rise to the increased β-galactosidase activity were mapped by PBS1 transduction (3) with the mapping kit of Dedonder et al. (4), each of the recipient strains having been lysogenized with phage ϕ105J43. The mutations in all but 2 of the 16 strains showed linkage to *hisA1* (about 45% cotransduction) and *thrA5* (about 10% cotransduction). When the nonlysogenic mapping strain QB917 (*hisA1 thrA5 trpC2*) was used as the recipient, it became apparent that the mutations linked to *hisA* and *thrA* were altering the regulation of an endogenous enzyme that hydrolyzed X-Gal and not regulation of the *spoIIIC-lacZ* fusion. All of the mutations that showed linkage to *hisA* and *thrA* behaved similarly. Mapping data for an isogenic derivative of one of the mutants, strain SG68, are shown in Table 2. The *lacR1* mutation clearly maps between *hisA1* and *thrA5* because these markers are virtually unlinked (~1% cotransduction; data not shown). Although 2 of the 16 mutations isolated did not map in this region, it was apparent that mutations affecting the regulation of the gene encoding the endogenous enzyme were likely to present a serious impediment to the general strategy of using *lacZ* fusions to probe for regulatory effector genes in *B. subtilis*. Thus, it seems to be useful to attempt to isolate mutations inactivating the structural gene for the endogenous enzyme.

Because the mutations were relatively abundant, it seemed most likely that they lay in a negative regulatory element (i.e., a repressor gene) controlling the expression of the structural gene for the β-galactosidase-like activity. We therefore designated the mutations *lacR*.

Isolation and characterization of *lacA* mutations. Strain SG63, a typical *lacR* mutant, was mutagenized with NTG as

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TABLE 1. *B. subtilis* strains used

Strain	Relevant genotype	Source or reference[a]
168	*trpC2*	Laboratory stock
168 (ϕ105J43)	*trpC2 spoIIIC'-lacZ cat*	13
CU267	*trpC2 ilvB2 leuB16*	S. A. Zahler
MB75	*lys-1 metC3 tal-1*	Laboratory stock
SG61	*trpC2 hisA1 thrA5 lacR1*	Mutagenesis of 168 (ϕ105J43)
SG62	*trpC2 hisA1 lacR1*	QB917 transduced to Thr^+, PBS1 lysate of SG61
SG63	*trpC2 thrA5 lacR1*	QB917 transduced to His^+, PBS1 lysate of SG61
SG64	*trpC2 lacR1 lacA17*	SG62 transformed to His^+, DNA from V17
SG68	*trpC2 lacR1*	CU267 transformed to Ilv^+ Leu^+, DNA from SG61
SG69	*lacR1 lacA17*	SG64 transformed to Trp^+, DNA from MB75
SG70	*lacR1*	SG68 transformed to Trp^+, DNA from SG69 (blue colony)
SG71	*lacR1*	SG68 transformed to Trp^+, DNA from SG69 (white colony)
V17	*trpC2 thrA5 lacR1 lacA17*	Mutagenesis of SG62
V17.1	*trpC2 lacR1 lacA17 (spo)*	SG62 transduced to His^+, PBS1 lysate of V17

[a] Methods for transduction (3) and transformation (1, 8) are described elsewhere.

described above and plated on nutrient agar plates containing X-Gal. From about 2,000 colonies, 14 white-colony mutants were isolated and purified.

In preliminary mapping crosses, the mutations in six of the strains were mapped by PBS1 transduction with strain SG62 as the recipient. All six showed linkage to *hisA1* (30 to 50%), putting the new mutations into the same region of the chromosome as the *lacR* class of regulatory mutations (data not shown). The mutants were then tested for the production

TABLE 2. PBS1 transduction analysis of the *lacR1* mutation

Donor	Recipient	Selected marker	% Cotransduction (no. of colonies scored)
SG68	QB917	Thr^+	17 (198)
trpC2 lacR1	*trpC2 hisA1 thrA5*	His^+	51 (208)

of β-galactosidase during sporulation and for the incidence of heat-resistant spores as described above. Unfortunately, the strains that showed the lowest β-galactosidase activities were asporogenous. It is possible that the asporogenous phenotypes were due to secondary mutations. If so, it should be possible to separate the mutations affecting the β-galactosidase (designated *lacA*) from the secondary *spo* mutations by genetic transfer. After transduction to His^+, most of the strains showed substantially improved sporulation (>20% of the wild-type level of heat-resistant spores), suggesting that the *lacA* and *spo* mutations were not linked. The derivative showing the lowest β-galactosidase activity, strain V17.1, was chosen for further purification by transformation. DNA from this strain was transformed into strain SG62 with selection for His^+. Six white transformant colonies were purified, induced to sporulate, and tested for β-galactosidase activity and incidence of heat-resistant spores as described above. All six strains showed approximately normal spore formation but virtually undetectable β-galactosidase (<3% of the level found in the wild-type strain, measured at 5 h after resuspension in the sporulation medium). A time course for β-galactosidase production by one of these transformants, strain SG64, is shown in Fig. 2.

Since the mutations were again relatively abundant (as judged by our experience of such mutagenesis experiments), it seemed likely that the new mutations had inactivated the structural gene encoding the endogenous β-galactosidase. The linkage results suggested that the regulatory gene and the structural gene might be closely linked. Thus, we measured the recombination index between mutations *lacR1* and *lacA17* by transforming strain MB75 with DNA from the isogenic strains SG70 and SG71. The recombination index between these mutations, 0.18, is consistent with their lying in adjacent genes (2) (Table 3). Of course, further work is needed to exclude the possibility that the mutations do not affect other functions such as positive regulatory elements.

In preliminary experiments, we have not detected reversion of the *lacA17* mutation, which suggests that the use of

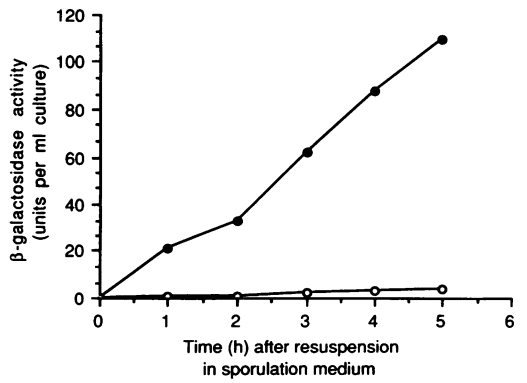

FIG. 1. Time courses for β-galactosidase activity during sporulation of strain 168 (ϕ105J43) (○) and derivative strain SG61 carrying the *lacR1* mutation (●).

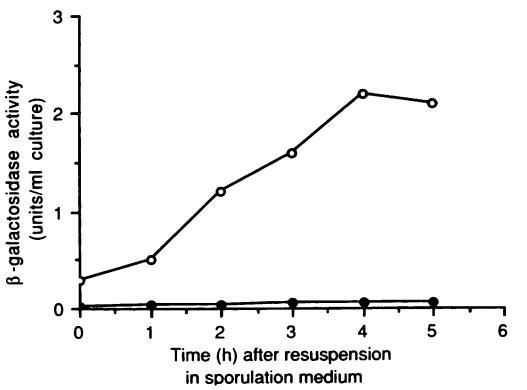

FIG. 2. Time courses for β-galactosidase activity during sporulation of wild-type strain 168 (○) and *lacR1 lacA17* double-mutant strain SG64 (●).

TABLE 3. Recombination index between *lacR1* and *lacA17* mutations

Donor strain	Marker(s)	% Congression (no. of Met^+ transformants scored)		Recombination index[a]
		Lys^+	Blue	
SG71	*lacR1 lacA17*	0.6	0.11 (865)	0.11/0.6
SG70	*lacR1*	1.0	1.0 (810)	1.0/1.0

[a] Scored in transformation crosses with MB75 *lys-1 metC3* as recipient and selecting for Met^+. Total recombination index = (0.11/0.6)/(1.0/1.0) = 0.18.

this mutation should virtually eliminate the problem posed by the endogenous β-galactosidase gene in experiments of the type described in the introductory paragraph. Indeed, we have used the strain successfully in the isolation of several mutations altering the expression of *spo-lacZ* fusions without encountering any revertants restoring the endogenous enzyme activity (S. R. Partidge and J. Errington, unpublished results). Of course, now that we have defined what is likely to be the structural gene for this enzyme, it is not difficult in principle to clone the gene and construct insertional mutations with a selectable marker. Such mutations could then easily be introduced into other genetic backgrounds whenever necessary.

We have deposited strain SG64 in the *Bacillus* Genetic Stock Center.

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