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5′-Noncoding Region *sacR* Is the Target of All Identified Regulation Affecting the Levansucrase Gene in *Bacillus subtilis*

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The regulation of the levansucrase gene *sacB* was studied in *Bacillus subtilis* strains. Fusions were constructed in which genes of cytoplasmic proteins such as *lacZ* were placed immediately downstream from *sacR*, the regulatory region located upstream from *sacB*. These fusions were introduced in mutants affected in *sacB* regulation. In all cases the marker gene was affected in the same way as *sacB* by the genetic context. This result is of particular interest for the *sacU* pleiotropic mutations, which affect *sacB* expression and other cellular functions such as the synthesis of several exocellular enzymes. We also showed that strains harboring *sacU*$^+$ or *sacU*-hyperproducing alleles contained different amounts of *sacB* mRNA, which was proportional to their levansucrase secretion. We concluded that the *sacU* gene does not affect *sacB* expression at the level of secretion but acts on a target within *sacR*. We discuss the possibility that *sacU* acts on a part of *sacR*, a homologous copy of which was found upstream from the gene of another *sacU*-dependent secreted enzyme of *B. subtilis*, β-glucanase.

The expression of *sacB*, the structural gene of the *Bacillus subtilis* exoenzyme levansucrase (LS), is controlled by several genes identified by mutations and unlinked to *sacB* (16, 17). Among them, the *sacU* gene is of particular interest because *sacU* mutations are pleiotropic. *sacU*-hyperproducing (*sacU*^{h}) mutations increase the synthesis of at least four exoenzymes (LS, neutral protease, serine protease, and amylase), while *sacU*$^-$ mutations reduce the synthesis of the two exocellular proteases and LS. These characters suggest that the *sacU* function is related to secretion or, at least, to the metabolism of the cell envelope. The hypothesis is supported by other observations. For example, *sacU*^{h} mutants are devoid of flagella and are poorly transformable. Other observations are harder to relate to envelope metabolism; for example, *sacU*^{h} mutants are altered in sporulation regulation (14).

Recently, it has been shown that the putative product of *sacU*, a 46-kilodalton protein which is absent in *sacU*$^-$ mutants and overproduced in *sacU*^{h} mutants, seems to be membrane bound. Furthermore, it has been reported that although *sacU*^{h}, *sacU*$^+$, and *sacU*$^-$ strains produce very different amounts of LS, they contain similar amounts of *sacB* mRNA. It was concluded that *sacU* mutations alter *sacB* expression at a posttranscriptional level, probably that of secretion (2).

We have cloned and sequenced *sacB* and its upstream *cis*-acting control region *sacR*. We have shown that sequences required for *sacB* expression are rather far from the ATG codon of the gene (9, 24). Strong arguments have been obtained by deletion analysis that the promoter is 230 base pairs (bp) upstream of *sacB* and that the double 30-bp palindromic region present within *sacR* is the target for *sacB* inducibility by sucrose (M. Steinmetz and S. Aymerich, Ann. Inst. Pasteur (Paris), in press).

What is the target of *sacU* regulation? Is it also within *sacR* or is it indeed a part of *sacB* related to secretion? To distinguish between these questions, we constructed in *Escherichia coli* fusions between the *sacR* region and genes of the cytoplasmic proteins *lacZ* and *nptI*, a neomycin phosphotransferase gene conferring kanamycin resistance. We introduced the fusions into *B. subtilis* strains altered in *sacB* regulation and found that *lacZ* and *nptI* expression is affected in the same way as *sacB* expression by the genetic context. We conclude that *sacR* is the target of all of the previously identified regulation that controls *sacB*. Moreover, we failed to reproduce the result that strains harboring different *sacU* alleles and secreting different amounts of LS contain similar amounts of *sacB* mRNA (2). Instead, we found that the *sacB* mRNA content of *sacU*$^+$ and *sacU*^{h} strains was approximately proportional to their LS secretion.

MATERIALS AND METHODS

Strains and plasmids. The strains of *B. subtilis* used in this study (Table 1) have been described previously or are prototrophic transformants of these strains (14, 16, 17). For the construction and production of the plasmids, *E. coli* HVC45 (24) was used, except when stated otherwise.

To obtain fusions we used Mu dIIPR3 (R. Ratet and F. V. Richaud, Gene, in press), a defective Mu prophage derived from Mu dII1734 prophage (6) which allows protein fusions. In Mu dIIPR3 the *lac* operon was substituted with the kanamycin resistance gene (*nptI*) from Tn*903*; it contained the chloramphenicol resistance gene (*cat*) from Tn*9* as a transposition marker (Fig. 1). Transpositions within *sacB* were selected as follows. A MC4100 (6) derivative harboring Mu dIIPR3 and Mu *cts* helper prophages inserted in the chromosome (Ratet and Richaud, in press) was transformed for ampicillin resistance by pLS50 (25). A transducing lysate was produced and used to infect the recipient strain M8820 Mu as described previously (6). Transductants were selected on LB-chloramphenicol-kanamycin-sucrose (25 μg/ml, 5 μg/ml, and 50 mg/ml, respectively). The presence of sucrose allowed the selection of pLS50 derivatives harboring insertions within the *sacB* gene (25). DNA from pLS50::Mu dIIPR3 plasmids was prepared and submitted to restriction

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TABLE 1. *sacB*, *npt*, and *lacZ* expression in transformed *sac* mutants

Recipient strain and genotype[a]	Qualitative tests[b]		*lacZ* and *sacB* expression (U/ml) in induced cultures of pLG131 transformants[c]	
	Without sucrose	With sucrose	β-Galactosidase	LS
QB128 *trpC2 leuA8*	0	m	5	0.2
QB136 *trpC2 leuA8 sacU*^{h}*32*	0	H	330	8
QB127 *trpC2 leuA8 sacU*^{h}*200*	0	H	130	9
QB150 *trpC2 metC3 sacQ*^{h}*36*	0	H	136	4
QB151 *trpC2 metC3*	0	m	16	0.4
QB1071 *sacA321 sacU52*	0	0	0.4	0.015
QB1072 *sacA321*	0	m	14	0.2
QB2033 *sacA321 sacS230*	0	0	<0.1	<0.01
QB2028 *sacA321 trpC2 sacS61*	0	0	0.1	<0.01
QB913 *sacA321 trpC2 sacS*^{h}*7*	0	H	—	—
QB165 *sacA321 trpC2 sacS*^{c}*37*	m	m	—	—

[a] *sacA321* mutation affects the gene of a second (endocellular) sucrase.

[b] *npt* and *lacZ* expression (by pLK131 and pLG131 transformants, respectively) and *sacB* expression of transformed and nontransformed mutants were estimated on solid medium as described in the text. 0, no (or low) expression; m, middle expression; H, high expression. For *npt* expression, 0, m, and H correspond to no resistance to 3 μg of kanamycin per ml and resistance to 10 μg and >50 μg of kanamycin per ml, respectively.

[c] pLG131 transformants were grown in liquid C mineral medium with glucose and sucrose as described in the text. —, Not determined.

analysis. Several plasmids with insertions at different points of *sacB* were characterized. The deletion of two *Pst*I fragments from the plasmids resulted in derivatives such as pLK131 (Fig. 1). In pLK131 the left end of Mu was very near the beginning of *sacB*. The *Bam*HI-*Hpa*II fragment from pLK131 which contained the fusion point was cloned in M13mp19 and sequenced. The following sequence was determined: . . .AAAAAAGGAGACATGAACG A::TG AAG CGG CGC ACG AAA AAC. . . (the underlined region corresponds to the Shine Dalgarno sequence of *sacB* [24], and the double colon refers to the point of fusion; the translation phase used in the left end of Mu is as indicated previously [6]). Thus, pLK131 appeared to be an ideal fusion because only one nucleotide of *sacB* was present. pLG131, a pLK131 derivative in which the *lacZ* gene is substituted for *nptI*, was constructed in three steps (Fig. 1). pFR97 (22) was used as the source of *lacZ*. Like the *nptI* gene present in Mu dIIPR3, the *lacZ* gene in pFR97 begins with a *Bam*HI site in the same phase. pFR97 DNA was prepared from a *dam*$^-$ strain (GM33 from M. Radman) to permit cutting at all *Cla*I sites. Strain 7118 (7), obtained from J. Messing, which allowed the selection of plasmids that express the 5′ part of *lacZ*, was used for the construction of pIC2. GC4468, a Δ*lac* strain supplied by R. D'Ari, was used as a recipient for the last step in the construction of pLG131.

For RNA assays two M13 derivatives constructed in strain 7118 were used. M13 *sacBEH* was an M13mp8 derivative containing the *Eco*RI-*Hin*dIII 1.15-kilobase internal fragment of *sacB* from pLS50. It should be noted that M13 *sacBEH* contains 80% of *sacB* but neither *sacR* nor the 5′ and 3′ ends of the gene. pEMBL*cat* was a pEMBL19 (7) derivative containing the *cat* gene present in pJH101 (8) derivatives, such as pLS50 and pLG131, and was obtained as follows. The *cat*-containing *Mbo*I-*Hpa*II fragment from pC194 (12) was cloned in pUC8, which was cut with *Bam*HI and *Acc*I; the *cat*-containing *Hin*dIII-*Eco*RI fragment from the resulting plasmid was cloned in pEMBL19, which was cut with the same enzymes. Single-stranded DNA from M13 *sacBEH* and pEMBL*cat* was prepared by the methods of Hu and Messing (13) and Dente et al. (7), respectively.

Transformation of *B. subtilis* strains and phenotypic characterization of the transformed clones. pLK131 and pLG131 were, like their parent pLS50, integrative plasmids that could transform *B. subtilis* strains for Cam^r. To avoid tandem integrations resulting from transformations by several plasmids, nonsaturating concentrations of DNA were added to competent cells (1). Transformants were selected on LB medium-chloramphenicol (3 μg/ml). Under these conditions, tandem integrations of two or three copies of the plasmid could still occur as a result of transformations by multimeric plasmids. The transformants and control clones containing one, two, and more than three integrated copies of pLS50 were picked on LB medium, grown at 37°C, and then replicated on LB medium-chloramphenicol (10 and 18 μg/ml). The transformants that contained only one copy could be distinguished because they showed a growth lag with 10 μg of chloramphenicol per ml and grew very poorly with 18 μg of chloramphenicol per ml (23a; unpublished data). These clones, containing one copy of *sacR-sacB* and one copy of *sacR::nptI* or *sacR::lacZ* in tandem (Fig. 1), were used for the following studies.

B. subtilis strains harboring different *sac* mutations and transformed by pLK131 and pLG131 were characterized on solid medium. LS synthesis was estimated as described previously (16). β-Galactosidase was estimated by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) hydrolysis with colonies grown on mineral medium (1) containing glucose (1 g/liter), auxotrophic requirements (100 mg/liter), X-gal (20 mg/liter), and, in some cases, sucrose (20 g/liter). *nptI* expression was estimated by replicating colonies on LB medium containing various concentrations of kanamycin with (20 g/liter) or without sucrose.

Quantitative studies of *sacB* and *lacZ* expression in liquid medium were performed as follows. Transformants were grown in C medium [70 mM K_2HPO_4, 30 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.01 mM $MnSO_4$, 22 mg of ferric ammonium citrate per liter] supplemented with glucose (10 g/liter), sucrose (20 g/liter), and auxotrophic requirements (100 mg/liter). Growth was monitored turbidimetrically with a Klett-Summerson colorimeter at 660 nm. Samples were taken at 150 Klett units (mid-exponential phase of growth). β-Galactosidase was assayed by the method of Miller (19) on cell extracts that were lysed with lysozyme and sonicated. LS was assayed by sucrose hydrolysis (14) on the whole extract (supernatant plus cells).

Glucanase activities were assayed on culture supernatants

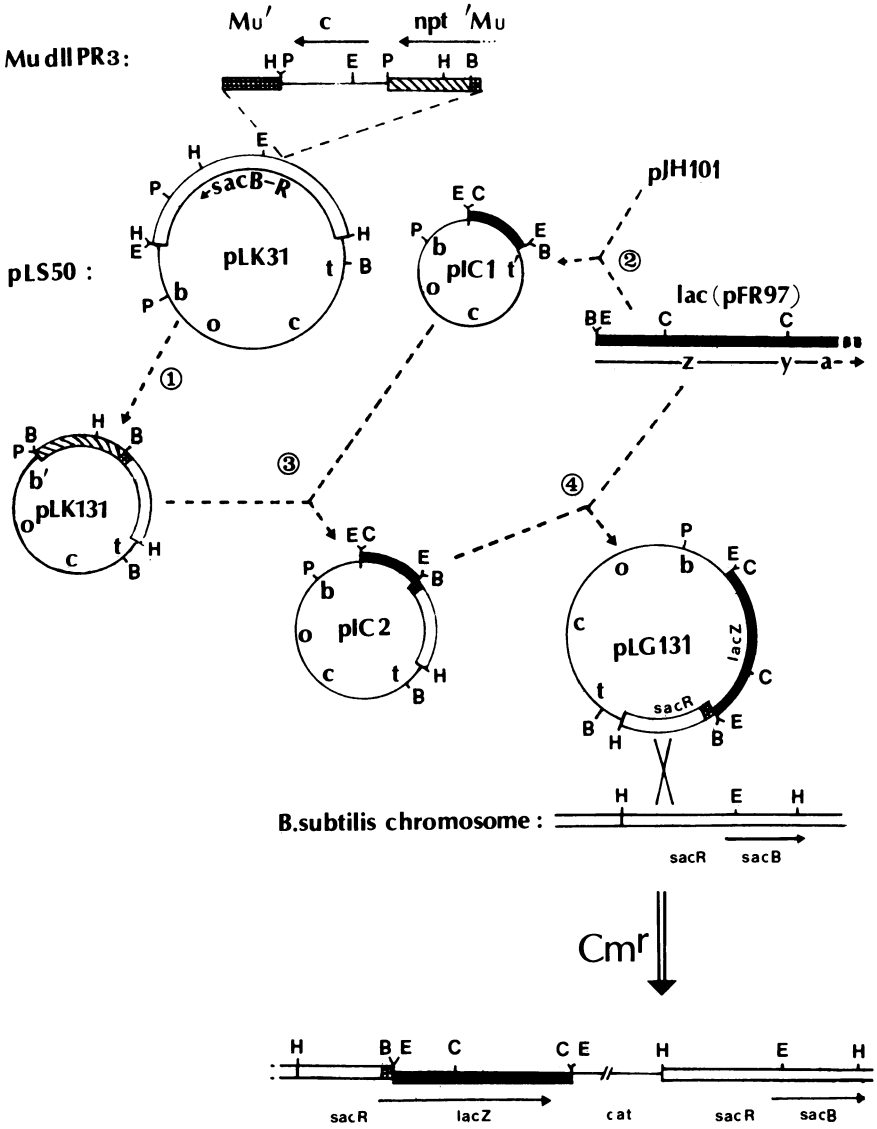

FIG. 1. Construction of the plasmids and their integration into the *B. subtilis* chromosome. pLK131 (14.6 kilobases) is a pLS50 (10.3 kilobases) derivative with an integrated Mu dIIPR3 prophage (4.3 kilobases). pJH101 (8) is a pBR322 derivative with a gram-positive *cat* gene inserted at the *Pvu*II site (the pLS50 *Hin*dIII-*Eco*RI large fragment comes from pJH101). Abbreviations: b, c, o, and t, β-lactamase gene, *cat* gene, *ori* gene from ColE1, and the tetracycline resistance gene, respectively; B, C, E, H, and P, restriction sites for *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Pst*I, respectively. Steps: 1, intramolecular deletion by *Pst*I; 2, cloning in pJH101 of the *Eco*RI-*Cla*I 5′ part of *lacZ*; 3, cloning of the *sacR* region (the *Bam*HI fragment from pLK131) upstream of the pIC1 *lacZ*′ gene; 4, cloning of the *lacZ* 3′ part (*Cla*I fragment) downstream of the pIC2 *lacZ* 5′ part.

by lichenan (Sigma Chemical Co., St. Louis, Mo.) hydrolysis by the method of Boriss et al. (3).

***sacB* and *cat* RNA assays.** RNA was extracted by the method of Glatron and Rapoport (10) from cells grown as described above in C medium with sucrose and harvested at 150 Klett units. After a second phenol-chloroform extraction and precipitation with cold ethanol, RNA was solubilized with a minimal volume of distilled water (solubilization was monitored by determining the A_{260}), and the remaining precipitate was discarded. This treatment eliminated viscous impurities such as polysaccharides that disturb dot blot analysis.

RNA was denatured with formaldehyde (18), and samples were dotted onto a Biodyne nylon membrane (Pall Ultrafine Filtration Corp.) with a Minifold TM apparatus (Schleicher & Schuell, Inc., Keene, N.H.). Filters were baked, prehybridized, hybridized, and washed under the conditions recommended by the supplier. Radioactive hybridization probes were prepared (13) from M13 *sacBEH* and pEMBL*cat* with sequencing primer (Biolabs). With this primer, radioactive DNA was obtained that was complementary to *sacB* and *cat* mRNA; these probes were denatured before hybridization.

RESULTS AND DISCUSSION

Expression of *lacZ* and *nptI* under the control of *sacR* in *B. subtilis* mutants. We constructed fusions in which the β-galactosidase or kanamycin resistance genes were placed immediately downstream of *sacR*, the regulatory 5′-

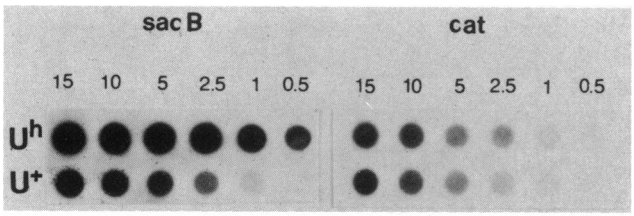

FIG. 2. Dot blot analysis of *sacB* and *cat* mRNA extracted from pLG131 transformants of strains QB128 (*sacU*$^+$) and QB136 (*sacU*^{h}). Various amounts of total RNA (in micrograms; indicated above the blots) were blotted, and the filters were successively hybridized with radioactive probes complementary to *sacB* and *cat* transcripts.

noncoding region of *sacB* (Fig. 1). pLG131 and pLK131, plasmids bearing the fusions, were used to transform strains carrying mutations that affect *sacB* regulation, i.e., the two kinds of *sacU* mutations described above, the different *sacS* mutations, and the *sacQ*^{h}*36* mutation. The *sacQ*^{h}*36* mutation is not linked to the *sacU* locus but confers a phenotype similar to that of the *sacU*^{h} mutants. The *sacS* locus is defined by three kinds of mutations that affect sucrose metabolism. Strains with *sacS*$^-$, *sacS*^{h}, and *sacS*^{c} mutations are, respectively, deficient, overproducing (only after induction by sucrose), and constitutive for LS synthesis. *sacS*^{h} and *sacS*$^-$ mutations resemble *sacU*^{h} and *sacU*$^-$ mutations, respectively, in their effect on LS synthesis, but they are not pleiotropic. Thus, the *sacS* locus seems to code for one or several diffusible components that control the sucrose induction of *sacB* as well as its level of expression (14, 16, 17).

The phenotypes of the strains transformed by pLK131 and pLG131 were characterized on solid medium. The results show that the regulation of *nptI* and *lacZ* expression is identical to that of *sacB*. Quantitative assays of *lacZ* expression in liquid medium confirmed this result. *lacZ* and *nptI* expression was inducible by sucrose in the wild-type strains; constitutive in the *sacS* constitutive (*sacS*^{c}) mutant; very low in the *sacU*$^-$ and *sacS*$^-$ mutants; and high but still inducible in the *sacU*^{h}, *sacQ*^{h}, and *sacS*^{h} mutants (Table 1). No significant titration effects were observed in these strains that contained two copies of *sacR* in their chromosome.

As mentioned above, the *sacR*::*nptI* and *sacR*::*lacZ* fusions contain only 1 bp from *sacB*. Between *sacR* and the *lacZ* or *nptI* genes a 113-bp segment from the Mu genome (6) is present, and this contains an open reading frame that is in phase with the downstream marker gene. The open reading frame showed no similarity to a secretion signal sequence, and its nucleotide sequence showed no homology with that of the beginning of *sacB*. Therefore, these observations show that the *sacR* locus is the target of all of the identified regulatory genes that affect *sacB* expression. Of particular interest for *sacU* regulation is the conclusion that its effect on LS synthesis is not related to an interaction of pre-LS or its nascent precursor with the secretory apparatus.

Assays of *sacB* mRNA. Results of preliminary experiments with RNA extracts from *sacU*^{h} and *sacU*$^+$ strains (QB136 and QB128, respectively) after induction with sucrose show that these strains contain different amounts of *sacB* mRNA. The discrepancy between our results and those reported previously (2) prompted us to introduce a control by assaying a constitutive mRNA in the extracts. RNA was extracted from strains QB128 and QB136 which were transformed with pLG131 (the integrated plasmid codes for a constitutive *cat* mRNA [4]). The filters were successively hybridized with probes complementary to *sacB* mRNA and *cat* mRNA.

A typical result is shown in Fig. 2. From the results of several independent experiments it was estimated that the *sacU*^{h} mutant contains 15- to 40-fold more *sacB* mRNA than the *sacU*$^+$ mutant. It is difficult to know why different results have been reported (2), because some experimental procedures (growth conditions, induction, etc.) have not been described. Our results show that the *sacU*^{h} mutant contains more *sacB* mRNA than the wild-type strain. Our upper estimate is a 40-fold increase in *sacB* mRNA, which is sufficient to account for the difference in LS synthesis (Table 1); however, our average estimate of a 25-fold increase would permit a slight additional regulation, for example, at the level of translation.

Supplementary data are required to determine whether the differences in the levels of *sacB* mRNA correspond to differences in transcription initiation or in RNA stability.

Homology between sequences upstream of *sacB* and β-glucanase gene. We have reported previously (23a) an important sequence homology (Fig. 3) between sequences of *sacR* that flank its palindromic sequence and sequences that flank another palindromic sequence located upstream of the *bgl* gene. *bgl* is the structural gene of β-glucanase, an enzyme secreted by *B. subtilis* (20). This observation led us to assay the glucanase activity secreted by the *sacU*^{h} and wild-type strains. We observed that when grown in LB medium, the *sacU*^{h} mutant strain QB136 produced 7 times more glucanase activity than the *sacU*$^+$ strain QB128 (Fig. 4). Estimates following growth on solid LB medium-lichenan (5) showed that the *sacQ*^{h}*36* mutant was also a glucanase overproducer and that the *sacU*$^-$ mutants were low producers, while glucanase production in the different *sacS* mutants was unaffected (data not shown).

We have shown by insertional inactivation and by deletion analysis (23a, 24) that the palindromic region and the sequences that flank it that are present upstream from *sacR* are involved in *sacB* regulation. Therefore, it might be suggested that the two homologous sequences found within *sacR* and upstream of *bgl* are targets of *sacU* regulation; however, supplementary data are required because we do not know whether the sequences found upstream of *bgl* are involved in its expression (20). Moreover, no similar sequences were

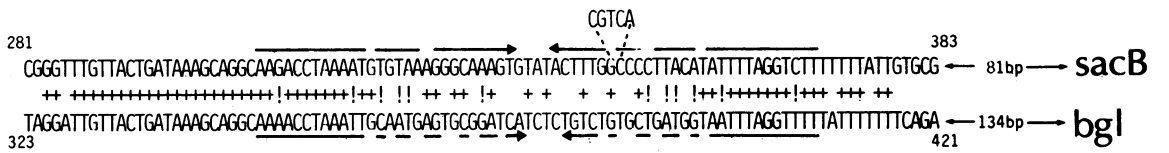

FIG. 3. Sequence homologies between *sacR* and a region located upstream of the β-glucanase gene (*bgl*). Nucleotide numbering is that used previously (20, 24). Symbols: +, homologies; !, nonhomologies which preserve the palindromic structure. The arrows indicate the inverse repeated sequences. The CGTCA pentanucleotide, an integral part of *sacR* sequence, was displaced to show the homology better. The distances to *sacB* and *bgl* are distances up to the ATG codon of the genes.

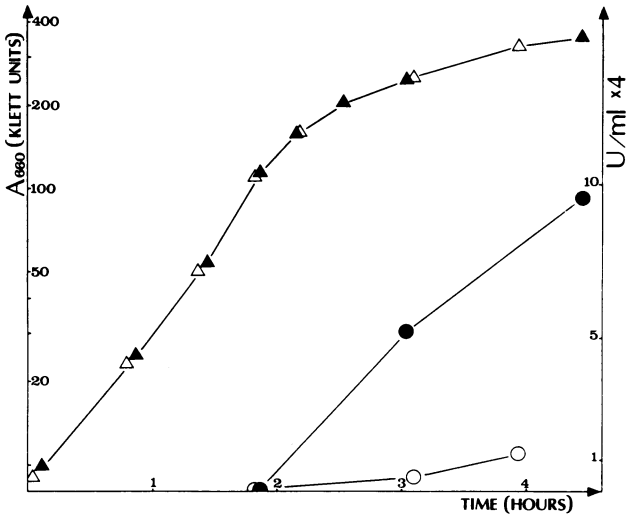

FIG. 4. Glucanase secreted by strains QB128 (*sacU*$^+$) (○) and QB136 (*sacU*^{h}) (●) grown in LB medium. Optical density is also indicated for strains QB128 (△) and QB136 (▲). One glucanase unit liberates 1 μmol of maltose equivalent per min (3).

found upstream of the other *sacU*-dependent genes sequenced thus far, such as the genes of secreted amylase and proteases (27–29). To identify the exact position of the *sacU* target in the *sacR* region, we are now using the *sacR*::*nptI* fusion to try to select mutations that confer expression that is independent of *sacU* control.

***sacU* regulation and the synthesis of exocellular proteins.** The product of the *sacU* gene, probably in association with the products of genes such as *sacQ*, seems to play a role in a complex system of regulation, the function and physiological role of which are still unknown. The effect of the *sacU* product on the synthesis of secreted proteins is important but not general. The *sacU*^{h} mutations enhance the synthesis of five exoenzymes but do not affect the synthesis of *B. subtilis* levanase (15), *Bacillus amyloliquefaciens* amylase, or *Bacillus lichenformis* penicillinase when the corresponding genes are cloned in *B. subtilis* (11, 23). On the other hand, the *sacU*^{h} mutations suppress sporulation inhibition by glucose (14) and increase the level of intracellular serine protease (M. Steinmetz, unpublished data). It is interesting that some cellular functions are affected both by the *sacU*^{h} mutations and by some *spoO* pleiotropic mutations. These effects are sometimes opposing, as in the case of endocellular and exocellular proteases and of sporulation commitment, and are sometimes similar, as in the case of transformability (14, 17). As reported recently (26), *SpoOA* and *SpoOF* proteins of *B. subtilis* are homologous to the *ompR* and *sfrA* proteins of *E. coli* which seem to regulate, at the transcription level, genes that are implicated in membrane biogenesis. The product of the *sacU* gene is perhaps also implicated in this kind of global regulation.

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