Expression of the *Bacillus subtilis* *sacB* Gene Leads to Sucrose Sensitivity in the Gram-Positive Bacterium *Corynebacterium glutamicum* but Not in *Streptomyces lividans*

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The expression of the structural gene (*sacB*) encoding *Bacillus subtilis* levansucrase in two gram-positive soil bacteria, *Corynebacterium glutamicum* ATCC 13032 and *Streptomyces lividans* 1326, was investigated. *sacB* expression in the presence of sucrose is lethal to *C. glutamicum* but not to *S. lividans*. While *S. lividans* secretes levansucrase into the medium, we could show that the enzyme is retained by *C. glutamicum* cells. Our results imply that the *sacB* gene can be used as a positive selection system in coryneform bacteria.

The *Bacillus subtilis* *sacB* gene encodes the enzyme levansucrase (EC 2.4.1.10), which is secreted by *B. subtilis* cells into the culture medium. Levansucrase is a transfructosylase catalyzing sucrose hydrolysis and levan synthesis. In addition, this enzyme is capable of adding fructosyl residues to a wide range of acceptor molecules (3). In *Escherichia coli* and other gram-negative bacteria such as *Rhizobium*, *Agrobacterium*, or *Cyanobacterium* species, expression of *sacB* is lethal in the presence of sucrose (2, 4, 15, 17). Therefore, in these gram-negative bacteria *sacB* has been widely used as a positive selection marker to entrap transposable elements by screening for *sacB* inactivation (4, 15). So far, the biochemical basis for toxicity of levansucrase action on sucrose has not readily been elucidated. In *E. coli*, it has been shown that levansucrase activity is located at least partly in the periplasm (17). On one hand, synthesis of levans might encumber the periplasm because levans are branched fructose polymers of high molecular weight. On the other hand, it is conceivable that the transfer of fructose units to unknown acceptor molecules has toxic effects on the cells (17).

Since gram-positive microorganisms lack an outer membrane, it was generally believed that the *sacB* system could not be applied successfully to a gram-positive bacterial strain. In this study, however, we show that the two gram-positive strains tested reveal striking differences in their phenotypes upon *sacB* expression. This is the first report showing that *sacB* expression is lethal to a gram-positive microorganism in the presence of sucrose.

We accomplished expression of the *sacB* gene in *Corynebacterium glutamicum* ATCC 13032 by using plasmid pWJ5 (Fig. 1), which is based on the mobilizable *E. coli*-*C. glutamicum* shuttle vector pECM2. pECM2 was obtained by deleting a 0.3-kb *SalI* restriction fragment from plasmid pECM1 which we described previously (14). pWJ5 carries the *sacB* gene downstream of the *npfII* gene of Tn5 (1), which lacks a transcriptional termination signal and is known to be efficiently expressed in corynebacteria (13). pWJ5 was transformed into the mobilizing strain *E. coli* S17-1 (16) and subsequently transferred to *C. glutamicum* by using RP4-mediated conjugal transfer as described previously (14).

Transconjugants were checked for their plasmid contents, and cells containing pWJ5 were tested for their growth behavior in the absence and presence of 10% sucrose in the medium. *C. glutamicum* harboring pWJ5 was not able to grow on media that contained sucrose, whereas cells containing pECM2 that lacks the *sacB* gene displayed normal growth in the presence of sucrose. Expression of *sacB* and the action of levansucrase on sucrose therefore turned out to be lethal to *C. glutamicum*.

We determined the activities of levansucrase in the cells and in the culture supernatant of *C. glutamicum*, carrying pWJ5 and pECM2, respectively, by using liquid cultures grown to exponential phase in sucrose-free medium. For this purpose the cells were harvested by centrifugation (15 min at 27,000 × *g*) at 4°C. The pellet (about 0.8 g [wet weight]) was washed with washing buffer (50 mM potassium phosphate, pH 6.0), concentrated in 2 ml of buffer, and disrupted in a French pressure cell (Aminco, Silver Spring, Md.) at a pressure of 1,070 lb/in². Finally, the cell debris was removed by centrifuging twice for 20 min at 30,000 × *g* and at 100,000 × *g*. The culture supernatant was collected and subjected to centrifugation (20 min at 100,000 × *g*) at 4°C. Conditions optimal for levansucrase were adjusted by dialysis for 4 h against washing buffer.

The protein concentrations in the cell extract were determined by using the Micro BCA-Kit (Pierce, Rockford, Ill.) according to the manufacturer's recommendations.

Levansucrase activity was determined by measuring the amount of glucose that is released by the action of levansucrase on sucrose (7) by using hexokinase and glucose 6-phosphate dehydrogenase. This enzymatic reaction leads to the reduction of NADP to NADPH, which can be quantified photometrically at a wavelength of 340 nm (6). As shown in Table 1, there was no detectable levansucrase activity in the supernatant of *C. glutamicum*(pWJ5) cultures. Levansucrase was completely associated with the cell fraction. By using *C. glutamicum*(pECM2) as a control we found negligible activity in the supernatant fraction, but activity was detected in the cell fraction, indicating that *C. glutamicum* possesses an endogenous sucrose-cleaving activity. However, this background activity was significantly raised upon introduction of pWJ5 and expression of *sacB*.

Although the majority of *C. glutamicum* cells expressing

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FIG. 1. Construction of plasmids pWJ1 and pWJ5 for expression of the *sacB* gene in *S. lividans* and *C. glutamicum*, respectively. Abbreviations: *oriV-E*, origin of vegetative replication in *E. coli*; *oriV-C*, origin of vegetative replication in *C. glutamicum*; *rep*, part of the plasmid which is necessary for vegetative replication in *S. lividans*; *oriT*, origin of transfer replication; *sacB*, gene encoding the enzyme levansucrase; *nptI*, neomycin phosphotransferase I; *nptII*, neomycin phosphotransferase II; *pNeo*, promoter of the *nptII* gene; *tsr*, thiostrepton resistance marker; *bla*, ampicillin resistance marker; *cat*, chloramphenicol acetyltransferase. Abbreviations for enzymes: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

TABLE 1. Levansucrase activities

Strain	Plasmid	Levansucrase activity ^a in:	
		Crude extract	Supernatant
<i>C. glutamicum</i> ATCC 13032	pWJ5 (<i>sacB</i>)	361 (±15)	0.0 (±1)
	pECM2	203 (±11)	1.4 (±2)
<i>S. lividans</i> 1326	pWJ1 (<i>sacB</i>)	7.9 (±2)	37.7 (±4)
	pGM9	1.6 (±1)	1.4 (±2)

^a Determined by sucrose hydrolysis. Methods are explained in the text. Activities in crude extract are shown as nanomoles of glucose per minute per milligram of protein; activities in supernatant are shown as nanomoles of glucose per minute per milliliter of supernatant. Values in parentheses are maximum deviations observed in three measurements.

sacB died on sucrose-containing agar, we found spontaneously resistant colonies arising after 2 to 3 days of incubation at 30°C. In these colonies we found insertions of around 1.5 kb that had occurred within the *sacB*-coding region of plasmid pWJ5 (data not shown). These insertions probably represent insertion sequence elements.

In parallel, *sacB* expression was also investigated by using *Streptomyces lividans* 1326 as a different gram-positive host. For this purpose we constructed plasmid pWJ1 by using pUM24 (12), pUC19 (10), and pGM9 (8) as shown in Fig. 1. Plasmid pWJ1 consists of the pSG5 replicon (9) for replication in *Streptomyces* strains, the thiostrepton resistance marker (18) suitable for selection, and the *sacB* gene cloned downstream of the promoter of the neomycin phosphotransferase gene (*nptII*) originating from Tn5 (1). *S. lividans* cells containing pWJ1 showed normal growth on sucrose-free medium, but in the presence of sucrose the colonies had a mucous phenotype. Normal growth could be restored by transferring the cells to sucrose-free medium. This slime formation phenotype was not observed with *S. lividans* carrying pGM9, which lacks the *sacB* gene as a control. As shown in Table 1, levansucrase activity is located mainly in the supernatant of the *S. lividans* culture. Only weak background activity was detected when testing *S. lividans* harboring pGM9.

The results obtained with *S. lividans* 1236 are therefore consistent with the data available for *B. subtilis* (17).

In this study, we have shown that expression of *sacB* in the presence of sucrose has different effects on the gram-positive bacterial species tested. *C. glutamicum* ATCC 13032 cannot tolerate *sacB* expression in the presence of sucrose. In this respect *C. glutamicum* behaves more like *E. coli* and other gram-negative bacteria. In *E. coli*, levansucrase is retained in the periplasm (17), where the generation of levans might interfere with essential transport processes across the membranes. We presume that in *C. glutamicum*, levansucrase is retained by some components of the corynebacterial cell wall. A very characteristic feature of the cell wall of coryneform bacteria from genera such as *Corynebacterium*, *Mycobacterium*, and *Nocardia* is the occurrence of mycolic acids (5) which are missing in *Streptomyces* and *Bacillus* species. These mycolic acids might constitute a hydrophobic barrier (5), and it is conceivable that levansucrase is retained by this lipid layer. In addition, an observation reported previously indicates the presence of an outer-membranelike structure in *C. glutamicum* consisting of lipidlike materials (11). We are therefore investigating other mycolic acid-containing coryneform strains with regard to their phenotypes on sucrose media upon *sacB* expression.

Another explanation for the lack of levansucrase secretion in *C. glutamicum* might be that the signal sequence of the enzyme is not or not exactly recognized by the *C. glutamicum* signal peptidase.

Since *sacB* expression in the presence of sucrose is lethal to *C. glutamicum*, the *sacB* gene should be a powerful tool to construct a positive selection system for insertional inactivation facilitating the isolation of transposable elements for this industrially important microorganism. Indeed, in our experiments, we found frequent *sacB* inactivation caused by insertion in *sacB* of DNA fragments with the size of typical insertion sequence elements. An investigation of these hypothetical insertion elements and a systematic screening for the occurrence of insertion sequence elements in coryneform strains by using the *sacB* system are in progress.

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REFERENCES

- Beck, E., G. Ludwig, E. A. Auerwald, and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19:327-336.
- Cai, Y., and C. P. Wolk. 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J. Bacteriol.* 172:3138-3145.
- Dedonder, R. 1966. Levansucrase from *Bacillus subtilis*. *Methods Enzymol.* 8:500-505.
- Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.* 164:918-921.
- Goodfellow, M., M. Mordarski, and S. T. Williams (ed.). 1984. The biology of the actinomycetes. Academic Press, London.
- Kunst, A., B. Draeger, and J. Ziegenhorn. 1984. UV-method with hexokinase and glucose-6-phosphate-dehydrogenase, p. 163-171. In H. C. Bergmeyer (ed.), *Methods of enzymatic analysis*, vol. 6: metabolites I—carbohydrates, third ed. Verlag Chemie, Deerfield Beach, Fla.
- Kunst, F., M. Pascal, J. Lepesant-Kejzarova, J.-A. Lepesant, A. Billaut, and R. Dedonder. 1974. Pleiotropic mutations affecting sporulation conditions and the synthesis of extracellular enzymes in *Bacillus subtilis* 168. *Biochimie* 56:1481-1489.
- Muth, G., B. Nußbaumer, W. Wohlleben, and A. Pühler. 1989. A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. *Mol. Gen. Genet.* 219:341-348.
- Muth, G., W. Wohlleben, and A. Pühler. 1988. The minimal replicon of *Streptomyces ghanaensis* plasmid pSG5 identified by subcloning and Tn5 mutagenesis. *Mol. Gen. Genet.* 211:424-429.
- Norrandner, J., T. Kempe, and J. Messing. 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. *Gene* 26:101-106.
- Richter, W., F. Hänel, and M. Hilliger. 1985. Freeze-fracture observations of *Corynebacterium glutamicum*: the occurrence of an outer membrane-like structure and the influence of temperature on the cytoplasmic membrane. *J. Basic Microbiol.* 25:527-536.
- Ried, J. L., and A. Collmer. 1985. An *nptII-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. *Gene* 57:239-246.

13. Santamaria, R., J. A. Gil, J. M. Mesas, and J. F. Martin. 1984. Characterization of an endogenous plasmid and development of a cloning vector and transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130**:2237–2246.
14. Schäfer, A., J. Kalinowski, R. Simon, A.-H. Seep-Feldhaus, and A. Pühler. 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. **172**:1663–1666.
15. Simon, R., B. Hötte, B. Klauke, and B. Kosier. 1991. Isolation and characterization of insertion sequence elements from gram-negative bacteria by using new broad-host-range, positive selection vectors. J. Bacteriol. **173**:1502–1508.
16. Simon, R., U. Prierer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1**:784–794.
17. Steinmetz, M., D. Le Coq, H. Ben Djemia, and P. Gay. 1983. Analyse genetique de *sacB*, gene de structure d'une enzyme secretee, la levansaccharase de *Bacillus subtilis* Marburg. Mol. Gen. Genet. **191**:138–144.
18. Thompson, C. J., J. M. Ward, and D. A. Hopwood. 1982. Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. **151**:668–677.