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NOTES

Expression of the *Bacillus subtilis sacB* Gene Confers Sucrose Sensitivity on Mycobacteria

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Expression in mycobacteria of the structural gene *sacB*, which encodes the *Bacillus subtilis* levansucrase, was investigated. *sacB* expression is lethal to *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG in the presence of 10% sucrose. *sacB* could thus be used as a counterselectable marker in mycobacteria.

The *Bacillus subtilis sacB* gene encodes the secreted enzyme levansucrase (sucrose: 2,6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10). The enzyme catalyzes hydrolysis of sucrose and synthesis of levans, which are high-molecular-weight fructose polymers (5). In the gram-negative bacteria *Escherichia coli*, *Erwinia chrysanthemi*, and *Legionella pneumophila*, expression of *sacB* in the presence of sucrose is lethal (4, 6, 16). In *E. coli*, levansucrase activity is mostly located in the periplasm (20). The molecular basis of the toxicity is still unclear, but the toxicity could be due to (i) an accumulation of levans which might encumber the periplasm because of their high molecular weight or (ii) a transfer of fructose residues to inappropriate acceptor molecules, which could thereafter have toxic effects on the bacterial cells (20).

Gram-positive bacteria lack an outer membrane, and therefore it was initially thought that sucrose selection did not apply to these species. However, it was recently shown that *sacB* expression in *Corynebacterium glutamicum* confers sucrose sensitivity to this gram-positive organism (9). It was suggested that the lethality was due to the particular organization of the corynebacterial cell wall, where the mycolic acids form an outer membrane-like structure. The molecular basis of the toxicity could therefore be the same as for gram-negative bacteria. Because of the phylogenetic relatedness between corynebacteria and mycobacteria (22), we investigated the possibility of using this counterselectable marker in mycobacteria.

To express *sacB* in mycobacteria, we constructed pPR2 (an *E. coli*-mycobacterium conjugative shuttle vector) by inserting the pAL5000 mycobacterial replicon of pB4 into the *Bam*HI site of pJQ200 (Fig. 1) (12, 13). pJQ200 is a versatile suicide vector carrying the *sacB* gene as a counterselectable marker and the *aacC1* gene from Tn*1696*, conferring resistance to gentamicin, as an antibiotic resistance marker (12). To our knowledge, gentamicin selection in mycobacteria has not been previously described. It was therefore necessary to verify whether it can be used in this genus. *Mycobacterium smegmatis* mc^2 155 (18) and *Mycobacterium bovis* BCG Pasteur were electroporated with pPR2 as previously described (7). On plates with gentamicin concentrations as low as 5 $\mu g \cdot ml^{-1}$, nontransformed *M. smegmatis* and *M. bovis* BCG yielded no colonies, whereas electroporation with pPR2 yielded gentamicin-resistant transformants at a frequency of $10^5/\mu g$ of plasmid DNA. This transformation efficiency was equivalent to that of a plasmid bearing a kanamycin resistance gene (18). The transformants were checked for their plasmid content by electroduction in *E. coli* (1). All gentamicin-resistant transformants were found to contain the plasmid. These results demonstrate that *M. smegmatis* and *M. bovis* BCG are fully sensitive to gentamicin and that *aacC1* expression in mycobacteria confers resistance to gentamicin, making it a useful selection marker.

Levansucrase activity can be demonstrated by detecting glucose release from sucrose hydrolysis, using the GOD-PAP colorimetric enzymatic test (Boehringer). It is a two-step reaction in which glucose oxidation (GOD) yields H_2O_2, which reacts with a chromogenic substrate (PAP). Clones producing levansucrase change the color of the suspension to red. We performed the test as described by Lepesant et al. (11) on liquid cultures, using the minimal medium MM63 with glycerol as the carbon source (20). One milliliter of culture was centrifuged, and the cell pellet was tested as described by the manufacturer. The result was first confirmed on *E. coli*. For the strain transformed with pPR2, there was an immediate change of the color of the suspension to red, whereas the suspension of the untransformed strain remained uncolored. *M. smegmatis* (pPR2) also generated a pink color after a few minutes, whereas the suspension of the untransformed strain remained uncolored, with only a faint coloration developing after longer incubations. These data show that levansucrase is produced and is active in *M. smegmatis*. The difference in intensity of color possibly reflects a difference of *sacB* expression between *E. coli* and *M. smegmatis* due to differences in plasmid copy number and promoter strength in these two organisms.

Mycobacterial cells harboring pPR2 were then tested for their growth on selective Luria-Bertani (or 7H10) medium in the presence or the absence of 10% sucrose. While untransformed mycobacteria grew normally on 10% sucrose, although the growth of *M. bovis* BCG was slowed down, transformants were not able to grow on sucrose-supplemented selective medium. *sacB* expression in the presence of sucrose is therefore lethal to mycobacteria. pPR2 was also introduced into *E. coli* by electroporation, and the selection efficiency on sucrose was compared with that of *M. smegmatis* and *M. bovis* BCG transformants (Table 1). Spontaneous sucrose-resistant colonies oc-

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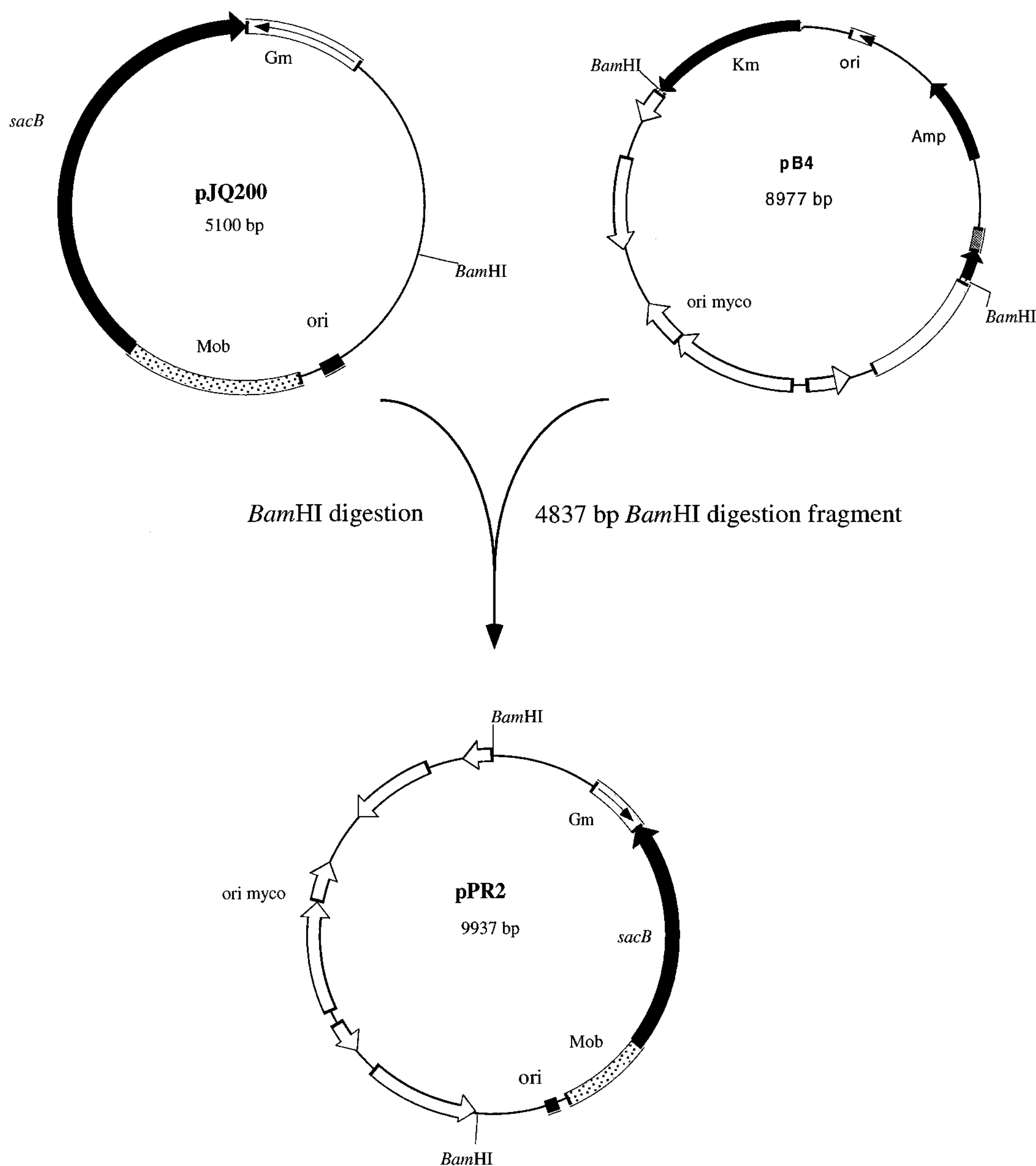

FIG. 1. Construction of plasmid pPR2 for *sacB* expression in mycobacteria. Abbreviations: Gm, *aacC1* gene coding for gentamicin resistance; ori, origin of replication in *E. coli*; Mob, RP4 origin of transfer; Km, Tn*903* gene encoding kanamycin resistance; Amp, gene encoding ampicillin resistance; ori myco, pAL5000 origin of replication functional in mycobacteria.

curred at a frequency of $\sim 5 \times 10^{-4}$ in *M. smegmatis*, compared with $\sim 10^{-5}$ in *E. coli*, which is in good agreement with previous reports (3). No sucrose-resistant colonies were detected for *M. bovis* BCG, even with a more concentrated inoculum: the efficiency of selection was higher than 10^{-6}. To our knowledge, this is the first counterselectable marker described for slowly growing mycobacteria.

This study demonstrates that the *B. subtilis sacB* gene can be expressed in mycobacteria from its own promoter despite the substantial phylogenetic distance between these species. Moreover, *sacB* expression leads to the death of transformed bacteria on 10% sucrose. Thus, mycobacteria behave like gram-negative bacteria (6). It was recently shown that *sacB* expression is also toxic to another gram-positive bacterium, *Corynebacterium glutamicum* (9), which is closely related to

TABLE 1. Effect of sucrose on growth of *M. smegmatis*, *M. bovis* BCG, and *E. coli* strains transformed with pPR2

Strain[a]	Selective medium[b]	
	Without sucrose	With 10% sucrose
mc^2 155	10^5	10^5
mc^2 155(pPR2)	10^5	~5
BCG	10^5	10^5
BCG(pPR2)	10^5	0
DH5α(pPR2)	10^5	~1

[a] Initial inoculum of 10^5 bacteria.

[b] Selective media were Luria-Bertani (or 7H10) medium for nontransformed bacteria and Luria-Bertani (or 7H10) medium with gentamicin for bacteria transformed with pPR2 (5 $\mu g \cdot ml^{-1}$ for mycobacteria and 20 $\mu g \cdot ml^{-1}$ for *E. coli*).

species of *Mycobacteria* and *Nocardia* (22). These genera present a particular cell wall organization with a layer of mycolic acids which may act as a hydrophobic barrier, analogous to the outer membrane in gram-negative bacteria (see reference 14 for a review). The space between the plasma membrane and the mycolic acid layer could thus be regarded as a "pseudo" periplasmic space (2). Therefore, the levans may be retained by the mycolic acid hydrophobic barrier, causing the toxicity of *sacB* in presence of sucrose (20). It seems likely by analogy that sucrose selection is also possible in *Nocardia* spp.

Mycobacteria comprise the causative agents of tuberculosis and leprosy. Genetic analysis of virulence determinants is hindered by a lack of efficient genetic tools (8). *sacB* could be used as a transposon trap, a method widely used (6) to isolate new transposable elements. Manipulations involving allelic exchange, though feasible, are relatively inefficient in mycobacteria (9, 15). Thus, the use of a sucrose counterselectable suicide vector should allow positive selection of double recombinants (21), and experiments involving allelic exchange events may be greatly facilitated (unpublished data). This approach has already been described for various gram-negative species, including *E. coli* and *Rhizobium meliloti* (2, 19). Moreover, it was recently shown that this strategy is very efficient in *M. smegmatis* when *rpsL* is used as a positive selection system for double recombinants (17). However, the *rpsL* system is limited because the recipient strain must be streptomycin resistant, and its use has not yet been described for slowly growing mycobacteria. *sacB* may thus be more useful for generating mutants by allelic exchange, because there is no prerequisite for an antibiotic-resistant strain. We expect that *sacB* will prove valuable as a counterselectable marker in experiments involving homologous recombination in mycobacteria.

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REFERENCES

1. **Baulard, A., C. Jourdan, A. Mercenier, and C. Locht.** 1992. Rapid mycobacterial plasmid analysis by electroduction between *Mycobacterium* spp. and *Escherichia coli*. Nucleic Acids Res. **20:**4105.
2. **Beveridge, T. J.** 1995. The periplasmic space and the periplasm in gram-positive and gram-negative bacteria. ASM News **61:**125–130.
3. **Blomfield, I. C., V. Vaughn, R. F. Rest, and B. I. Eisenstein.** 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature sensitive pSC101 replicon. Mol. Microbiol. **5:**1447–1457.
4. **Cianciotto, N. P., R. Long, B. I. Einsenstein, and N. C. Engleberg.** 1988. Site-specific mutagenesis in *Legionella pneumophila* by allelic exchange using counterselectable colE1 vectors. FEMS Microbiol. Lett. **56:**203–208.
5. **Dedonder, R.** 1966. Levansucrase from *Bacillus subtilis*. Methods Enzymol. **8:**500–505.
6. **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.
7. **Guilhot, C., B. Gicquel, and C. Martin.** 1992. Temperature-sensitive mutants of the *Mycobacterium* plasmid pAL5000. FEMS Microbiol. Lett. **98:**181–186.
8. **Jacobs, W. R.** 1992. Advances in mycobacterial genetics: new promises for old diseases. Immunobiology **184:**147–156.
9. **Jäger, W., A. Schäfer, A. Pühler, G. Labes, and W. Wohlleben.** 1992. Expression of the *Bacillus subtilis sacB* gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. J. Bacteriol. **174:**5462–5465.
10. **Kalpana, G. V., B. R. Bloom, and W. R. Jacobs, Jr.** 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA **88:**5433–5437.
11. **Lepesant, J. A., F. Kunst, J. Lepesant-Kejzlarovà, and R. Dedonder.** 1972. Chromosomal location of mutations affecting sucrose metabolism in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **118:**135–160.
12. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene **127:** 15–21.
13. **Ranes, M. G., J. Rauzier, M. Lagranderie, M. Gheorghiu, and B. Gicquel.** 1990. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: construction of a "mini" mycobacterium-*Escherichia coli* shuttle vector. J. Bacteriol. **172:**2793–2797.
14. **Rastogi, N.** 1991. Structure and functions of the cell envelope in relation to mycobacterial virulence, pathogenicity and multiple drug resistance. 7th Forum in Microbiology. Res. Microbiol. **142:**419–481.
15. **Reyrat, J. M., F. X. Berthet, and B. Gicquel.** 1995. The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in *Mycobacterium bovis* bacillus Calmette-Guérin. Proc. Natl. Acad. Sci. USA **92:**8768–8772.
16. **Ried, J. L., and A. Collmer.** 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-eviction mutagenesis. Gene **57:**239–246.
17. **Sander, P., A. Meier, and E. C. Böttger.** 1995. *rpsL*+: a dominant selectable marker for gene replacement in mycobacteria. Mol. Microbiol. **16:**991–1000.
18. **Snapper, S. B., R. E. Melton, S. Mustapha, T. Kieser, and W. R. Jacobs.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
19. **Soupène, E., M. Foussard, P. Boistard, G. Truchet, and J. Batut.** 1995. Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. USA **92:**3759–3763.
20. **Steinmetz, M., D. Le Coq, H. B. Djemia, and P. Gay.** 1983. Analyse génétique de *sacB*, gène de structure d'une enzyme secrétée, la lévane-saccharase de *Bacillus subtilis* Marburg. Mol. Gen. Genet. **191:**138–144.
21. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
22. **Wayne, L. G., and G. P. Kubica.** 1986. Mycobacteria, p. 1435–1457. *In* P. A. Sneath (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore.