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Cloning Structural Gene *sacB*, Which Codes for Exoenzyme Levansucrase of *Bacillus subtilis*: Expression of the Gene in *Escherichia coli*

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A clone bearing the structural gene *sacB*, coding for the exoenzyme levansucrase, was isolated from a library of *Bacillus subtilis* DNA that was cloned in phage λ charon 4A on the basis of the transforming activity of the chimeric DNA. This λ clone also was found to contain the *sacR* and *smo* loci. Subcloning the *sacB-sacR* region in plasmid pBR325 resulted in a clone which directed levansucrase synthesis in *Escherichia coli*. The nucleotide sequence coding for the secreted protein was localized on the physical map of the cloned DNA.

Levansucrase (sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferase; E.C. 2.4.1.10) is an enzyme secreted in culture medium by *Bacillus subtilis* after induction by sucrose. The enzyme catalyzes mainly the following transfructosylation reaction: sucrose + acceptor → glucose + acceptor-fructose.

Since the enzyme was purified (17), it has been extensively investigated. Physical properties of the protein have been described by Gonzy-Treboul et al. (20) and Berthou et al. (2). The enzyme consists of a single polypeptide chain of 50,000 daltons. The sequence of the 444 amino acids of the secreted enzyme was recently elucidated by A. Delfour (unpublished data). A model of the three-dimensional structure at 3.8-Å resolution was proposed by Le Brun and Van Rappenbusch (27). Detailed enzymological studies by Chambert and Gonzy-Treboul showed that the enzyme obeys a ping-pong mechanism which involves a covalent fructosyl-enzyme intermediate which has been isolated (10–12).

The genetic analysis of sucrose metabolism in *B. subtilis* by Lepesant and co-workers led to the identification of several loci related to levansucrase synthesis (29). The stuctural gene (*sacB*) coding for the enzyme was defined by mutations that affected either the thermostability of levansucrase or its ability to synthesize levans (31). A regulatory locus (*sacR*) was defined by mutations resulting in constitutive synthesis of the enzyme. The two loci, *sacB* and *sacR*, mapped close to one another and were localized between the *hisA* and *smo* markers (29).

We took advantage of the *sacB* mutants isolated by Lepesant et al. (29, 31) to screen for the presence of wild-type *sacB* alleles in the library of *B. subtilis* DNA cloned in λ charon 4A (described in a previous paper [19]). A clone bearing the *sacB*, *sacR*, and *smo* genes was isolated. This communication describes the isolation of this clone, the reconstitution of the *sacB-sacR* locus in a plasmid, and the identification of levansucrase synthesized by the *E. coli* host of the plasmid.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The strains and vectors used are listed in Table 1. Plasmid pH4 is a derivative of pHV33 (37) obtained by partial *Hha*I digestion. It has lost the pC194 functions and the *Eco*RI site of the pBR322 moiety of pHV33. The nomenclature concerning the various phenotypes related to levansucrase synthesis was taken from Lepesant et al. (29). Lvs^+/Lvs^- stands for the ability/inability to synthesize levansucrase as characterized by its saccharolytic activity. Lvs^c/Lvs^i stands for the constitutive/inducible synthesis of the enzyme. Lev^+/Lev^- stands for the ability/inability of levansucrase to synthesize levans on appropriate solid medium. For example, strain QB2060 (*sacR2 sacB204 cysB3*) constitutively synthesizes a levansucrase, the activity of which can hydrolyze sucrose, but it is poorly able to polymerize fructose (31). Its phenotype is Lvs^+ Lvs^c Lev^- Cys^-. The gene responsible for the smooth/rough phenotype was cloned in our experiments. In most laboratories, the reference strain 168M carries a *smo* allele, the origin of which is not clearly identified. It is noteworthy that the wild-type phenotype is rough (21).

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TABLE 1. List of the strains and cloning vectors used

Bacterial strain or cloning vector	Genotype	Relevant phenotype	Origin
B. subtilis			
168M	*trpC2 smo*	Trp^- Smo	
QB1058	*sacA321 sacB197 sacS32 trpC2 smo*	Trp^- Lev^- Smo	IRBM[a]
PG790	*sacA321 sacB197 sacS32 smo*	Lev^- Smo	IRBM
QB2060	*sacA321 sacB204 sacR2 cysB3*	Lvs^+ Lvs^c Lev^- Rou Cys^-	IRBM
PG795	*sacA321 sacB204 sacR2*	Lvs^+ Lvs^c Lev^- Rou	IRBM
E. coli cloning hosts			
DP50 *supF*	F^- *dapD8 lacY* Δ(*gal-uvrB*) Δ*thyA nalA hdsS supII supIII*		F. R. Blattner
HVC45	*thrA1 leu-6 thi-1 lacY1 supE44 hsdR rpsL tonA1*	Amp^s Cm^s Tet^s	S. D. Ehrlich
Cloning vectors			
λ charon 4A			F. R. Blattner (4)
λ charon 4A derivatives			
λ LSC66			This paper
λ *gutF38*			This paper
pBR325		Amp^r Cm^r Tet^r	J. Doly (see reference 6)
pBR325 derivatives			
pLS1, pLS2, pLS3, pLS4		Amp^r Cm^s Tet^r	This paper
pLS8, pLS10		Amp^r Cm^s Tet^s	This paper
pH4		Amp^r Tet^r	I. Jones
pH4 derivatives			
pLS5, pLS8		Amp^r Tet^s	This paper

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Media. The multiplication of λ charon phage was supported by *Escherichia coli* strain DP50 (*supF*) grown in a medium containing: tryptone (Difco Laboratories), 20 g/liter; yeast extract (Difco), 10 g/liter; NaCl, 10 g/liter; $MgCl_2$, 10 mM; diaminopimelic acid (Sigma Chemical Co.), 100 mg/liter; thymidine (Sigma), 50 mg/liter. LB medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; water, 1,000 ml) was used as a solid or liquid medium to support the growth of other *E. coli* strains.

B. subtilis was grown on tryptose blood agar base (Difco) plates or in MM (1) mineral medium. SG stands for MM mineral medium supplemented by sucrose (20 g/liter) and glucose (1 g/liter).

DNA isolation and purification. (i) Bacteriophage λ DNA. Phage lysates were concentrated by precipitation (PEG 6000 [J. T. Baker Chemical Co.], 100 g/liter; NaCl, 0.5 M). Phages were then purified by CsCl gradient centrifugation. DNA was extracted twice with phenol, and residual phenol was extracted with ether. After ethanol precipitation, the DNA was solubilized in TE (Tris-hydrochloride, 10 mM [pH 7.5]; EDTA, 1 mM).

(ii) Plasmid DNA. For large-scale isolation, strains carrying plasmids were grown according to the method of Bolivar et al. (7), and plasmid amplification was obtained by the addition of either chloramphenicol (170 mg/liter) or spectinomycin (300 mg/liter) (6). DNA was extracted according to the method of Humphreys et al. (24). Plasmid DNA was purified by CsCl banding or hydroxyapatite chromatography according to the method of Colman et al. (14). For small-scale isolation, we used the rapid alkaline procedure of Birnboim and Doly (3).

B. subtilis transforming DNA was extracted according to the method of Marmur (32).

DNA analysis and cloning. Enzymes *Bam*HI, *Eco*RI, *Hin*dIII, and bacteriophage T4 DNA ligase were prepared at the Research Institute of Scripps Clinic by Alain Meier. Enzymes *Kpn*I and *Pvu*II were from Bethesda Research Laboratories, *Cla*I was from New England Biolabs, and *Hpa*II was from Boehringer Mannheim Corp. They were used according to the directions of the manufacturers.

Slab gel electrophoresis (Sigma agarose, catalog no. A 6877) was run in Tris-acetate buffer (40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA, adjusted to pH 7.9 with acetic acid). Charon 4A DNA, doubly digested with *Eco*RI and *Bam*HI, or pBR322 DNA, digested with *Hpa*II, was used as the molecular weight standard.

Genetic procedures. *E. coli* strains were transformed according to the method of Dagert and Erhlich (15). Cm^r, Tet^r, and Amp^r colonies were selected on LB plates supplemented with the related antibiotic(s): chloramphenicol, 15 mg/liter; tetracycline, 15 mg/liter; and ampicillin, 200 mg/liter, respectively.

B. subtilis strains were transformed according to the method of Anagnostopoulos and Spizizen (1), including the use of the MG I and MG II media of Borenstein and Ephrati-Elizur (8). Prototrophic transformants were selected on MM plates lacking either tryptophan or cysteine. The Smo/Rou phenotype was scored on TBAB plates by replica plating of patched colonies.

Levansucrase-related phenotypes were scored according to the method of Lepesant et al. (29). The Lev$^+$ phenotype was identified by the synthesis of levan, which surrounded the colonies on SG plates. The Lvs^c/Lvs^i phenotype was identified on colonies patched on MM plates supplemented with glycerol instead of glucose. After 24 h of growth at 37°C, the plates were sprayed with a mixture of chloramphenicol (2 mg/ml) and sucrose (500 mg/ml), incubated at 37°C for an additional 90 min, and then sprayed with GOD Perid reagent (Boehringer Mannheim). The constitutive phenotype results in a green halo surrounding the colonies.

Bacterial extracts and levansucrase assays. *E. coli* cells treated with EDTA (1 mM in Tris-hydrochloride, 100 mM, pH 7.5) were sedimented and suspended in 50 mM potassium phosphate buffer, pH 6.0. Lysozyme (1 mg/ml) was added, and the cells were incubated at 0°C for 30 min. Lysis was achieved by freezing and thawing. Extracts were treated with DNase (50 μg/ml) to avoid excess viscosity.

Levansucrase was estimated according to the method of Chambert et al. (12). Uniformly labeled [^{14}C]sucrose and [^{14}C]glucose were obtained from Amersham Corp. They were purified by paper chromatography before use. Transfructosylation on glucose (exchange reaction) was assayed in the following mixture: [^{14}C]glucose, 200 mM (0.1 μCi/μmol); sucrose, 100 mM; potassium phosphate buffer, 50 mM, pH 6.0. Transfructosylation on levan was characterized in the following mixture: [^{14}C]sucrose, 240 mM; purified levan (molecular weight, 10,000), 10 mg/ml; potassium phosphate buffer, 50 mM, pH 6.0. In the absence of acceptor levan, incubation of levansucrase in this mixture resulted mainly in sucrose hydrolysis.

In all cases, portions of the reaction mixtures were subjected to paper chromatography (washed paper, Schleicher & Schuell catalog no. 2043a; solvent, *n*-butanol-acetic acid-water, 4:1:1 [vol/vol/vol). The radioactive spots were localized by autoradiography. The radioactivity of each spot was estimated directly from the paper immersed in scintillation fluid.

RESULTS

Isolation of a clone carrying the *sacB* gene from a λ charon 4A library. The construction of a library of *B. subtilis* DNA originating from strain 168M (*trpC smo*) cloned in the *Eco*RI sites of λ charon 4A was described previously. When this library was tested for transforming activity on competent *B. subtilis*, 70% of the markers tested were found in a sample of 1,710 plaques (19). The same sample was tested for the presence of clones that carried the structural gene coding for the exoenzyme levansucrase. Transformation of *sacB* mutants to wild-type does not allow a direct selection of recombinants. However, wild-type Lev$^+$ colonies can easily be distinguished from the Lev$^-$ background on SG medium since they are surrounded by levan. To identify Lev$^+$ colonies in the transformed population, transformants were selected by congression with an unlinked auxotrophic marker.

A competent culture of strain QB1058 (*sacB197 trpC2 sacS2*) was exposed to a subsaturating amount of DNA from strain PG790 (*sacB197 sacS2*) and plated on SG medium to get less than 5,000 Trp$^+$ colonies per plate (the appropriate concentration of PG790 DNA was previously determined by a saturation curve). DNA originating from each of 19 subpools of about 90 plaques of λ recombinant phages was spotted on the lawn of competent cells. Among the Trp$^+$ colonies thus selected, Lev$^+$ colonies were found in two spots, indicating that some recombinant clones carrying *sacB* alleles were present in two subpools. Crude lysates were prepared from each plaque of one subpool and were used further as DNA sources in a transformation experiment as described above. This allowed the identification and isolation of the λ LSC66 plaque, the DNA of which transforms the *sacB197* allele. The clone was purified twice and then was grown in large amounts for further studies.

Genetic mapping of λ LSC66 insert. Previous work by Lepesant et al. (29) showed that the *sacB* gene mapped close to the regulatory locus *sacR*, not far from the *smo* marker (position 305 in the map compiled by Henner and Hoch [23]). *hisA*, *sacB*, *sacR*, *smo*, and *cysB* were further found by PBS1 transduction to be linked in this order (M. Steinmetz, unpublished data). The presence of *sacB*, *sacR*, and *smo* markers in the insert of λ LSC66 was tested with strain QB2060 (*sacA321 sacB204 sacR2 cysB3*) as the recipient and a mixture of DNA originating from strain PG795 (*sacA321 sacB204 sacR2*) and λ LSC66 as the donor DNA. The patched Cys$^+$ recombinants were checked for unselected markers on appropriate media. Table 2 shows that the alleles *sacB204*, *sacR2*, and *smo* were transformed. Moreover, the recombination frequencies between the markers were consistent with the order found in transduction experiments.

Subcloning the *B. subtilis* DNA insert of λ LSC66. A preliminary physical map of the *B. subtilis* DNA insert of λ LSC66 was established with regard to *Eco*RI and *Hin*dIII sites (Fig. 1). It showed that five *Eco*RI sites delimited four pieces of 2.1, 3.4, 6.0, and 5.1 kilobases (kb), respectively, in this order. Isolation of these fragments from agarose gels allowed us to localize the *sacB* transforming activity on the 2.1-kb piece, localized at the left end of the insert (data not shown).

An *Eco*RI digest of λ LSC66 DNA was ligated in the *Eco*RI site of plasmid pBR325 (6), and the ligation mixture was used to transform *E. coli* strain HVC45. Among the Tet^r, Amp^r, Cm^s colonies, recombinant plasmids carrying each *Eco*RI piece were found (Fig. 1). The plasmid DNAs were used to transform the QB2060 strain as described above. The results (Table 2)

TABLE 2. Genetic characterization of the markers cloned in λ LSC66 and the derived subclones[a]

Cross	Relevant donor DNA[b]	No. in Cys⁺ recombinant classes[c]								Alleles detected
		Lev⁺				Lev⁻				
		Lvs[i]		Lvs[c]		Lvs[i]		Lsv[c]		
		Smo	Rou	Smo	Rou	Smo	Rou	Smo	Rou[d]	
1	λ LSC66	27	17	2	3	8	5	37	105	*sacB sacR smo*
2	λ LSC66	47	40	6	5	NS[e]	NS	NS	NS	*sacB sacR smo*
3	pLS1	0	0	0	16	0	0	0	192	*sacB*
4	pLS2	0	0	0	0	0	22	0	186	*sacR*
5	pLS4	0	0	0	0	0	0	35	173	*smo*
6	pLS5	0	39	0	13	0	40	0	408	*sacB sacR*

[a] Recipient strain, QB2060: *sacA321 sacB204 sacR2 cysB3* Lsv⁺ Lsv[c] Lev⁻ Rou Cys⁻.
[b] The DNA cloned in λ LSC66 and the derived subclones was from strain 168M (*trpC2 smo*).
[c] The congression of the *cysB* marker was utilized only to select the competent cells (see text).
[d] Phenotype of the recipient strain; no recombination detected in the locus investigated.
[e] NS, Not scored.

showed that transforming activities for *sacB*, *sacR*, and *smo* were present, respectively, in pLS1, pLS2, and pLS4, thus giving an unambiguous map of this region of the *B. subtilis* chromosome.

Expression of the *sacB* gene in λ LSC66 and reconstitution of the *sacB sacR* loci in a plasmid. We looked for a potential expression of *sacB* in *E. coli* cells infected by λ LSC66. The presence of the highly specific exchange activity of levansucrase (fructosyl transfer from sucrose to glucose) was tested in either crude lysates or in *E. coli* cells harvested in the stationary phase that precedes lysis. Both crude lysates and infected cells were found to contain approximately 0.015 U of enzyme per ml of culture, whereas no detectable activity (less than 0.001 U) was found under the same conditions in cells infected by another clone, λ *gut*F38 (a clone which contains part of the *gut* operon; P. Gay, unpublished data). This result suggested that λ LSC66 contained a functional *sacB* gene.

No levansucrase activity was found in *E. coli* cells carrying plasmid pLS1, suggesting that the 2.1-kb *Eco*RI fragment contained in pLS1 was insufficient to direct levansucrase synthesis. To obtain a plasmid with an insert capable of coding for the synthesis of levansucrase, *Hin*dIII fragments of λ LSC66 were subcloned. DNA of λ LSC66 was digested by *Hin*dIII and ligated in the *Hin*dIII site of plasmid pH4. The selection of Amp[r] Tet[s] transformants in *E. coli* allowed the isolation of a clone carrying plasmid pLS5, the insert of which overlaps both the right end of pLS1 and the left end of pLS2 inserts (Fig. 1). Table 2 shows that DNA of pLS5 transformed both *sacB204* and *sacR2* alleles. All the alleles tested further, *sacB91*, *sacB182*, *sacB194*, *sacB236*, *sacR37*, *sacR41*, and *sacR44* (29), were also transformed by pLS5 DNA (data not shown). However pLS5 did not direct the synthesis of levansucrase activity in *E. coli* hosts. We assumed that pLS5 was lacking one end of the *sacB* gene. Thus, we attempted to construct a plasmid sharing inserts of both pLS1 and pLS5. To generate this plasmid, λ LSC66 DNA

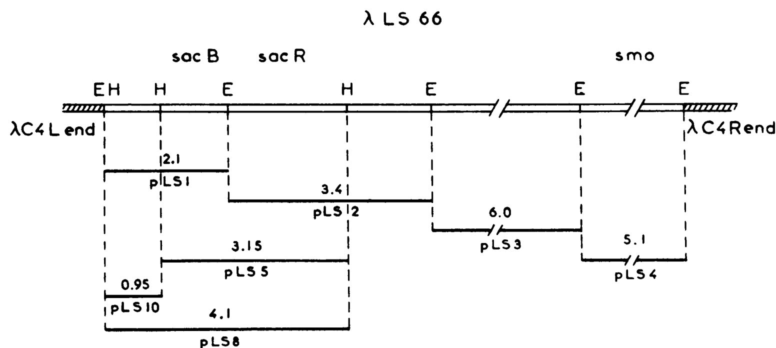

FIG. 1. Organization of the *B. subtilis* DNA inserts of λ LSC66 and of the derived subclones. E and H stand, respectively, for *Eco*RI and *Hin*dIII restriction sites. Five additional *Hin*dIII sites present in pLS4 were not mapped.

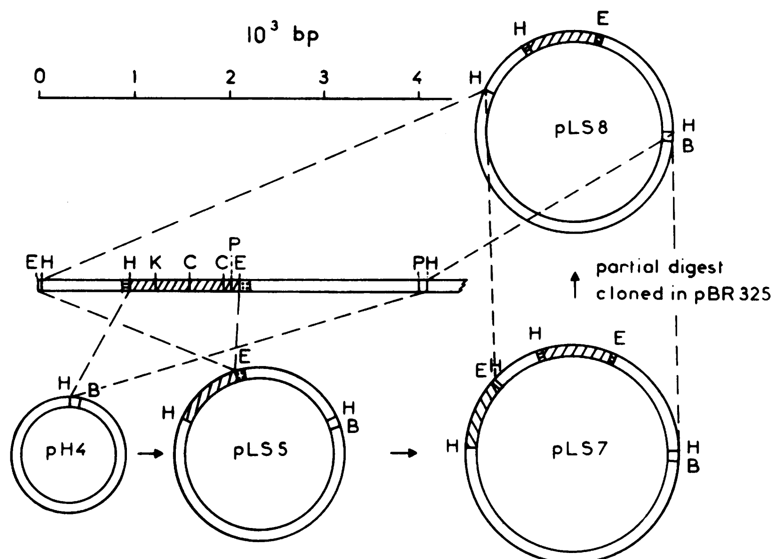

FIG. 2. Physical map of the *sacB-sacR* region and schematic diagram of the reconstitution of a functional *sacB* gene in pLS7 and pLS8 plasmids. The 1,332 base pairs (bp) encoding the known amino acid sequence of the secreted levansucrase are indicated as follows: dotted area, origin of the gene; oblique hatching, middle part of gene; horizontal hatching, terminus of the gene. C, E, H, K, and P stand, respectively, for *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, and *Pvu*II restriction sites.

digested by *Eco*RI was ligated in the unique *Eco*RI site present in the insert of pLS5 (Fig. 2). The recombinant plasmid pLS7 was identified by systematic restriction analysis of the DNA of Amp^r transformants. The 5.5-kb insert of this plasmid contains 4.1 kb of the left end of λ LSC66, part of this material being duplicated.

Levansucrase was found to be synthesized by pLS7 hosts. However, we observed that Lvs$^+$ colonies of *E. coli* segregated Lvs$^-$ colonies at a relatively high rate. These Lvs$^-$ colonies were found to carry a plasmid similar to pLS5, probably originating from internal recombination between the duplicated segments (data not shown).

The availability of both precise physical mapping of the *sacB-sacR* region and the sequence of the protein allowed a precise location of the *sacB* gene. A. Delfour (personal communication) elucidated the complete sequence of the 444 amino acids of the secreted levansucrase of *B. subtilis*. The localization of 11 restriction sites on the physical map of the *sacB* region (6 of them indicated in Fig. 2) was found to be perfectly consistent with the amino acid sequence of the protein. This comparison provided an unambiguous positioning of the nucleotide sequence coding for the secreted form of the enzyme. This sequence indicated that pLS1 and pLS5 plasmids were lacking, respectively, the origin and the terminus of the gene, which was consistent with the absence of expression of *sacB* in their hosts.

It appeared that a convenient way to clone a shorter insert that would allow *sacB* expression was to start from a partial *Hin*dIII digestion of either λ LSC66 or pLS7. Such a partial digestion of pLS7 DNA was cloned in the *Hin*dIII site of pBR325. The experiment allowed the isolation of plasmid pLS8 in which the *tet* gene of pBR325 was inactivated (Fig. 2).

Identification of levansucrase in *E. coli* hosts of pLS8. A crude extract of strain HVC45 carrying pLS8 was obtained as described above. The fructosyl transferase activities of levansucrase synthesized by this strain were characterized by three different reactions (Fig. 3): (i) the exchange reaction (fructosyl transfer from sucrose to radioactive glucose); (ii) the hydrolysis of sucrose; and (iii) the fructosyl transfer to acceptor levan. Since neither exchange reaction nor levan synthesis could be shown in *E. coli* strains carrying vectors that did not contain *sacB* alleles, these experiments identify the expression of the *sacB* gene. The specific activity of the enzyme was found to be constant during exponential growth, 0.8 U per mg of bacterial protein.

DISCUSSION

The use of a library of *B. subtilis* DNA cloned in λ charon 4A (4) allowed us to isolate a DNA fragment which included the structural gene *sacB* coding for the exoenzyme levansucrase and the regulatory locus *sacR*. It is worthy to

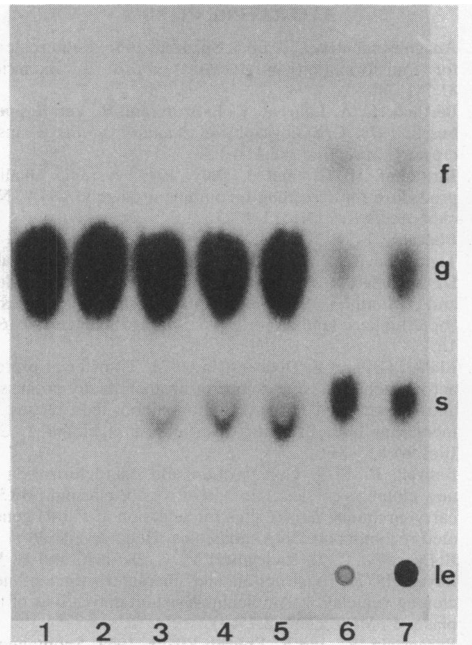

FIG. 3. Characterization of levansucrase synthesized by the *E. coli* strain HVC45 carrying the plasmid pLS8. Bacterial extracts were prepared and incubated in the relevant reaction mixtures (total volume, 0.1 ml) as described in the text. The abbreviations le, s, g, and f stand, respectively, for levan, sucrose, glucose, and fructose. Tracks 1 to 5, assays of the exchange reaction with extracts originating from: lane 1, strain HVC45; lane 2 strain HVC45(pBR325); lanes 3 to 5, strain HVC45(pLS8). To determine the kinetics of the reaction, samples were taken after 20, 40, and 60 min of incubation. Tracks 6 and 7, assays of sucrose hydrolysis and levan synthesis catalyzed by the same extract of strain HVC45(pLS8). A comparison of the two tracks shows that the accumulation of radioactivity in the levan spot is correlated with the low radioactivity of the fructose spot.

note the ease with which cloning in λ charon phages allows a genetic screening of clones inserting *B. subtilis* DNA, since the high transforming activity of either the purified DNA of λ chimera or the DNA present in crude lysates can be used to transform relevant alleles even when this transformation does not result in a selective phenotype.

The presence of the *sacB* gene in the insert cloned in λ LSC66 and cloned further in plasmid pLS8 was identified on the basis of three criteria: (i) the transforming activity of the cloned DNA tested on various mutants; (ii) the correlation between the physical map of the DNA and the amino acid sequence of levansucrase; and (iii) the expression of the *sacB* gene in *E. coli*.

All the *sacB* and *sacR* alleles tested were found to be transformed by λ LSC66 DNA. The subcloning experiments allowed us to localize in the pLS1 insert the wild-type allele of the *sacB204* mutation, which is responsible for the synthesis of modified enzyme (31), whereas the wild-type alleles of *sacR* mutations were found in the pLS2 insert. Furthermore, the DNA of plasmid pLS5 was found to transform all the *sacB* and *sacR* alleles tested. The localization of *sacR* was not as precise as *sacB* since, up until now, few restriction sites convenient for cloning were found in this area. This localization does not bring much more information concerning *sacR* function than is already available. It is consistent with an operator role, as suggested by Lepesant et al. (30), but does not exclude another function. It is known that transformation by DNA of plasmids that do not replicate in *B. subtilis* involves mostly integration of the complete plasmid sequence in the chromosome (18, 22). Extensive studies of such integrations of LS plasmids bearing a resistance marker which is expressed in *B. subtilis* are in progress. They should provide more information concerning the *sacR* region and its function.

On the basis of (i) the genetic localization, (ii) the expression of *sacB* in pLS8 and the lack of expression of the gene in pLS1 and pLS5, and (iii) the known size of the protein (2), the position of the *sacB* gene could be determined within a 1,600-base pair segment, which included the *Hin*dIII-*Eco*RI segment shared by pLS1 and pLS5 (see Fig. 1). On the basis of the amino acid sequence of the secreted enzyme, the position of the gene was exactly determined on the physical map of the cloned DNA. The occurrence of a signal peptide (5, 16) involved in the secretion of levansucrase is still questioned. Evidence for such amino-terminus extensions in proteins secreted by *Bacillus* sp. was obtained by Neugebauer et al. (33) and Palva et al. (34), who elucidated the nucleotide sequences of the genes coding, respectively, for the penicillinase of *Bacillus licheniformis* and the α-amylase of *Bacillus amyloliquefaciens*. Although some observations by Petit-Glatron and Chambert (35) suggest that the secretion of levansucrase may involve a membrane-bound form of the enzyme, the very small amount of material detected did not allow structural analysis. Progress in sequencing the *sacB* gene will provide more precise data about this problem.

Levansucrase synthesis was characterized in the *E. coli* hosts of either λ LSC66 or pLS7 and pLS8. The specific activity of the enzyme in the crude extract of HVC45(pLS8) strain was approximately 0.8 U per mg of protein. This corresponds to a rate of synthesis of approximately 10^{-11} U of enzyme per cell per min. This rate obviously depends upon the number of *sacB*

gene copies in the cells. pLS8 was derived from pBR325, and it has a ColE1 mode of replication. Clewell and Helinski (13) found that plasmid ColE1 was present in *E. coli* to the extent of approximately 24 copies per cell. From the yield of pLS8 DNA extractions without spectinomycin amplification, we may roughly estimate for this plasmid a number of copies of the same order of magnitude. In *B. subtilis*, the levansucrase rate of synthesis measured in mineral medium varies from 10^{-12} U per cell per min in the reference strain 168M to 1.5×10^{-10} U in the high producer QB112, which differs from 168M by the presence of the *sacU32* mutation unlinked to *sacB* (26, 36). Therefore, the rate of synthesis of levansucrase in *E. coli* related to the gene copy number may be compared to the rate measured in the 168M strain of *B. subtilis*.

It is not unlikely that levansucrase synthesis in *E. coli* depends upon the *sacB* promotor itself. One may assume that the 2,000 base pairs that precede the origin of the gene in pLS8 includes the promotor. The orientation of *sacB* in pLS8, opposite to the *tet* gene orientation, is consistent with this assumption. In the case of the expression of the cloned *penP* gene of *B. licheniformis*, Brammar et al. (9) and Imanaka et al. (25) discussed the occurrence of *Bacillus* promotor recognition by *E. coli* RNA polymerase and concluded that the transcription of *penP* was promoted by its own promotor. Moreover, it is known that some *Bacillus* promotors have significant homologies with *E. coli* promotors (28).

Preliminary experiments did not allow us to conclude clearly whether *E. coli* secretes levansucrase in the periplasmic space. Osmotic shocks carried out with various *E. coli* hosts of plasmid pLS8 showed that levansucrase was partially liberated in the shock fluid. However, in some cases we found that some β-galactosidase, supposedly a strictly intracellular enzyme and thus chosen as a control, was also present in the shock fluid. A similar phenomenon was observed in some hosts of a cloned α-amylase gene (K. Willemot and M. Shwartz, personal communication). This suggests that the synthesis of relatively large amounts of a foreign secreted protein results in fragility of the *E. coli* envelopes. How such fragility may be related to the secretion process is to be investigated.

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Biohazards associated with the experiments described in this publication have been examined previously by the French National Committee.

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