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Cloning, Sequencing, and Disruption of a Levanase Gene of *Bacillus polymyxa* CF43

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The *Bacillus polymyxa* CF43 *lelA* gene, expressing both sucrose and fructan hydrolase activities, was isolated from a genomic library of *B. polymyxa* screened in *Bacillus subtilis*. The gene was detected as expressing sucrose hydrolase activity; *B. subtilis* transformants did not secrete the *lelA* gene product (LelA) into the extracellular medium. A 1.7-kb DNA fragment sufficient for *lelA* expression in *Escherichia coli* was sequenced. It contains a 548-codon open reading frame. The deduced amino acid sequence shows 54% identity with mature *B. subtilis* levanase and is similar to other fructanases and sucrases (β-D-fructosyltransferases). Multiple-sequence alignment of 14 of these proteins revealed several previously unreported features. LelA appears to be a 512-amino-acid polypeptide containing no canonical signal peptide. The hydrolytic activities of LelA on sucrose, levan, and inulin were compared with those of *B. subtilis* levanase and sucrase, confirming that LelA is indeed a fructanase. The *lelA* gene in the chromosome of *B. polymyxa* was disrupted with a chloramphenicol resistance gene (*cat*) by "inter-gramic" conjugation: the *lelA*::*cat* insertion on a mobilizable plasmid was transferred from an *E. coli* transformant to *B. polymyxa* CF43, and *B. polymyxa* transconjugants containing the *lelA*::*cat* construct replacing the wild-type *lelA* gene in their chromosomes were selected directly. The growth of the mutant strain on levan, inulin, and sucrose was not affected.

Exopolysaccharides appear to play a role in the early steps of plant colonization by symbiotic or phytopathogenic bacteria such as *Rhizobium* spp. or *Agrobacterium* spp., respectively (23). Free-living rhizobacteria belonging to the genus *Bacillus* have been isolated from the rhizosphere of *Graminae* spp., including wheat and maize (7, 24, 26, 32). In the wheat rhizosphere, *Bacillus polymyxa* frequently constitutes the dominant population among the free-living nitrogen-fixing bacteria (29). *B. polymyxa* CF43 was isolated from a wheat rhizosphere (15). Inoculation by *B. polymyxa* CF43 has been shown to stimulate the yield of spring wheat (6). This and other observations (16, 17) have confirmed the plant growth-promoting rhizobacterial character of *B. polymyxa.*

The presence of *B. polymyxa* in the wheat rhizosphere correlates with the presence of polysaccharide mucus on the root surface (14). Sucrose has been reported to be exuded by wheat roots (13). Like numerous species of the *Bacillus* genus, *B. polymyxa* is able to utilize sucrose, both as a source of carbon and energy and to synthesize levan, a fructosyl polymer composed of residues linked 2-6 with branches linked 2-1; *B. polymyxa* can also degrade two fructans, levan and inulin, a fructosyl polymer composed of 2-1-linked residues (27). The *Bacillus subtilis* enzymes (β-D-fructosyltransferases) catalyzing these reactions and the corresponding genes have been extensively studied. Three β-D-fructosyltransferases are synthesized by *B. subtilis* (37): an intracellular sucrose-6-phosphate hydrolase, an extracellular levansucrase, and an extracellular levanase. In addition to their sucrose hydrolase activities, levansucrase synthesizes levan and levanase hydrolyzes both levan and inulin.

We investigated the role of fructans in wheat rhizosphere colonization by *B. polymyxa*. To address this issue, we cloned *B. polymyxa* CF43 genes involved in the metabolism of fructan polymers such as levan and inulin. The cloning of the levansucrase gene will be reported elsewhere (7a). Here we report the cloning and sequencing of a *B. polymyxa* CF43 gene, *lelA*, expressing levanase activity and a preliminary characterization of its product. We also constructed a *B. polymyxa* CF43 derivative in which the *lelA* gene has been disrupted by a reverse genetic procedure by using the "inter-gramic" conjugation system developed by Trieu-Cuot et al. (41).

MATERIALS AND METHODS

Bacterial strains. *B. polymyxa* CF43 has previously been described (15). *Escherichia coli* TGI (28) was used for cloning and preparation of plasmid and single-stranded DNA. *E. coli* DKI (22) was used for cloning the 5′ upstream region of *lelA*. *E. coli* JM83 (42) was used as a donor strain for intergramic conjugation. *B. subtilis* SA100 (*sacBΔ23 trpC2 leuB6* r^- m^-) was derived from strain MT119 (19); *sacBΔ23* is a deletion mutation of *sacB* (5). *B. subtilis* GM108 (*sacB′*::*nptI sacA321*) is a *his*$^+$ transformant of GM107 (4), and GM639 (*sacB′*::*lacZ sacT30*) was derived from GM122 (39) by introducing the *sacT30* allele (37). *B. subtilis* GM1068 (*sacB′*::*lacZ sacA′*::*tet sacL6*) was derived from GM122 (39) by introducing the *sacL6* allele (21) and disrupting *sacA* with a tetracycline resistance cassette (38).

Growth media and phenotypic characterization. Luria broth was used for *E. coli* and *B. subtilis* cultures, except as otherwise indicated. *B. subtilis* saccharolytic activity was tested by using duplicated colonies as described previously (21). Sucrose utilization by *E. coli* transformants was detected by the red color of the colonies after plating on MacConkey medium supplemented with 1% (wt/vol) sucrose and appropriate antibiotics. Brain heart infusion (BHI) medium (Difco) was used for *B. polymyxa* growth and for conjugation experiments. Utilization of carbon sources by *B. polymyxa* strains was tested on MCBA médium (MM mineral medium [1] containing 22 mg of amo-

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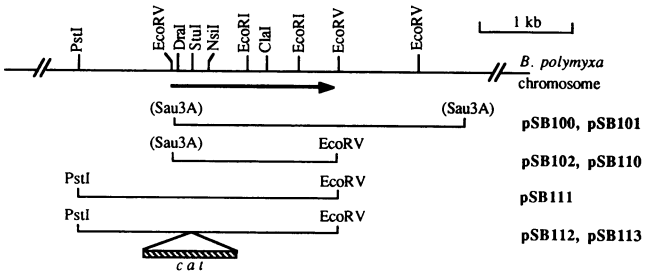

FIG. 1. Restriction map of the *lelA* region and overlapping *B. polymyxa* inserts present in the pSB series of plasmids. pSB101 is a pSB100 derivative constructed by deletion of the *Hin*dIII fragment containing the replication origin of pHV1432 functional in gram-positive bacteria (pAMβI *ori*). pSB102 was constructed from pSB101 by deletion of the fragment present between the central *Eco*RV site of the insert in pSB101 and the *Eco*RV site of the vector to the left of the insert. pSB110 was constructed by inserting the *lelA*-containing *Bam*HI-*Nru*I fragment of pSB102 into the *Sma*I site of pUC18 (44); it thus contains the entire *B. polymyxa* insert in pSB102 plus the 276- and 321-bp fragments of the vectors that border it on the left and right, respectively. pSB111 was constructed from pSB110 by inserting the *Pst*I-*Cla*I fragment of the *B. polymyxa* chromosome containing the 5′ region of *lelA* between the *Pst*I (in the vector part) and the *Cla*I sites of pSB110. pSB112 was constructed by the insertion of a chloramphenicol resistance cassette (*cat*), the small *Sma*I fragment of pBEST401 (18), into the *Stu*I site of pSB111. pSB113 was constructed by inserting the *Kpn*I *lelA*::*cat*-containing fragment of pSB112 into the *Kpn*I site of pAT187. The *B. polymyxa lelA* ORF is represented by the arrow.

niaco-ferric citrate per liter, 0.001% (wt/vol) thiamine, and agar) supplemented with 0.1% (wt/vol) of the carbon source.

Transformation and conjugation. *E. coli* was transformed by the calcium shock procedure. *B. subtilis* was transformed by using natural competence (1). Slight modifications of the method of Trieu-Cuot et al. (41) for intergramic conjugation were used: donor and recipient strains for conjugation were grown till saturation in BHI medium supplemented with relevant antibiotics; the cells were then washed twice with BHI and mated in a donor/recipient ratio of 5:1 on nitrocellulose filters laid on BHI plates overnight at 37°C; filters were immersed in 5 ml of BHI and gently vortexed till the resuspension of the cells, and dilutions were plated on selective medium for conjugants.

Plasmids. The pSB plasmids are described in Fig. 1. pHV1432 is a *B. subtilis/E. coli* shuttle vector constructed by Jannière et al. (19). pAT187 is a mobilizable shuttle vector constructed by Trieu-Cuot et al. (41); it contains the replication origins of pAMβI and pBR322, the *aphA3* gene conferring kanamycin resistance, and the origin of transfer of the IncP plasmid RK2. pRK24 is a self-transferable IncP plasmid (42) able to mobilize pAT187 from *E. coli*.

Genomic library of *B. polymyxa* CF43. DNA extracted from *B. polymyxa* CF43 was partially digested with the restriction enzyme *Sau*3A. The 3- to 6-kb fragments were purified and treated with the Klenow fragment of *E. coli* DNA polymerase in the presence of dATP and dGTP, and pHV1432 linearized with *Sal*I restriction enzyme was treated with the Klenow fragment in the presence of dTTP and dCTP: the extremities of the vector are thus cohesive only with the extremities of the inserts (45). Inserts and vectors were ligated at high concentrations to favor formation of concatemers.

Cloning of the 5′ upstream region of *lelA*. A *Pst*I restriction site, 2.1 kb upstream of the *Cla*I site in *lelA*, was mapped on the *B. polymyxa* chromosome by Southern hybridization. *B. polymyxa* genomic DNA was digested by *Pst*I and *Cla*I; 2- to 2.2-kb fragments were purified and ligated with the large *Pst*I-*Cla*I fragment of pSB110 (Fig. 1); the ligation mixture was used to transform *E. coli* DKI. The transformants were selected on MacConkey sucrose plates containing ampicillin; plasmid DNA was extracted from two red clones. The two plasmids were identical, containing a 2.1-kb *Pst*I-*Cla*I insert, the restriction map of which matched that determined by the Southern hybridization. One of these plasmids, pSB111 (Fig. 1), was used to determine the nucleotide sequence of the 5′ upstream region of *lelA*.

DNA sequencing. Fragments of pSB plasmids inserted into pBluescript (see the legend of Fig. 2) were sequenced by the method of Sanger et al. (33a) with a Sequenase (U.S. Biochemicals Corp.) kit using single-stranded templates prepared by the method of Messing as described by Sambrook et al. (33).

Extracts and enzymatic assays. GM1068 was cultured in minimal C medium (3) containing 0.25% (wt/vol) casein hydrolysate and 1% (wt/vol) glutamate. GM108(pSB100) and GM639 were cultured in CHg medium (5) containing chloramphenicol (4 μg/ml) and in CHg medium, respectively. Washed GM108(pSB100) and GM639 cells and untreated GM1068 culture samples were lysed with lysozyme in 0.05 M potassium phosphate buffer, pH 6. The extracts were assayed for sucrose and inulin hydrolase activities by the method of Kunst et al. (21) with a fructose detection kit from Boehringer; the activities are expressed in nanomoles of fructose liberated per minute per milliliter of culture at an optical density at 600 nm (OD_{600}) of 1. The extracts were assayed for sucrose and levan hydrolase activities with ^{14}C-radiolabelled substrates by the method of Chambert et al. (11); sucrase and levanase activities are expressed in nanomoles of glucose liberated and in percent of levan hydrolyzed, respectively, per minute per milliliter of culture at an OD_{600} of 1.

Nucleotide sequence accession number. The nucleotide sequence of the *lelA* gene of *B. polymyxa* CF43 has been entered into the EMBL Data Library under the accession number Z26651.

RESULTS

Cloning of a *B. polymyxa* DNA fragment encoding sucrose hydrolase activity in *B. subtilis* and *E. coli*. A genomic library of *B. polymyxa* CF43 was constructed in the *B. subtilis/E. coli* shuttle vector pHV1432 (19). The ligation mixture was used to transform *B. subtilis* SA100. About 2,000 transformants were tested on plates for sucrose hydrolase activity. One clone was positive. It contained a plasmid, pSB100, consisting of a 3-kb fragment inserted into pHV1432; the restriction map of the insert was established (Fig. 1); and the presence and the relative positions of the *Eco*RI and *Eco*RV sites were checked on the *B. polymyxa* chromosome by Southern hybridization (data not shown). pSB101 and pSB102, two pSB100 deletion derivatives (Fig. 1), were introduced into *E. coli* TGI and shown to confer sucrose utilization on this host. It was thus concluded that the cloned fragment contained a *B. polymyxa* structural gene encoding a sucrose hydrolase.

Nucleotide sequence determination. The sequence of the 1,664-nucleotide *B. polymyxa* insert in pSB102 was determined. An open reading frame (ORF) starting from the 5′ extremity of the fragment and ending just upstream from the *Eco*RV site was found (Fig. 2). Thus, it was possible that the *B. polymyxa* gene in this fragment was not complete, although the fragment was sufficient to encode sucrose hydrolase activity in *E. coli*. Therefore, we cloned (see Materials and Methods) and sequenced a *B. polymyxa* genomic fragment overlapping the

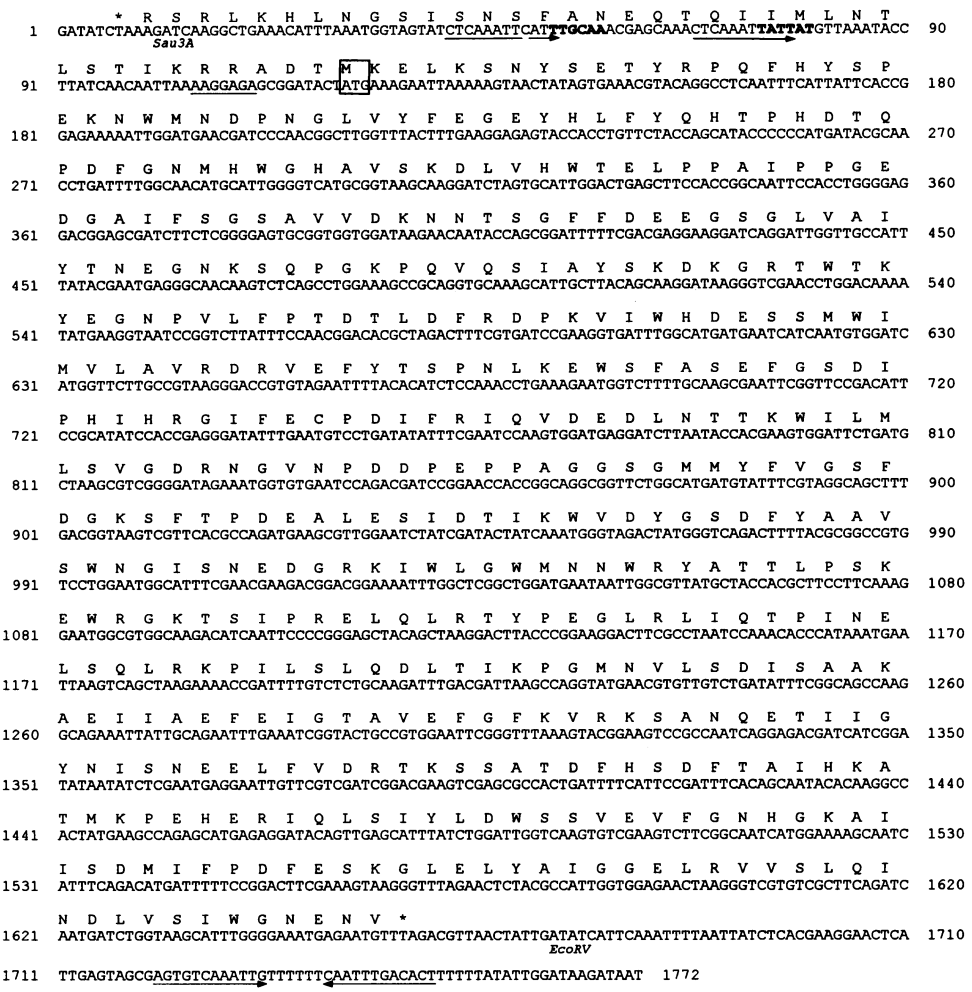

FIG. 2. Nucleotide sequence of the *B. polymyxa lelA* region. The deduced amino acid sequence of the ORF is shown. The putative ATG initiation codon is boxed. Nucleotides constituting the potential *lelA* promoter and the Shine-Dalgarno sequence are in boldface letters and underlined, respectively. Direct-repeated and inverted-repeated sequences are indicated by arrows. Only one *Sau*3A site and the *Eco*RV site that border the insert present in pSB102 are shown. The sequence was determined on both strands in fragments of pSB101, pSB102, and pSB111 cloned into pBluescript.

5′-terminal part of the sequenced fragment; the sequence (Fig. 2) revealed an in-frame nonsense codon just upstream of the pSB102 fragment.

We looked for putative transcription and translation initiation signals in the 5′ part of the sequence. The first 60 codons of the ORF include two ATG codons and one CTG codon. The ATG at position 121 (37th codon of the ORF) is preceded by a possible ribosome binding site (43) and might be the translation initiation codon. Sequence comparisons further strengthened the argument in favor of this being the initiation codon (see below). Forty-two base pairs upstream of this ATG are putative −10 and −35 promoter sequences, as indicated by their similarity with the consensus sequence recognized by the major vegetative RNA polymerase in *E. coli* and *B. subtilis* (30). A directly repeated (11 of 12 identical bp) sequence is present immediately upstream of the putative −35 and −10 promoter regions. The ORF ends only 15 bp upstream of the *Eco*RV site bordering the *B. polymyxa* DNA fragment in pSB102. The sequence downstream of the *Eco*RV site was determined; there is a putative transcription terminator (inverted repeated sequence followed by a stretch of T's) 61 bp downstream of the ORF stop codon (Fig. 2). Assuming that the ATG at position 121 is the initiation codon, the cloned fragment would encode a 512-amino-acid polypeptide.

Similarity with other saccharolytic enzymes. Fasta and Blast programs were used to screen sequence data banks for similarities to the amino acid sequence deduced from the cloned fragment. Significant similarity was found with various microbial β-D-fructosyltransferases. The most similar, calculated over the entire length of the *B. polymyxa* ORF, was *B. subtilis* levanase (28); alignment (using the Gap algorithm from the Genetics Computer Group package; Genetics Computer, Inc.) of the two sequences showed 54% identity. The score reached 70% when classes of homologous amino acids were taken into account (Fig. 3). Weaker similarities were found with three other fructan depolymerizing enzymes: 42% identity with *Bacteroides fragilis* levanase (8), 37% identity with the central part (residues 432 to 920) of *Streptococcus mutans* fructanase (10), and 31% identity with *Kluyveromyces marxianus* inulinase (GenBank). Significant similarities were also found with the yeast invertases: 36% identity with *Schwanniomyces occidentalis* invertase (20) and 33% identity with that of *Saccharomy-*

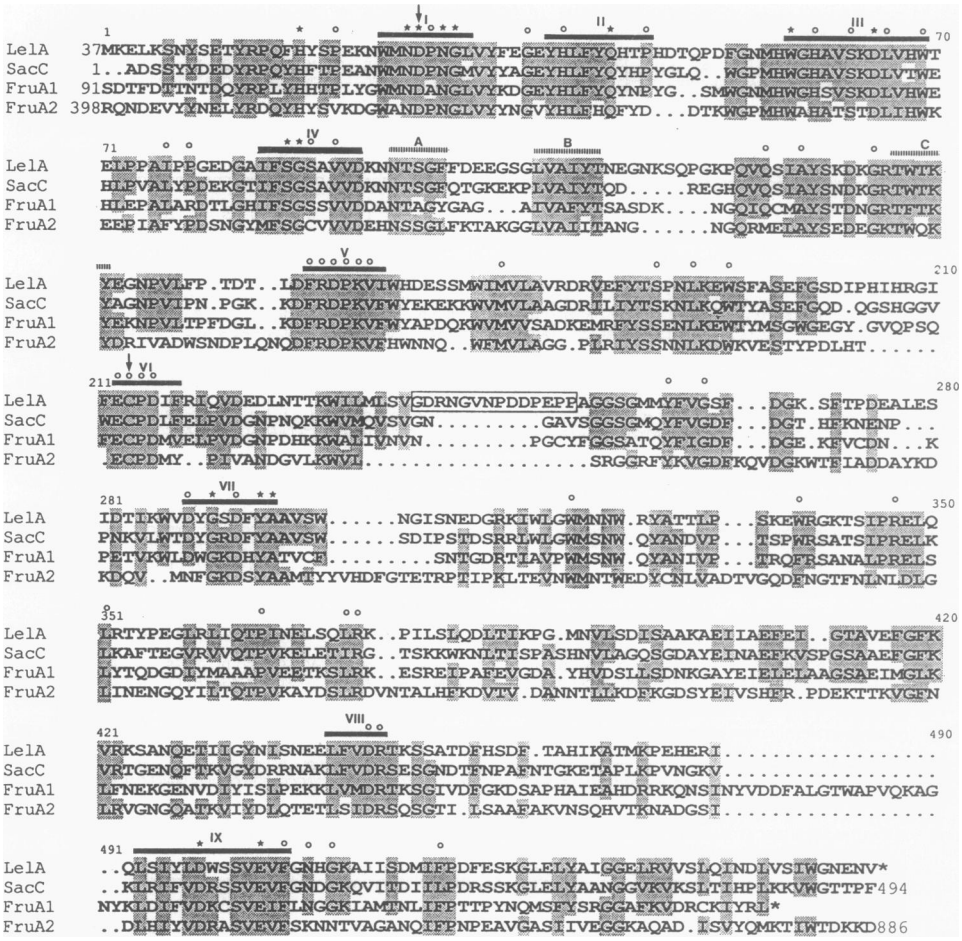

FIG. 3. Multiple-sequence alignment of *B. polymyxa* LelA, *B. subtilis* levanase (SacC), *B. fragilis* fructanase (FruA1), and *S. mutans* fructanase (FruA2). The alignment was produced with the Pileup program (Genetics Computer Group package; Genetics Computer, Inc.) and verified by hydrophobic cluster analysis (25). Residues identical or belonging to the same functional class (F, W/S, T/D, E/N, Q/L, I, V, F/R, K) in at least three of the four sequences are boxed. The nine regions that are conserved in 10 other bacterial or yeast sucrases and inulinase are overlined and numbered from I to IX (see text); the published sequences of *B. subtilis* sucrase (12) and *Schwanniomyces occidentalis* invertase (20) drastically diverge from the consensus in regions V and VI, respectively (in each case, a one-nucleotide insertion and a one-nucleotide deletion a few base pairs ahead could reestablish the consensus). ★, identical residues in the 14 sequences; ○, identical residues in at least 11 of the 14 sequences. The three regions that are conserved among bacterial fructanases, yeast invertases, and inulinase alone are overlined with dashed lines and lettered from A to C. The Gly- and Pro-rich segment of LelA that has no counterpart in any of the other 13 sequences is boxed. The Asp and Cys residues possibly involved in the active site are indicated by an arrow (see Discussion); the SVEVF pentapeptide mentioned by Burne and Penders (10) is part of region IX (see Discussion). Numbering refers to the entire ORF for LelA and to the mature forms of the enzymes for the others.

ces cerevisiae (40). Still weaker similarities were found with bacterial sucrases and sucrose-6-phosphate hydrolases: 34% identity with *Zymomonas mobilis* sucrase (9); 33% identity with RafD, the sucrase from the plasmid-borne genes mediating raffinose utilization in *E. coli* (2); and 26 to 28% identity with the endocellular sucrose-6-phosphate hydrolases of *B. subtilis*, *Klebsiella pneumoniae*, *Vibrio alginolyticus*, *Staphylococcus xylosus*, and *S. mutans* (9, 37).

The ATG codon that is the 37th codon of the *B. polymyxa* ORF, i.e., the putative initiation codon, aligns with the putative first amino acid of the mature forms of *B. subtilis* levanase (codon 25 of the SacC ORF, Fig. 3) and yeast invertases (data not shown). There was no significant similarity upstream of the 37th codon of the *B. polymyxa* ORF with any of the above-mentioned proteins; similarity started at codon 44. None of the typical features of a bacterial signal peptide (31) are found in the first 44 codons of the *B. polymyxa* ORF. Nine discrete regions are particularly well conserved between the *B. polymyxa* sequence and those of all the above-mentioned fructanases, invertases, and sucrases (Fig. 3). There is no similarity between the sequences of levansucrases and the *B. polymyxa* sequence, except a previously described (34) 5-amino-acid motif corresponding to a part of conserved region IV (Fig. 3).

These data suggested that the cloned *B. polymyxa* DNA fragment encodes an enzyme with an activity similar to that of *B. subtilis* levanase; the *B. polymyxa* gene was called *lelA*, for levanaselike gene. However, the C-terminal 159-amino-acid sequence of levanase has no counterpart in LelA, and the *B. polymyxa* LelA ORF does not possess a classical bacterial signal peptide.

Characterization of the enzymatic activity of the *lelA* gene product. *lelA* was cloned on the basis of its capacity to encode a sucrose hydrolase activity, and sequence comparison suggested that *lelA* encodes an enzyme of the fructanase class. To

TABLE 1. Hydrolytic activities of LelA, SacC, and SacA extracts on sucrose, inulin, and levan

Extracts	Hydrolytic activity, method I[a]		Inu/Suc[b]	Hydrolytic activity, method II[c]		Lev/Suc[d]
	Sucrose	Inulin		Sucrose	Levan	
LelA	110	14	143	99	1	25
SacC	190	17	100	100	4	100
SacA	280	<0.6	<3	ND[e]	ND	ND

[a] Extracts were assayed according to the method of Kunst et al. (21); one unit corresponds to 1 nmol of fructose liberated per min per ml at an OD_{600} of 1.

[b] Inu/Suc, the ratio of hydrolase activity on inulin to the hydrolase activity on sucrose relative to that of SacC arbitrarily defined as 100.

[c] Extracts were assayed with radiolabelled substrates according to the method of Chambert et al. (11). One unit of sucrose hydrolase activity corresponds to 1 nmol of glucose liberated per min per ml at an OD_{600} of 1, and one unit of levan hydrolase activity corresponds to 0.01% of levan hydrolyzed per min per ml at an OD_{600} of 1.

[d] Lev/Suc, the ratio of the hydrolase activity on levan to the hydrolase activity on sucrose relative to that of SacC arbitrarily defined as 100.

[e] ND, not determined.

demonstrate that it does actually express fructanase activity, we compared the hydrolase activities of LelA on sucrose, levan, and inulin with those of *B. subtilis* sucrase and levanase.

LelA-containing extracts were prepared from *B. subtilis* GM108 (Δ*sacB sacA*) transformed by pSB100 (Fig. 1). *B. subtilis* sucrase (SacA)-containing extracts were prepared from strain GM639 (*sacT30*), which synthesizes SacA constitutively because of the *sacT30* mutation. *B. subtilis* levanase (SacC)-containing extracts were prepared from strain GM1068 (*sacL6*), which synthesizes SacC constitutively because of the *sacL6* mutation.

The ratio of sucrose hydrolysis to inulin hydrolysis of LelA extracts was similar to that of SacC extracts. The SacA extract showed no detectable activity on inulin. SacC appeared more active than LelA on levan (Table 1). These results indicate that *lelA* encodes a β-D-fructosyltransferase of the fructanase subfamily able to hydrolyze sucrose and both fructan polymers, levan and inulin.

LelA-containing extracts also showed a low but significant transferase activity: exchange activity (synthesis of labelled sucrose from labelled glucose and unlabelled sucrose) (11) could be detected (data not shown).

Insertional inactivation of *lelA* in *B. polymyxa*. To investigate the role of LelA in *B. polymyxa*, the *lelA* gene was inactivated in the chromosome. In the absence of any transformation and genetic marker exchange experiments reported for *B. polymyxa*, we decided to use the intergramic conjugation system developed by Trieu-Cuot et al. (41). The conditions for conjugation between *E. coli* and *B. polymyxa* and for genetic marker exchange between the conjugation vector and the recipient chromosome were optimized by using the *B. polymyxa* levansucrase gene (7a).

A chloramphenicol resistance cassette (*cat*) was inserted into pSB111 at the *StuI* site of the *lelA* gene to produce pSB112 (Fig. 1). The *lelA*::*cat*-containing fragment of pSB112 was inserted into the conjugative vector pAT187 (harboring a kanamycin resistance gene) to create pSB113 (Fig. 1). An *E. coli* JM83 transformant carrying both pSB113 and pRK24 was mated with *B. polymyxa* CF43. Transconjugants were selected on BHI plates containing 5 mg of chloramphenicol per liter and 20 mg of colistin per liter to counterselect *E. coli* donors. About 4% of the *B. polymyxa* chloramphenicol-resistant transconjugants were kanamycin sensitive, suggesting that their *lelA* gene has been replaced by the *lelA*::*cat* cassette. One conjugant was chosen for further examination. The correct integration of *cat* into *lelA* in the transconjugant and the absence of vector sequences were confirmed by Southern hybridization (data not shown).

The *lelA*::*cat B. polymyxa* mutant, designated CD003, was tested for levan and inulin utilization on plates containing levan or inulin as the sole carbon source: CD003 and CF43 were streaked on MCBA plates supplemented with levan or inulin. No difference between the growth of the mutant and that of the parental strain could be detected. The same result was obtained with inulin dialyzed against water to eliminate possible contamination by fructose and oligofructomers. Thus, although it possesses fructanase activity, LelA is not essential for the assimilation of fructans as a carbon source for growth.

DISCUSSION

A genomic library of *B. polymyxa* CF43 was constructed in a *B. subtilis*/*E. coli* shuttle vector and screened in *B. subtilis* for the expression of sucrose hydrolysis activity. A positive clone was shown to contain a 3-kb insert. The nucleotide sequence of a 1,772-bp segment of the insert, sufficient to express sucrose hydrolysis activity in *E. coli*, was determined. It revealed the presence of an ORF of 548 codons that exhibits 54% identity with *B. subtilis* levanase and weaker similarities with other fructosylfuranosidases. This suggested that the sequenced gene encodes a fructanase; the gene was called *lelA*, for levanaselike gene. LelA-containing extracts were prepared from a *B. subtilis* transformant and assayed along with *B. subtilis* sucrose-6-phosphate hydrolase (SacA) and levanase (SacC) extracts for hydrolysis activities on sucrose, inulin, and levan (see Results). This confirmed that LelA is a fructanase.

SacC is secreted by *B. subtilis*. Conversely, when *lelA* was expressed in this host, LelA activity was not recovered in the extracellular medium (data not shown). However, *lelA* conferred on *E. coli* a sucrose-plus phenotype on MacConkey sucrose plates. Since sucrose does not enter into *E. coli* cytoplasm (9), this phenotype suggested that LelA was, at least partially, noncytoplasmic when expressed in this host. At least two saccharolytic enzymes which are clearly cytoplasmic in their natural hosts, SacA from *B. subtilis* and ScrB from *V. alginolyticus*, also confer a sucrose-plus phenotype (red colonies on MacConkey sucrose plates) on *E. coli* tranformants expressing the relevant genes (35, 38). Since the typical features of bacterial secretion signal peptides cannot be found in the N-terminal parts of the LelA ORF, SacA, and ScrB, the apparent exportation of these enzymes out of the *E. coli* cytoplasm is unexplained. It is possible that these enzymes, structurally related to true exoenzymes such as SacC and other fructanases, are able to interact with the *E. coli* secretory apparatus, although they are devoid of signal peptides. It remains unknown whether, when expressed in its natural host, LelA is cytoplasmic or exported by a signal peptide-independent mechanism.

The sequences of seven bacterial sucrases and sucrose-6-phosphate hydrolases, four bacterial fructanases (including LelA), two yeast invertases, and a yeast inulinase were aligned (Results and data not shown). Conserved motifs were detected in the 14 enzymes. In addition to the seven conserved regions and putative essential residues previously described (9), we found two new conserved segments; this alignment also indicates possible sequencing errors (or recent pairs of mutations) in two of these genes (legend of Fig. 3). The comparison identified three segments characteristic of fructanases and yeast invertases in the N-terminal parts of the proteins. Con-

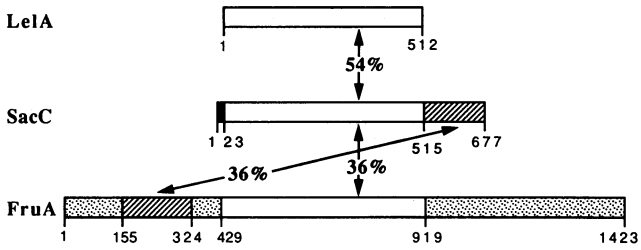

FIG. 4. Homologous segments in *B. polymyxa* LelA, *B. subtilis* levanase (SacC), and *S. mutans* fructanase (FruA). Percent identity between segments is indicated. Amino acids bordering the different segments are numbered.

versely, the pentapeptide, SVEVF, claimed to be characteristic of fructanases by Burne and Penders (10) is in fact conserved in all 14 enzymes (Fig. 3). This comparison also showed that the 159-amino-acid C-terminal segment of *B. subtilis* levanase has no counterpart in LelA. Interestingly, we found that this segment is homologous to an N-terminal region (residues 160 to 320) of *S. mutans* fructanase (Fig. 4); the similarities between these segments and that between the N-terminal part of SacC and the central part of FruA, i.e., the domains conserved in the 14 fructofuranidases are the same (36% identity). This suggests that this additional segment present in both SacC and FruA is a functional domain. No similarity between this domain and other proteins in the data banks was found. Schörgendorfer et al. (36) previously reported that a partial deletion of this domain in SacC (114-amino-acid deletion of the C-terminal end) did not affect inulinase activity. These observations suggest that the domain conserved in both FruA and SacC (and absent in LelA) plays a role although clearly inessential for sucrose and fructan hydrolysis.

To investigate the role of LelA in fructan metabolism, we constructed a *B. polymyxa* mutant in which the chromosomal *lelA* was inactivated. This was achieved using intergramic conjugation (41). To the best of our knowledge, this constitutes the first reported reverse genetic experiment with *B. polymyxa*. The abilities of the mutant and parental strains to grow on levan or inulin as the sole carbon source were tested on plates. No difference between the two strains could be detected. This suggests that LelA might not be involved in the assimilation of extracellular fructan as a carbon source; alternatively, *B. polymyxa* might possess a second enzyme with this function. If LelA is cytoplasmic, as suggested by the absence of a signal peptide, it might be involved in intracellular catabolism of endogenously synthesized fructans or small fructose oligomers transported into *B. polymyxa* cells. Finally, a role for LelA in fructan anabolism cannot be ruled out. As briefly mentioned in Results, LelA was also shown to have fructosyltransferase activity; this activity, which appeared to be weak under the conditions tested, might be higher in specific cytoplasmic conditions, for example, in the presence of specific fructosyl acceptors and/or fructosyl donors other than sucrose. To our knowledge, none of these hypothetical intracellular pathways of fructan metabolism in bacteria have been described. Further investigations on the role and the localization of LelA are required.

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