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Levanase Operon of *Bacillus subtilis* Includes a Fructose-specific Phosphotransferase System Regulating the Expression of the Operon

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The levanase gene (*sacC*) of *Bacillus subtilis* is the distal gene of a fructose-inducible operon containing five genes. The complete nucleotide sequence of this operon was determined. The first four genes *levD*, *levE*, *levF* and *levG* encode polypeptides that are similar to proteins of the mannose phosphotransferase system of *Escherichia coli*. The *levD* and *levE* gene products are homologous to the N and C-terminal part of the enzyme III^{Man}, respectively, whereas the *levF* and *levG* gene products have similarities with the enzymes II^{Man}. Surprisingly, the polypeptides encoded by the *levD*, *levE*, *levF* and *levG* genes are not involved in mannose uptake, but form a fructose phosphotransferase system in *B. subtilis*. This transport is dependent on the enzyme I of the phosphotransferase system (PTS) and is abolished by deletion of *levF* or *levG* and by mutations in either *levD* or *levE*. Four regulatory mutations (*sacL*) leading to constitutive expression of the levanase operon were mapped using recombination experiments. Three of them were characterized at the molecular level and were located within *levD* and *levE*. The *levD* and *levE* gene products that form part of a fructose uptake PTS act as negative regulators of the operon. These two gene products may be involved in a PTS-mediated phosphorylation of a regulator, as in the *bgl* operon of *E. coli*.

1. Introduction

The phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS‡) is responsible for the uptake and concomitant phosphorylation of a number of sugars in both Gram-positive and Gram-negative bacteria (for a review, see Postma & Lengeler, 1985). In addition, the PTS is implicated in several regulation mechanisms such as chemotaxis, metabolic control and transcriptional regulation (for a review, see Saier, 1989).

During uptake by the PTS, a phosphoryl group is transferred from phosphoenolpyruvate to the different carbohydrates *via* a number of proteins transiently phosphorylated. Enzyme I of the PTS and histidine-containing phosphocarrier protein (Hpr), which are cytoplasmic proteins, are required for the transport and phosphorylation of all PTS sugars and are therefore called general proteins. Sugar specificity in each uptake system is determined by specific proteins: a membrane bound enzyme II and, in some cases, an associated soluble enzyme III.

It has been established that the PTS is involved in the vectorial transport of mono- and disaccharides in *Bacillus subtilis*. These sugars include glucose, fructose, mannose, mannitol and sucrose (Gay *et al.*, 1973). Enzyme I, Hpr and several enzyme II types have been characterized genetically or biochemically in this micro-organism (for a review, see Fouet *et al.*, 1989). In particular, *fruA* mutants specifically affected in fructose uptake have been characterized (Gay *et al.*, 1970). These mutants are, however, able to take up fructose, but only when grown under catabolite derepressed conditions with prior induction by fructose. Gay & Delobbe (1977) proposed the existence in *B. subtilis*

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‡ Abbreviations used: PTS, phosphotransferase system(s); Hpr, histidine-containing phosphocarrier protein; kb, 10^3 bases or base-pairs; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; Cm^r, chloramphenicol resistance; bp, base-pair(s); Am^r ampicillin resistance; Em^r, erythromycin resistance; PCR, polymerase chain reaction; ORF, open reading frame(s); Cm^s, chloramphenicol sensitive; Km, kanamycin.

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of at least two fructose-PTS whose expression is fructose inducible.

Postma & Lengeler (1985) pointed out that the structural genes for the enzyme II types are not part of a *pts* regulon but form units with enzymes involved in the corresponding sugar's metabolism. Most of these enzyme II associated operons are induced by the relevant sugar. Here, we describe another system in which an enzyme, levanase, able to hydrolyze sucrose and to degrade fructose polymers, is associated with a fructose-PTS.

The levanase structural gene, *sacC*, has been cloned and sequenced (Martin *et al.*, 1987). Using β-galactosidase fusions, it was shown that in *B. subtilis* the expression of the *sacC* gene encoding levanase is inducible by fructose and subject to catabolite repression (Martin *et al.*, 1989). A fructose inducible vegetative promoter has been characterized 2·7 kb upstream from the *sacC* gene. The data obtained suggest that the *sacC* gene is the distal gene of a 5 kb operon (Martin *et al.*, 1989). The DNA sequence of the region between *sacC* and the promoter was determined with a view to the characterization of the intervening genes. The results show that there are five genes in the operon, four of which are involved in a fructose-PTS.

The expression of the levanase operon is inducible by fructose. Presumably regulatory proteins are involved in this regulation. *sacL* mutants isolated by Kunst *et al.* (1977) constitutively express the levanase operon (Martin *et al.*, 1989). The corresponding mutations were mapped on the chromosome of *B. subtilis* by PBS1 transduction between *leuA* and *aroD* near the structural gene for levanase. Here, four *sacL* mutations were mapped more precisely using recombination techniques. Three of them were characterized at the molecular level and located by DNA sequencing within the first two genes of the levanase operon. The corresponding gene products act as negative regulators of the levanase operon. A model for the regulation of transcription of the operon is presented.

Table 1

B. subtilis *strains and parental plasmids used in this study*

Strain or plasmid	Genotype or description	Source
Strains		
168	*trpC2*	Laboratory stock
QB166	*trpC2 sacL5*	Kunst *et al.*, 1977
QB168	*trpC2 sacL7*	Kunst *et al.*, 1977
QB169	*trpC2 sacL8*	Kunst *et al.*, 1977
QB2018	*leuA8 hisA1sacA321 sacL6*	Kunst *et al.*, 1977
QB5030	*trpC2 sacC′-lacZ*$^+$ *erm*	Martin *et al.*, 1989
QB5031	*hisA1 leuA8 sacA321 sacL6 sacC′-lacZ*$^+$ *erm*	Martin *et al.*, 1989
QB5050	*trpC2 sacL8 sacC′-lacZ*$^+$ *erm*	QB5030 $\xrightarrow{TF}$ QB169
QB5051	*trpC2 metC3 ptsI6 sacC′-lacZ*$^+$ *erm*	QB5030 $\xrightarrow{TF}$ PG554
QB5054	*trpC2 sacL8 levG′-lacZ*$^+$ *erm*	pJC26† $\xrightarrow{TF}$ QB169
QB5055	*trpC2 sacL8 levF′*::pJC27	pJC27† $\xrightarrow{TF}$ QB169‡
QB5058	*trpC2 levG′-lacZ*$^+$ *erm*	pJC26† $\xrightarrow{TF}$ 168
QB5061	*trpC2 metC3 ptsI6 sacL8 sacC′-lacZ*$^+$ *erm*	QB5050 $\xrightarrow{TD}$ PG554
QB5064	*trpC2 sacL8 levG′-lacZ*$^+$ *erm amyE*::(Pf-*levG-lacZ*$^+$ *cat*)	pIC2† $\xrightarrow{TF}$ QB5054
QB5065	*trpC2 sacL8 levF′*::pJC27 *amyE*::(Pf-*levG-lacZ*$^+$ *aphA3*)	pIC3† $\xrightarrow{TF}$ QB5055
QB5066	*hisA1 leuA8 sacA321 sacI6 sacC′-lacZ*$^+$ *erm amyE*::(Pf-*levD-levE-levF-levG* Δ*lacZ cat*)	pIC5† $\xrightarrow{TF}$ QB5031
QB5067	*trpC2 levG′-lacZ*$^+$ *erm amyE*::(Pf-*levD-levE-levF-levG* Δ*lacZ cat*)	pIC5† $\xrightarrow{TF}$ QB5058
PG554	*trpC2 metC3 ptsI6*	Gay *et al.*, 1973
Plasmids	*B. subtilis* chromosomal DNA insert	
pJC1	6 kb fragment§ carrying the levanase operon (Fig. 1)	Martin *et al.*, 1987
pJC7	1·2 kb fragment carrying the promoter of the levanase operon and the 123 first codons of the *levD* gene (Fig. 1)	Martin *et al.*, 1989
pJC14	See Fig. 1	Martin *et al.*, 1989

TF, transformation; TD, transduction; *cat*, the pC194 chloramphenicol acetyl transferase gene (Ehrlich, 1977); *erm*, the Tn*917* erythromycin resistance gene (Shaw & Clewell, 1985); *aphA3*, the *Streptococcus faecalis* kanamycin resistance gene (Trieu-Cuot & Courvalin, 1983); Pf, the fructose inducible promoter of the levanase operon; Δ*lacZ*, the *lacZ* gene is partially deleted (see Fig. 1(b)).

† Plasmid described in the text.

‡ Plasmid pJC27 was integrated into the chromosome of the recipient strain by a Campbell-type mechanism.

§ Obtained from a chromosomal DNA *Sau*3A1 partial digest.

2. Materials and Methods

(a) *Bacterial strains*

The *B. subtilis* strains used in this work are listed in Table 1. *E. coli* strain TG1 (K12 Δ(*lac pro*) *supE thi hsd5*/F′ *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* ΔM15) was used for cloning experiments (Gibson, 1984).

(b) *Culture media*

E. coli was grown in L broth (10 g tryptone/l, 5 g yeast extract/l, 5 g NaCl/l). *B. subtilis* was grown in SP medium (8 g nutrient broth/l, 1 mM-$MgSO_4$, 10 mM-KCl, 0·5 mM-$CaCl_2$, 10 μM-$MnCl_2$, 2 μM-$FeSO_4$) or in C medium (70 mM-$K_2HPO_4 \cdot 3H_2O$, 30 mM-KH_2PO_4, 25 mM-$(NH_4)_2SO_4$, 0·5 mM-$MgSO_4$, 10 μM-$MnSO_4$, 22 mg ferric ammonium citrate/l, auxotrophic requirements 100 mg/l). CSK medium is C medium supplemented with potassium succinate (6 g/l) and potassium glutamate (8 g/l).

L or SP-plates were prepared by the addition of 17 g Bacto-Agar (Difco)/l to L broth or SP medium containing chloramphenicol (5 μg/ml), kanamycin (5 μg/ml) and erythromycin plus lincomycin (1 and 25 μg/ml, respectively), as appropriate. MMHC plates were obtained by the addition of 17 g Bacto-agar/l to MMHC medium (60 mM-$K_2HPO_4 \cdot 3H_2O$, 44 mM-KH_2PO_4, 15 mM-$(NH_4)_2SO_4$, 3 mM trisodium citrate, 2 mM-$MgSO_4$, 22 mg ferric ammonium citrate/l, 0·05% (w/v) casein hydrolysate, 100 mg auxotrophic requirements/l).

(c) *Transformation and characterization of phenotypes*

E. coli and *B. subtilis* strains were transformed as described by Cohen *et al.* (1972) and Anagnostopoulos & Spizizen (1961). *B. subtilis* and *E. coli* transformants were selected as described (Martin *et al.*, 1989; Msadek *et al.*, 1990).

Hydrolysis of sucrose by levanase was tested on SP plates. The release of glucose was detected with the GOD-Perid reagent (Boehringer-Mannheim Biochemicals, F.R.G.), as described by Lepesant *et al.* (1972).

In *B. subtilis*, amylase activity was detected after growth on tryptose blood agar base (TBAB, Difco) supplemented with 10 g hydrolyzed starch (Connaught)/l. Starch degradation was detected by sublimating iodine on to the plates.

β-Galactosidase activity was estimated by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) hydrolysis using colonies grown on MMCH plates. β-Galactosidase assays were performed as described by Martin *et al.* (1989). One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol of *o*-nitrophenol/min at 28 °C.

(d) *DNA purification and manipulations*

Standard procedures were used for extracting plasmid DNA from *E. coli* (Birnboim & Doly, 1979). Restriction enzymes, Klenow polymerase, *Taq* DNA polymerase and phage T4 DNA ligase were used according to the manufacturers recommendations. DNA fragments were recovered from agarose gels by using either electroelution or Gene Clean (Bio101, La Jolla, CA).

Chromosomal DNA was prepared as described by Msadek *et al.* (1990).

(e) *Plasmids and cloning procedure*

Parental plasmids pJC1, pJC7 and pJC14 are listed in Table 1 and are described in Fig. 1(a). The vector pJH101 (Ferrari *et al.*, 1983) contains a *cat* gene conferring chloramphenicol resistance (Cm^r) and replicates in *E. coli* but not in *B. subtilis*. Plasmids derived from pJH101 and containing *B. subtilis* DNA integrate into the chromosome of *B. subtilis* by homologous recombination.

Plasmid pAF1, a derivative of ptrpBG1 (Shimotsu & Henner, 1986) was a gift from A. Fouet (Fouet & Sonenshein, 1990). This plasmid carries the *cat* resistance determinant and a promoterless *lacZ* gene between 2 fragments of the *B. subtilis amyE* gene, allowing direct selection of single copy integration of β-galactosidase fusions at the *B. subtilis amyE* locus.

Plasmid pJC6 was constructed as follows: plasmid pJC1 (Martin *et al.*, 1987, 1989) contains 2 *Pst*I restriction sites, 1 of which is located in the vector pJH101 (Ferrari *et al.*, 1983). Plasmid pJC6 was obtained by circularizing the large *Pst*I fragment of pJC1 (Fig. 1(a)). In plasmid pJC6, the ampicillin resistance gene is inactivated and a 3·5 kb *B. subtilis* DNA fragment is present.

Plasmid pJC26 (Fig. 1(a)) was constructed as follows: a 4·5 kb *Sma*I–*Kpn*I fragment of pTV32 (Perkins & Youngman, 1986) containing the *lacZ* and *erm* genes was purified and the ends made blunt with T4 DNA polymerase. This DNA fragment was inserted into the unique *Bst*BI site of pJC6 (Fig. 1(a)). The ligated DNA mixture was used to transform *E. coli* TG1. Chloramphenicol resistant clones were selected on L Cm-X-gal plates, and the plasmid structure was verified by restriction mapping.

Plasmid pJC27 was constructed by cloning a 310 bp *Ava*I–*Hin*dIII DNA fragment, purified from pJC6, into the vector pJH101 digested with *Eco*RV and *Hin*dIII (Fig. 1(a)). Plasmid pJC27 contains an internal part of *levF*.

Plasmid pJC28 (Fig. 1(a)) was obtained by cloning a 1·1 kb DNA fragment containing the *levG* gene into the plasmid pJC7 (Table 1, Fig. 1(a)). A *Dra*I–*Pst*I fragment was purified from pJC6 and cloned into pJC7 digested by *Eco*RV and *Pst*I. In plasmid pJC28, the ampicillin resistance (Am^r) gene is inactivated and the *levG* gene is transcribed under the control of the levanase operon promoter.

Plasmid pIC2 (Fig. 1(b)) was constructed as follows: pJC28 was digested with *Cla*I and *Pst*I. The ends of the *Cla*I–*Pst*I fragment carrying the promoter of the levanase operon and the *levG* gene (Fig. 1(a)), were made blunt using T4 DNA polymerase and Klenow enzyme and the fragment was purified. It was ligated into the unique *Hin*dIII site of pAF1 made blunt using Klenow enzyme (Fig. 1(b)).

Plasmid pIC5 was obtained by cloning a DNA fragment containing the promoter and the 4 proximal genes of the levanase operon into the plasmid pAF1 (Fig. 1(b)). A 3·5 kb *Cla*I–*Pst*I fragment was obtained from pJC1 (Fig. 1(a)), treated with T4 DNA polymerase and Klenow enzyme and then purified. It was cloned at the unique *Hin*dIII site of pAF1 made blunt using Klenow enzyme.

Plasmid pIC3 (Fig. 1(b)) was constructed by replacing the *cat* determinant of pIC2 by the kanamycin resistance gene *aphA3* from *Streptococcus faecalis* (Trieu-Cuot & Courvalin, 1983). A 1·5 kb *Cla*I fragment, carrying the *aphA3* gene made blunt with Klenow fragment, was cloned into the pIC2 plasmid digested with *Stu*I and *Eco*RI and made blunt with Klenow enzyme.

(f) *Construction of* B. subtilis *strains*

Plasmid pJC26 (Fig. 1(a)) was linearized with *Stu*I and used to transform *B. subtilis* 168 and QB169 (*sacL8*).

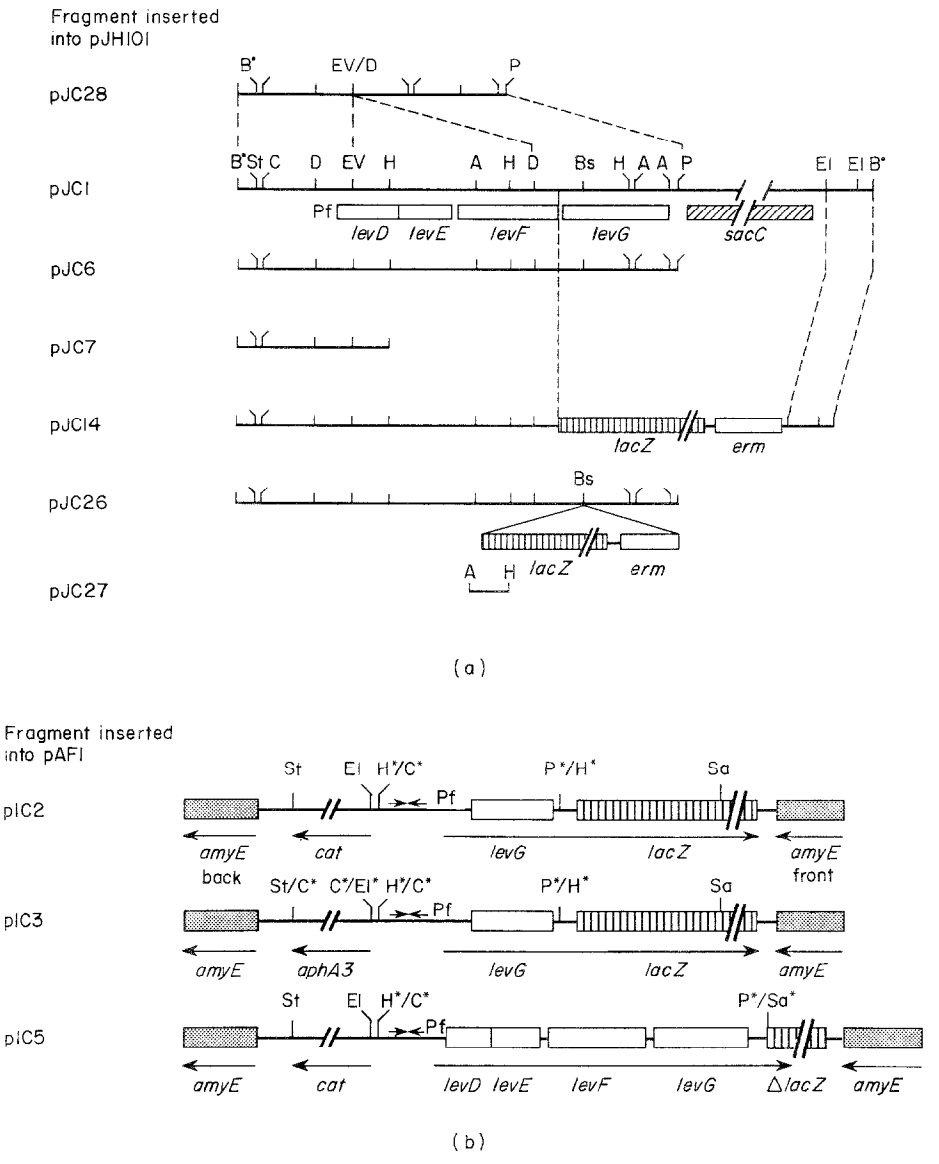


Figure 1. Simplified restriction maps of plasmids used in this study. The positions of *levD*, *levE*, *levF*, *levG* and *sacC* are shown. Pf, the fructose inducible promoter of the levanase operon; A, *Ava*I; Bs, *Bst*BI; C, *Cla*I; D, *Dra*I; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pts*I; Sa, *Sac*I; St, *Stu*I; X*, indicates an X restriction site made blunt using Klenow enzyme or phage T4 DNA polymerase; B•, indicates that the *Bam*HI restriction sites were not regenerated during the cloning of a partial *Sau*3A fragment. (a) Restriction map of DNA fragments inserted into the integrative pJH101 vector. (b) Restriction map of DNA fragments inserted into the vector pAF1.

Erythromycin resistant (Em^r) chloramphenicol sensitive (Cm^s) integrants were isolated; they resulted from the integration of *lacZ* and *erm* into the chromosome by a double cross-over. The resulting strains, QB5058 derived from strain 168 and QB5054 derived from strain QB169, contain an insertion of *lacZ* and *erm* into *levG* as a transcriptional *levG'–lacZ*$^+$ fusion. The presence of the *sacL8* mutation in strain QB5054 was tested by verifying constitutive *lacZ* expression.

Strain QB5050 and QB5051 were constructed by transformation of strains QB169 (*sacL8*) and PG554 (*ptsI6*) with the DNA of strain QB5030 (*saC'–lacZ*$^+$, *erm*). Transformants carrying a *lacZ* and *erm* insertion into *sacC* were obtained by selection for Em^r.

Strain QB5061 was constructed as follows: a PBS1 transducing lysate of strain QB5050 carrying a *sacL8* mutation and a *sacC'–lacZ*$^+$ fusion was used to transduce the recipient strain PG554 (*ptsI6*; Gay *et al.*, 1973), following the technique described by Lepesant *et al.* (1972). Em^r recombinants were selected on SP plates containing erythromycin and lincomycin. The resulting strain, QB5061, carried the *ptsI6* and *sacL8* mutations and a *sacC'–lacZ*$^+$ fusion. Back-cross experiments were used to confirm the presence of the *sacL8* mutation.

The DNA fragment present in pAF1 derivative plasmids (pIC2, pIC3 and pIC5) was inserted into the *B. subtilis* chromosome as follows: these plasmids were linearized at their unique *Pst*I sites and the DNA fragments located between the 2 regions of *B. subtiliz amyE* gene were integrated by homologous recombination at the *amyE* locus, using Cm or kanamycin (Km) selection. The amylase-minus phenotype of strains QB5064, QB5065, QB5066 and QB5067 was verified on TBAB plates containing soluble starch.

(g) *DNA sequencing*

DNA sequences were determined by the dideoxy termination method (Sanger *et al.*, 1977) and modified phage T7 polymerase (Sequenase, USB, Cleveland, OH). An *Eco*RV–*Pst*I DNA fragment from pJC1 (Fig. 1(a)) was purified and cloned into the replicative forms M13mp19 and M13mp18 digested with *Sma*I and *Pst*I. Overlapping deletions of the *Eco*RV–*Pst*I fragment inserted into M13mp18 were obtained using the technique described by Dale *et al.* (1985), and the nucleotide sequence was thereby determined for 1 DNA strand. The nucleotide sequence of the 2nd DNA strand was determined using a series of synthetic oligonucleotides that prime at intervals of 200 nucleotides. To eliminate gel compression, dITP was used rather than dGTP according to the manufacturer's instructions (Sequenase, USB, Cleveland, OH). The sequence located between the levanase operon promoter and the *Eco*RV restriction site was determined as described by Martin *et al.* (1989). The 157 bp sequence 3′ to the *Pst*I site has already been published (Martin *et al.*, 1987).

The oligonucleotide 5′ CCACAGTAGTTCACCACC 3′ complementary to the *spoVG* DNA fragment present in *lacZ* fusions was used as the primer in the determination of the position of the fusion junction with the levanase operon in plasmid pJC14 (Fig. 2).

(h) *Amplification of DNA fragment of* sacL *mutants: cloning and sequencing*

A 1·8 kb DNA fragment (see Fig. 5) containing the promoter and the 1st 3 genes of the levanase operon was amplified by the polymerase chain reaction (PCR) technique (Mullis & Faloona, 1987). This procedure was repeated independently with DNA from *sacL5*, *sacL6*, *sacL7* and *sacL8* strains. Two 28 bp oligonucleotide primers, 5′-AGGAATTCTTGCTATTGGCTGAAATAAC-3′ and 5′-AGGCCCGGGCATCAAGATCATCATCTCG-3′, were used for DNA amplification and included mismatches to the wild-type sequence leading to the creation of *Eco*RI and *Sma*I restriction sites. PCRs were carried out using 1 μg of DNA matrix and thermostable *Thermus aquaticus* DNA polymerase, as recommended by New England Biolabs, Inc. After an initial denaturation step of 15 min at 95 °C, amplification was carried out for 25 cycles. The DNA was denatured at 95 °C for 1 min, annealed at 50 °C for 1 min and extended at 72 °C. The extension period was 4 min for the first 10 cycles and 6 min for the last 15. Samples were then successively extracted with phenol and chloroform, precipitated with ethanol, digested with *Eco*RI and *Sma*I restriction enzymes and gel purified before cloning into M13mp18 or M13mp19 digested with *Eco*RI and *Sma*I. The DNA sequence was determined using oligonucleotides. Since DNA amplification by this method consistently led to misincorporation errors of 0·1%, 3 separate templates were sequenced for each mutant strain to identify errors due to the amplification procedure (Dunning *et al.*, 1988).

(i) *Radioactive sugar uptake* in vivo

B. subtilis strains were grown in CSK medium in the presence or absence of fructose as appropriate. It has been shown that the expression of the levanase operon was derepressed in this medium (Kunst *et al.*, 1977; Martin *et al.*, 1989). Sugar uptake assays were performed as described by Delobbe *et al.* (1971). Medium C supplemented with 10 g glycerol/l, 250 mg chloramphenicol/l was used for incorporation. Exponentially growing cells were harvested by centrifugation at an A_{600} of 0·6 to 0·8 and washed once with the incorporation medium. Cells were incubated at 37 °C with shaking. Labeled [^{14}C]fructose (37 kBq/ml) and non-labeled fructose (final concentration 0·4 mM) were added. Samples (1 ml) were withdrawn at intervals and filtered through Millipore membranes (HA 0·45 μm). Filters were washed twice with 10 ml of the incorporation medium at 37 °C. After drying, radioactivity was measured by scintillation counting.

3. Results

(a) *Organization of the levanase operon*

(i) *Nucleotide sequence of the levanase operon*

The levanase gene, *sacC*, is the distal gene of an operon and is located 2·7 kb downstream from the promoter (Martin *et al.*, 1989). The nucleotide sequence of this 2·7 kb DNA fragment was determined as described in Materials and Methods. Four open reading frames, ORF1, ORF2, ORF3 and ORF4, were found 5′ of the levanase gene on the same DNA strand (Fig. 2). These were preceded by putative ribosome binding sites at optimal distances from the initiation codons (Fig. 2), respectively, GAAAGGAG ($\Delta G = -77$ kJ/mol), AAGAGAGGATGA ($\Delta G = -81$ kJ/mol), AAGGGGATGA ($\Delta G = -81$ kJ/mol) and AGGGGGA ($\Delta G = -77$ kJ/mol) (Tinoco *et al.*, 1973; Hager & Rabinowitz, 1985). ORF1 encodes a 146 amino acid residue polypeptide with an M_r of 16,238. ORF2 encodes a 163 amino acid residue polypeptide with an M_r of 18,194. ORF3 and ORF4 code for 269 and 275 amino acid residue polypeptides with M_r values 28,071 and 30,051, respectively. The polypeptides deduced from these four ORF were designated P16, P18, P28 and P30 according to their calculated molecular weight (see Fig. 5). The corresponding genes were designated *levD*, *levE*, *levF* and *levG* (see Fig. 5).

The nucleotide sequence of the first gene overlaps that of the second gene at the sequence ATGA, where ATG is the initiation codon of *levE* and TGA is the stop codon of *levD* (Fig. 2). The intercistronic distances between *levE* and *levF*, and *levF* and *levG* are 19 bp and 23 bp, respectively. The distance between *levG* and *sacC* is 159 bp.

(ii) *Comparison of the product of the first four genes of the levanase operon with that of known proteins*

A computer search for similarities with other proteins revealed that P16, P18, P28 and P30 share homology with the three specific proteins of the *E. coli* mannose phosphotransferase system, III^{Man}, IIM^{Man} and IIP^{Man} (Erni *et al.*, 1987). The polypeptides P16 and P18 (*levD* and *levE*) are similar to the N and C-terminal parts of the enzyme III^{Man} of *E. coli*, respectively. The combined molecular weights of P16 and P18 is 34,432, which approximates to the molecular weight of enzyme III^{Man}, 35,016. The percentage of identical residues between the P16/P18 pair and the enzyme III^{Man} is about 40% (Fig 3(a)). The *levF* gene product, P28, is

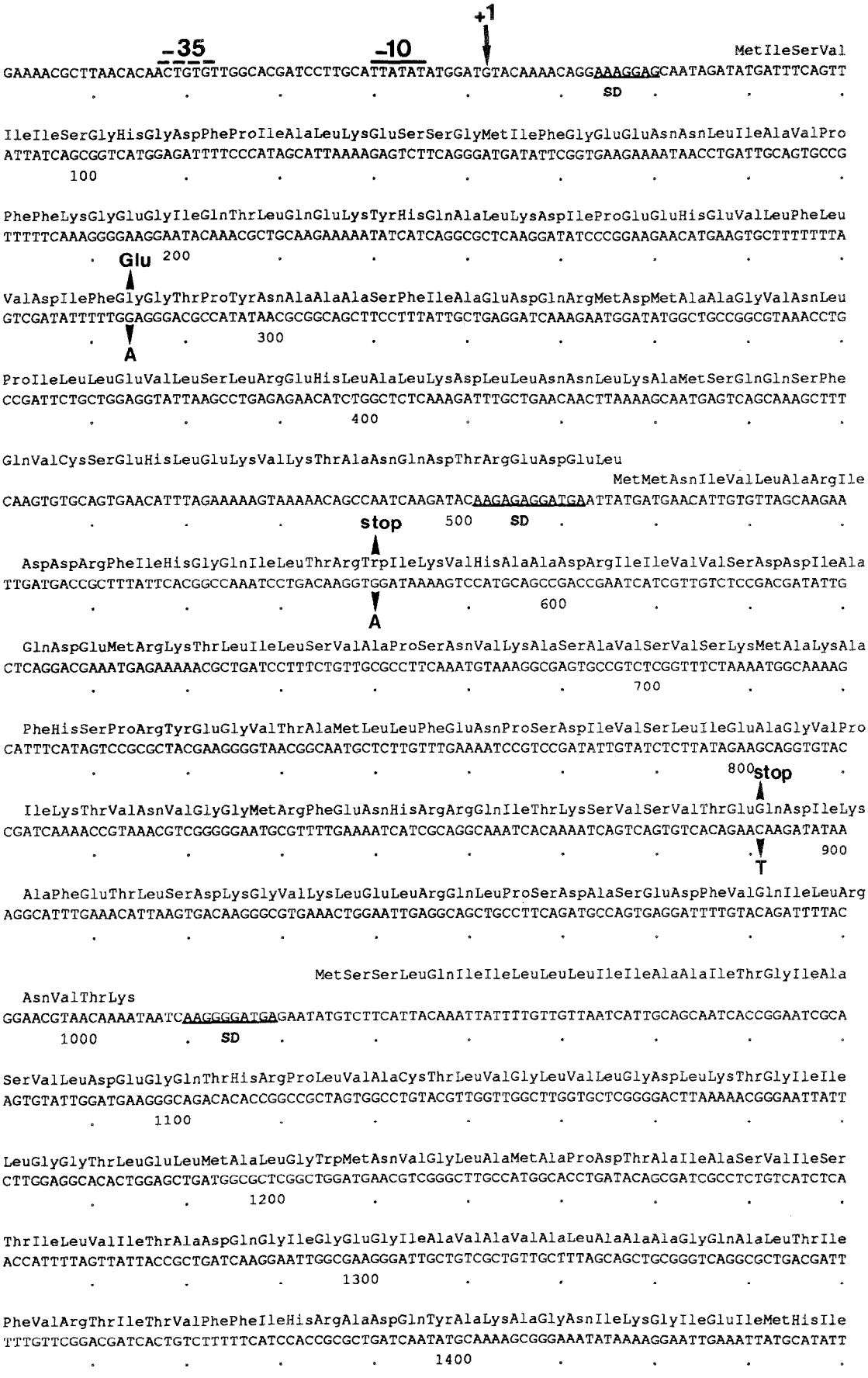

-35
-10
+1
MetIleSerVal
GAAAACGCTTAACACAACTGTGTTGGCACGATCCTTGCATTATATATGGATGTACAAAACAGGAAAGGAGCAATAGATATGATTTCAGTT
SD
IleIleSerGlyHisGlyAspPheProIleAlaLeuLysGluSerSerGlyMetIlePheGlyGluGluAsnAsnLeuIleAlaValPro
ATTATCAGCGGTCATGGAGATTTTCCCATAGCATTAAAAGAGTCTTCAGGGATGATATTCGGTGAAGAAAATAACCTGATTGCAGTGCCG
100
PhePheLysGlyGluGlyIleGlnThrLeuGlnGluLysTyrHisGlnAlaLeuLysAspIleProGluGluHisGluValLeuPheLeu
TTTTTCAAAGGGGAAGGAATACAAACGCTGCAAGAAAAATATCATCAGGCGCTCAAGGATATCCCGGAAGAACATGAAGTGCTTTTTTTA
Glu 200
ValAspIlePheGlyGlyThrProTyrAsnAlaAlaAlaSerPheIleAlaGluAspGlnArgMetAspMetAlaAlaGlyValAsnLeu
GTCGATATTTTTGGAGGGACGCCATATAACGCGGCAGCTTCCTTTATTGCTGAGGATCAAAGAATGGATATGGCTGCCGGCGTAAACCTG
A
300
ProIleLeuLeuGluValLeuSerLeuArgGluHisLeuAlaLeuLysAspLeuLeuAsnAsnLeuLysAlaMetSerGlnGlnSerPhe
CCGATTCTGCTGGAGGTATTAAGCCTGAGAGAACATCTGGCTCTCAAAGATTTGCTGAACAACTTAAAAGCAATGAGTCAGCAAAGCTTT
400
GlnValCysSerGluHisLeuGluLysValLysThrAlaAsnGlnAspThrArgGluAspGluLeu
MetMetAsnIleValLeuAlaArgIle
CAAGTGTGCAGTGAACATTTAGAAAAAGTAAAAACAGCCAATCAAGATACAAGAGAGGATGAATTATGATGAACATTGTGTTAGCAAGAA
stop 500 SD
AspAspArgPheIleHisGlyGlnIleLeuThrArgTrpIleLysValHisAlaAlaAspArgIleIleValValSerAspAspIleAla
TTGATGACCGCTTTATTCACGGCCAAATCCTGACAAGGTGGATAAAAGTCCATGCAGCCGACCGAATCATCGTTGTCTCCGACGATATTG
A
600
GlnAspGluMetArgLysThrLeuIleLeuSerValAlaProSerAsnValLysAlaSerAlaValSerValSerLysMetAlaLysAla
CTCAGGACGAAATGAGAAAAACGCTGATCCTTTCTGTTGCGCCTTCAAATGTAAAGGCGAGTGCCGTCTCGGTTTCTAAAATGGCAAAAG
700
PheHisSerProArgTyrGluGlyValThrAlaMetLeuLeuPheGluAsnProSerAspIleValSerLeuIleGluAlaGlyValPro
CATTTCATAGTCCGCGCTACGAAGGGGTAACGGCAATGCTCTTGTTTGAAAATCCGTCCGATATTGTATCTCTTATAGAAGCAGGTGTAC
800stop
IleLysThrValAsnValGlyGlyMetArgPheGluAsnHisArgArgGlnIleThrLysSerValSerValThrGluGlnAspIleLys
CGATCAAAACCGTAAACGTCGGGGGAATGCGTTTTGAAAATCATCGCAGGCAAATCACAAAATCAGTCAGTGTCACAGAACAAGATATAA
T
900
AlaPheGluThrLeuSerAspLysGlyValLysLeuGluLeuArgGlnLeuProSerAspAlaSerGluAspPheValGlnIleLeuArg
AGGCATTTGAAACATTAAGTGACAAGGGCGTGAAACTGGAATTGAGGCAGCTGCCTTCAGATGCCAGTGAGGATTTTGTACAGATTTTAC
MetSerSerLeuGlnIleIleLeuLeuLeuIleIleAlaAlaIleThrGlyIleAla
AsnValThrLys
GGAACGTAACAAAATAATCAAGGGGATGAGAATATGTCTTCATTACAAATTATTTTGTTGTTAATCATTGCAGCAATCACCGGAATCGCA
1000 SD
SerValLeuAspGluGlyGlnThrHisArgProLeuValAlaCysThrLeuValGlyLeuValLeuGlyAspLeuLysThrGlyIleIle
AGTGTATTGGATGAAGGGCAGACACACCGGCCGCTAGTGGCCTGTACGTTGGTTGGCTTGGTGCTCGGGGACTTAAAAACGGGAATTATT
1100
LeuGlyGlyThrLeuGluLeuMetAlaLeuGlyTrpMetAsnValGlyLeuAlaMetAlaProAspThrAlaIleAlaSerValIleSer
CTTGGAGGCACACTGGAGCTGATGGCGCTCGGCTGGATGAACGTCGGGCTTGCCATGGCACCTGATACAGCGATCGCCTCTGTCATCTCA
1200
ThrIleLeuValIleThrAlaAspGlnGlyIleGlyGluGlyIleAlaValAlaValAlaLeuAlaAlaAlaGlyGlnAlaLeuThrIle
ACCATTTTAGTTATTACCGCTGATCAAGGAATTGGCGAAGGGATTGCTGTCGCTGTTGCTTTAGCAGCTGCGGGTCAGGCGCTGACGATT
1300
PheValArgThrIleThrValPhePheIleHisArgAlaAspGlnTyrAlaLysAlaGlyAsnIleLysGlyIleGluIleMetHisIle
TTTGTTCGGACGATCACTGTCTTTTTCATCCACCGCGCTGATCAATATGCAAAAGCGGGAAATATAAAAGGAATTGAAATTATGCATATT
1400

Fig. 2.

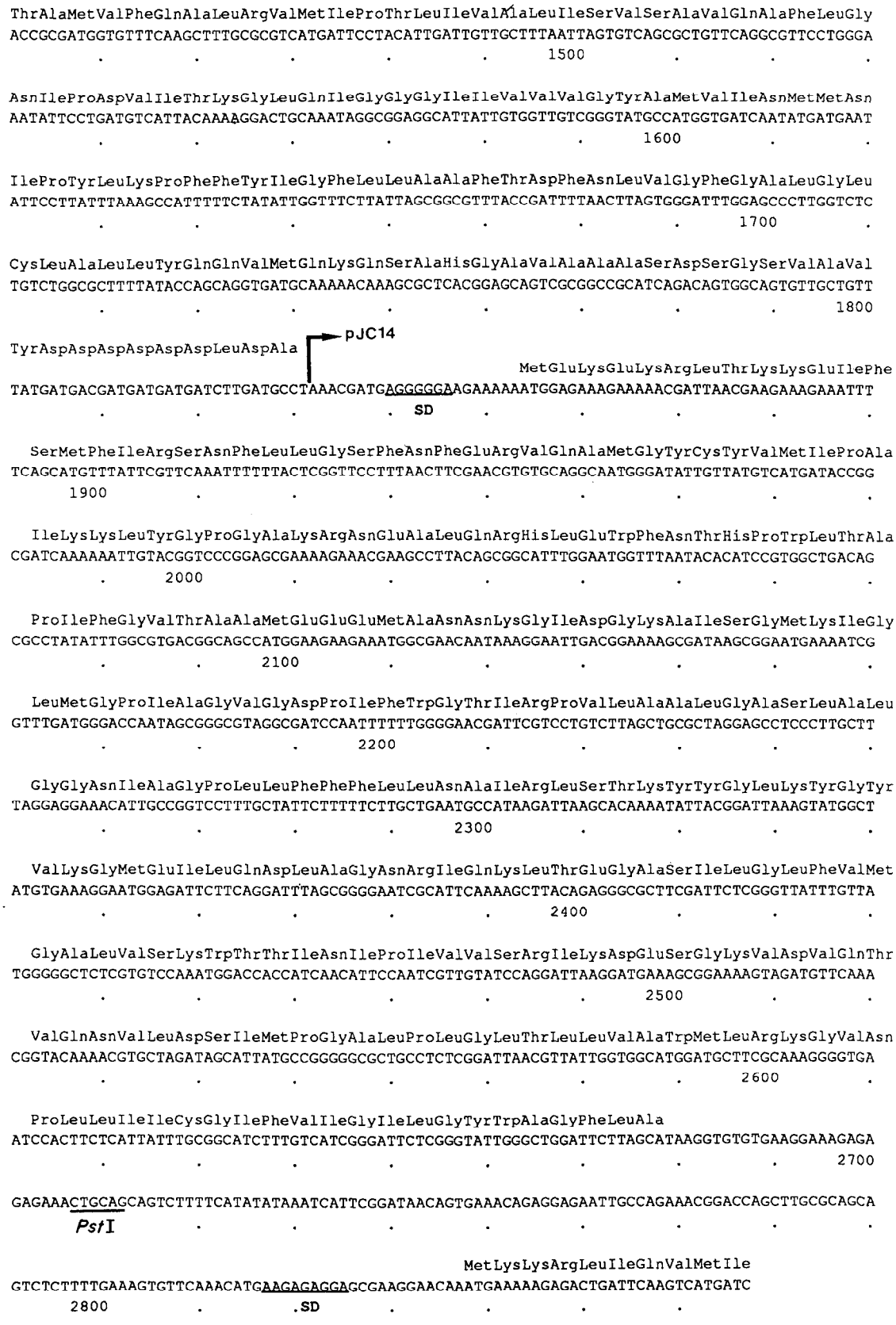
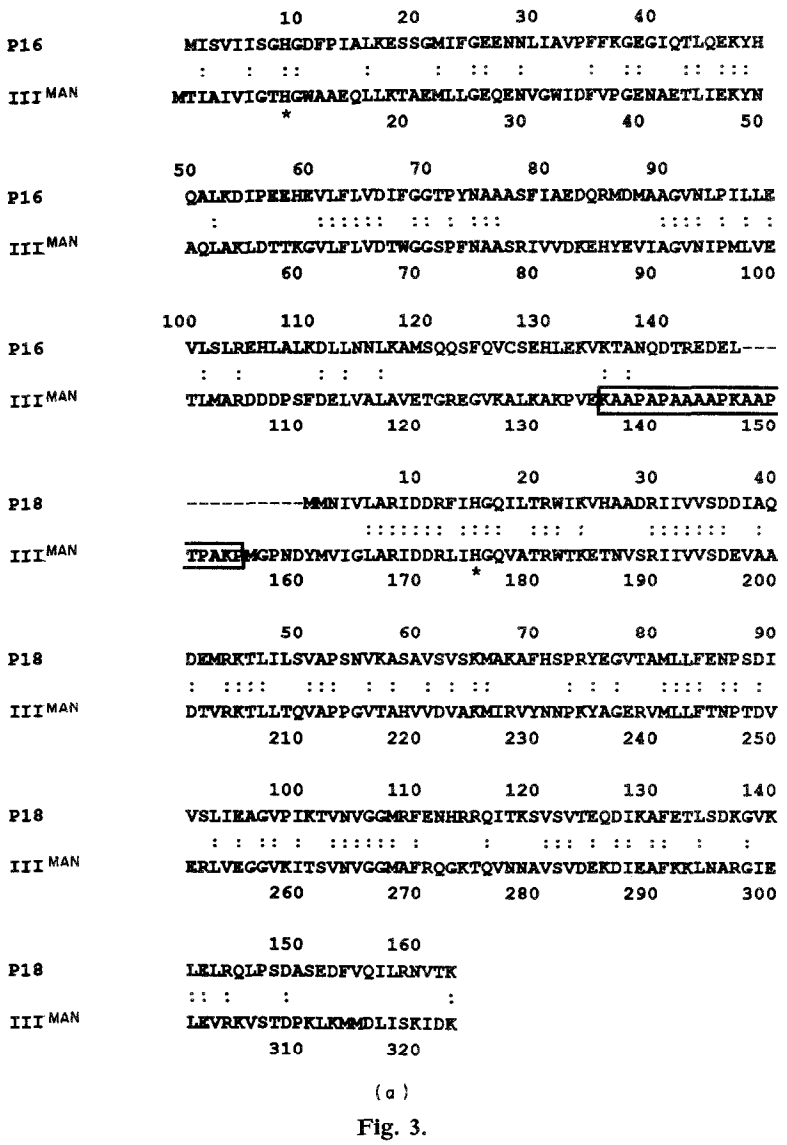


Figure 2. Nucleotide sequence of the 4 proximal genes of the levanase operon. The sequence of the 2867 bp DNA fragment containing the promoter, the 1st 4 genes of the levanase operon and part of the *sacC* gene is shown. The 4 ORFs are indicated. Putative ribosome binding sites (**SD**) are underlined (Hager & Rabinowitz, 1985). A vertical arrow indicates the position of the transcription start point (+1; Martin *et al.*, 1989). The corresponding −10 and −35 regions are overlined. The *lacZ* fusion joint of plasmid pJC14 is indicated by an arrow. *sacL6* (position 284), *sacL7* (position 580) and *sacL5* (position 891) mutations are indicated in the nucleotide sequence.



(a)

Fig. 3.

similar to the enzyme IIP of the mannose-PTS of *E. coli*, with 57% identical residues (Fig. 3(b)). Likewise, the *levG* gene product, P30, shows extensive similarity (60% identical residues) to the enzyme IIM^{Man} (Fig. 3(c)).

P16 and P18 are hydrophilic proteins with an average hydrophobicity (Kyte & Doolittle, 1982) of −0·08 and −0·07 and are therefore probably cytoplasmic proteins (data not shown). The polypeptides P28 and P30 are hydrophobic proteins with an average hydrophobicity (Kyte & Doolittle, 1982) of 1·02 and 0·45, respectively. Moreover, the hydropathy profiles of P28 and P30 are similar to those of IIP^{Man} and IIM^{Man} and the putative transmembrane domains present in IIM^{Man} and IIP^{Man} are conserved (data not shown).

(b) *The role of the products of the first four genes of the levanase operon in fructose-PTS uptake by* B. subtilis

The deduced amino acid sequences of *levD*, *levE*, *levF* and *levG* gene products are very similar to those of the mannose-PTS of *E. coli*. However, *in vivo* [^{14}C]mannose uptake experiments showed that P16, P18, P28 and P30 are not involved in mannose uptake in *B. subtilis* (data not shown).

The product of the fifth gene of the operon, levanase, is involved in the degradation of fructose polymers and the expression of the levanase operon is inducible by fructose. It appeared possible, therefore, that P16, P18, P28 and P30 were involved in fructose uptake. *B. subtilis* contains several fructose-PTS whose expression are inducible by fructose (Gay & Delobbe, 1977). To distinguish fructose uptake due to P16, P18, P28 and P30 from that of other systems, *sacL* mutants were used. In these mutants, the expression of the levanase operon is constitutive (Martin *et al.*, 1989). Moreover, in these mutants we may suppose that the levanase operon is expressed in the absence of fructose but the other fructose-PTS are not, as they require induction (Gay & Delobbe, 1977).

Strains QB166 (*sacL5*), QB169 (*sacL8*) and QB2018 (*sacL6*) were grown in CSK medium in the absence of fructose. Assays of [^{14}C]fructose uptake

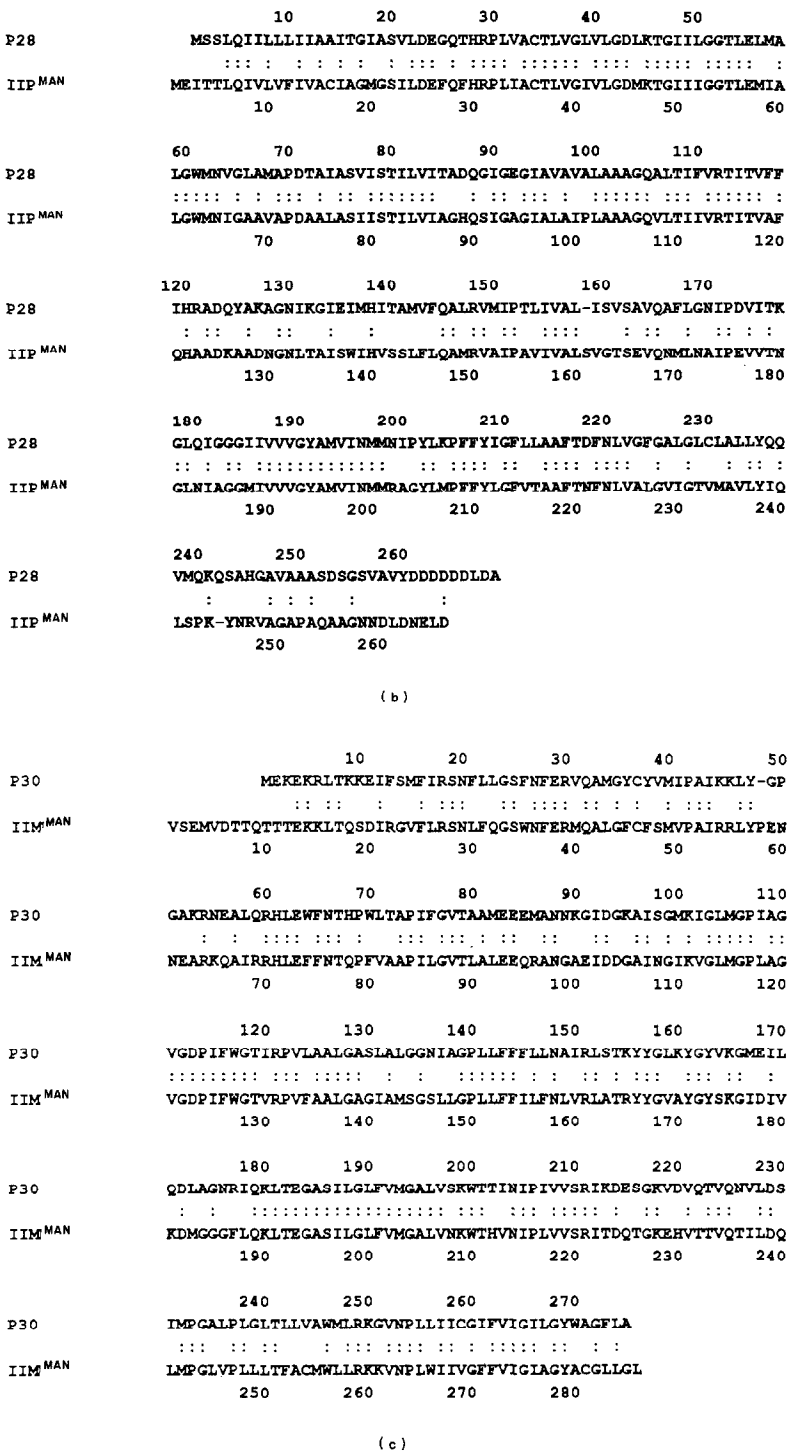


Figure 3. Comparison of the amino acid sequences of the polypeptides encoded by the 4 proximal genes of the levanase operon with that of the 3 specific enzymes of the mannose phosphotransferase system of *E. coli*. (:) Identical residues. Numbers indicate the position in the amino acid sequence of the relevant protein. (a) Alignment of P16 and P18 with the *E. coli* enzyme III^{Man}. Ala-Pro rich putative hinge peptide linking the 2 domains of III^{Man} is boxed (Erni, 1989). The 2 His residues (His10 and His175), phosphorylated in III^{Man} are indicated by stars. (b) Alignment of P28 and the *E. coli* IIP^{Man}. (c) Alignment of P30 and the *E. coli* IIM^{Man}.

were performed in these strains, as indicated in Materials and Methods. The results obtained are shown in Figure 4(a). Constitutive fructose uptake was observed in strain QB169 (*sacL8*). However, in strains QB166 (*sacL5*) and QB2018 (*sacL6*), fructose uptake was much lower and similar to that observed in an uninduced wild-type strain (Fig. 4(a)). These results will be considered in Discussion, in the light of the identification of the *sacL* mutations at the molecular level (see section (c), below).

The role of the *levF* and *levG* gene products in the fructose uptake observed in strain QB169 (*sacL8*)

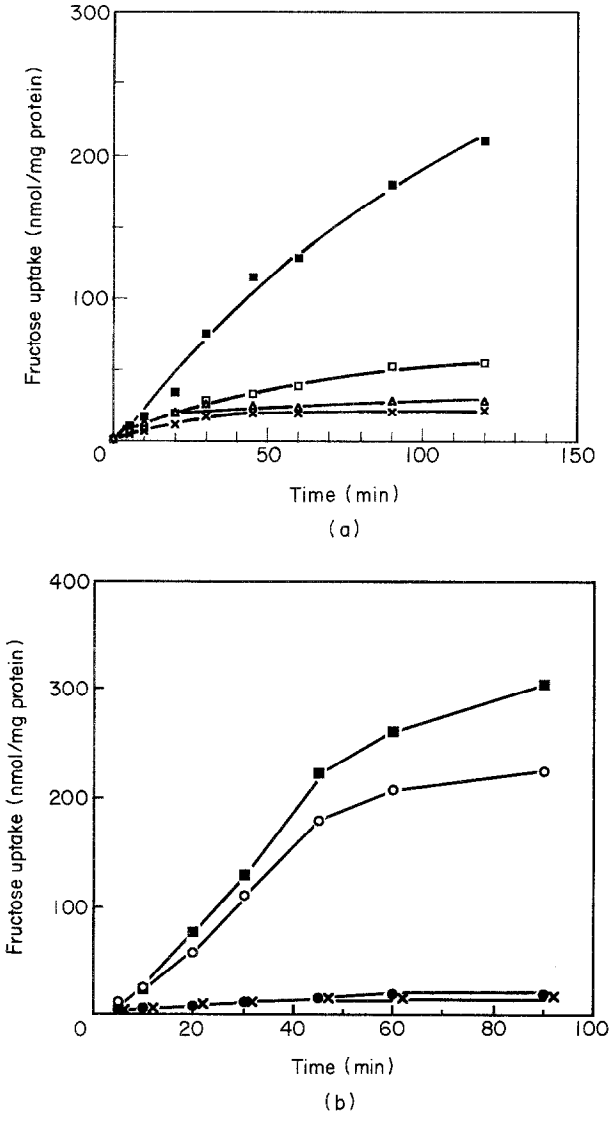


Figure 4. Fructose uptake in different *B. subtilis* strains. (a) Constitutive fructose uptake in *sacL* mutants (b) Involvement of the *levG* gene product in fructose uptake. Measurements of [^{14}C]fructose uptake were performed as described in Materials and Methods. The strains were grown in CSK in the absence of fructose. (■) QB169 (*sacL8*), (□) QB2018 (*sacL6*), (△) QB166 (*sacL5*), (●) QB5054 (*sacL8*, *levG'-lacZ*$^+$, *erm*), (○) QB5064 (*sacL8*, *levG'-lacZ*$^+$, *erm*, *amyE*::Pf-*levG*), (X) 168.

was tested. The *levF* and *levG* genes in the *sacL8* mutant were inactivated by insertion mutagenesis and [^{14}C]fructose uptake was measured. The constitutive fructose uptake by a *sacL8* mutant (strain QB169) was reduced to that observed in the wild-type strain when a *lacZ-erm* cassette was introduced into *levG* to give strain QB5054 (Fig. 4(b)). The *levG* gene product, P30, therefore takes part in fructose uptake. This result was corroborated by the following observation: constitutive fructose uptake was restored when a functional *levG* gene transcribed from the levanase operon promoter was introduced at the *amyE* locus of strain QB5054 to generate strain QB5064. The constitutive fructose uptake measured in strain QB5064 corresponded to 80% of that observed in a *sacL8* mutant (Fig. 4(b)). An insertion into the *levG* gene may therefore be partially complemented by an intact copy in *trans*.

A *levF* disruption was obtained by integration of the plasmid pJC27 containing an internal part of *levF* into the chromosome of a *sacL8* mutant (strain QB169). This integration was realized by a Campbell-type mechanism *via* homology of the plasmid insert with the chromosomal DNA giving strain QB5055 (Table 1). However, a polar effect upon *levG* transcription could not be ruled out in this strain. Therefore, a QB5055 derivative strain, QB5065, which contains the *levG* gene transcribed from its own promoter at the *amyE* locus, was constructed. The abolition of the fructose uptake in strain QB5065 as compared to the *sacL8* mutant, suggests that the *levF* gene product, P28, is in fructose transport (data not shown). In conclusion, the levanase operon is involved in fructose transport in a *sacL8* mutant grown under conditions of catabolic derepression.

The constitutive fructose uptake observed in a *sacL8* mutant may be dependent on the general proteins of the PTS, enzyme I and Hpr. To examine this possibility, the strain QB5061 carrying the *sacL8* and *ptsI6* mutations was constructed, and fructose uptake was measured in this strain. The inactivation of enzyme I due to the presence of the *ptsI6* mutation led to the abolition of constitutive fructose uptake in strain QB5061 (data not shown). This result confirms that the fructose transport observed in a *sacL8* mutant is dependent on the PTS.

(c) *Regulation of expression of the levanase operon*

(i) *Mapping of* sacL *mutations*

In *sacL* mutants, the genes of the levanase operon are constitutively expressed. The *sacC* and *sacL* loci map to the same region of the chromosome as determined by PBS1 transduction (Martin *et al.*, 1987). Also, two *sacL* mutations, *sacL6* and *sacL8*, have been shown to be closely linked by transformation to the *sacC* gene (Martin *et al.*, 1989). Here, the *sacL5* and *sacL7* mutations were located using chromosomal DNA of strain QB5030 as described by Martin *et al.* (1989). The *sacL5* and *sacL7* mutations were shown to be linked by transformation to the *sacC* gene with a percentage of co-transformation of 67 and 63, respectively.

In order to localize *sacL* mutations, plasmids pJC6 and pJC7 (Fig. 1(a)) were integrated by a Campbell-type mechanism into independently isolated *sacL* mutants. Plasmid pJC7 contained the wild-type promoter region on a 1·2 kb fragment and pJC6 carried the same promoter region as well as the first four genes of the levanase operon (Fig. 1(a)). Plasmids pJC6 and pJC7 were used to transform different *sacL* mutants and the percentage of wild-type integrants was determined for each mutant (Table 2).

If the mutations are in the region corresponding to DNA fragments inserted in pJC6 and pJC7, a proportion of the integrants will be transformed to wild-type. *sacL6* and *sacL8* mutations have been mapped by this technique to the promoter proximal region present in pJC7 (Martin *et al.*, 1989). Moreover, the transformation of QB2018 (*sacL6*) and QB169 (*sacL8*) with pJC6 gave 10% and 98% wild-type integrants, respectively. These results, in agreement with those obtained with pJC7, suggest that the *sacL6* and *sacL8* mutations are independent mutations at different sites.

All the pJC7 integrants in mutants QB166 (*sacL5*) and QB168 (*sacL7*) were constitutive (Table 2), indicating that neither mutation is in the 1·2 kb DNA fragment of pJC7. However, transformation of strains QB166 (*sacL5*) and QB168 (*sacL7*) with plasmid pJC6 gave 100% and 96% of wild-type integrants, suggesting that *sacL5* and *sacL7* mutations are located in the DNA fragment inserted into pJC6. To map the position of *sacL5* and *sacL7* mutations more closely, plasmid pJC14 (Fig. 1(a)) was linearized and used to transform strains QB166 (*sacL5*) and QB168 (*sacL7*). Spontaneous Em^r Cm^s transformants were obtained by a double cross-over event leading to the integration of the *levF'-lacZ*$^+$ fusion and the *erm* resistance determinant into the chromosome. With the *sacL5* and *sacL7* mutations, 51% and 46% of the integrants, respectively, constitutively expressed the transcriptional *levF'-lacZ*$^+$ fusion. These two mutations are located in the DNA fragment inserted into plasmid pJC14 but not in that carried by pJC7. These mutations are therefore in the levanase operon between the *Hin*dIII restriction site of *levD* and the end of *levF* (Fig. 5).

(ii) *Characterization of the* sacL *mutations*

Having located the *sacL5*, *sacL6*, *sacL7* and *sacL8* mutations, the nucleotide sequence of the corresponding DNA fragments was determined.

For this purpose a 1·8 kb DNA fragment, from position −55 to the end of the *levF* gene, was amplified using chromosomal DNA from each of the four *sacL* mutants and the PCR technique as described in Materials and Methods (Fig. 5).

Table 2
Mapping of sacL *mutations by integrative recombination*

Strain	Percentage of wild-type integrants with plasmid	
	pJC7	pJC6
QB166 (*sacL5*)	0	100
QB168 (*sacL7*)	0	96
QB169 (*sacL8*)	22	10
QB2018 (*sacL6*)	57	98

Strains QB166, QB168, QB169 and QB2018 were transformed with the integrative plasmids pJC6 and pJC7 (Fig. 1(a)). Cm^r transformants containing integrated plasmids (Campbell-type integration) were selected. Levanase synthesis of each transformant was tested on plates and their phenotype (wild-type or constitutive) was assessed. The percentage of wild-type integrants is indicated for each *sacL* mutant.

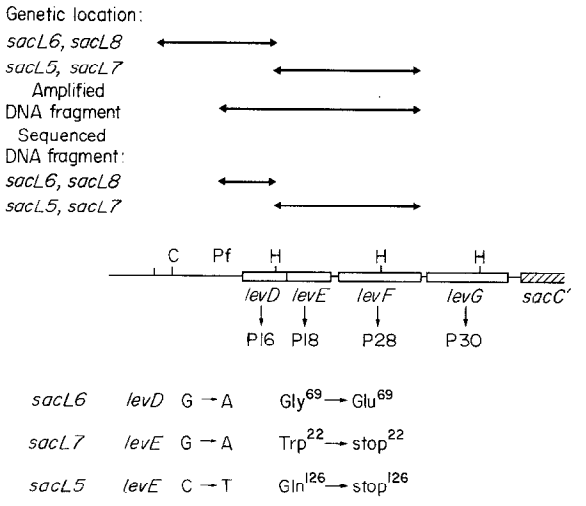


Figure 5. Mapping and characterization of *sacL* mutations. The position of *sacL5*, *sacL6*, *sacL7* and *sacL8* mutations, as determined by recombination experiments, is shown. The DNA fragments amplified by PCR, or sequenced, to identify the mutations are shown as double-headed arrows. The base changes corresponding to the mutations are shown. Pf, denotes a fructose inducible promoter. C, *Cla*I; H, *Hin*dIII.

Restriction sites introduced during amplification were used to clone fragments into phage M13 replicative form for sequence analysis. For each mutation the sequenced DNA fragment is indicated in Figure 5.

The *sacL8* mutation was not located between the −55 region of the promoter and the *Hin*dIII restriction site of pJC7. This mutation is therefore upstream from the levanase operon promoter in the 1·2 kb fragment of pJC7.

The *sacL5* and *sacL7* mutations were both located in the *levE* gene. Strain QB168 carries the *sacL7* mutation, a G to A transition, leading to the replacement of Trp22 by a stop codon. The *sacL5* mutation corresponds to a C to T transition in the 126th codon of *levE* replacing Gln126 by a stop codon (Figs 2 and 5). These two nonsense mutations in *levE* led to the constitutive expression of the levanase operon.

The *sacL6* mutation is a G to A transition in the *levD* gene. This mutation changes Gly69 to Glu. To test the dominance or recessivity of a wild-type copy over the *sacL6* allele, a merodiploid strain was constructed. The strain QB5066, containing a *sacC'-lacZ*$^+$ fusion, a *sacL6* mutation and a wild-type copy of the promoter and the four proximal genes of the levanase operon was obtained (Table 1). β-Galactosidase activity was tested on colonies of strain QB5066, grown on MMCH-X-gal plates in the presence or absence of 14 mM-fructose. The expression of the *sacC'-lacZ*$^+$ fusion was inducible

by fructose as in the wild-type strain. The *sacL6* allele was therefore recessive over the wild-type allele.

All the *sacL* mutations characterized in this work at the molecular level by DNA sequencing are located in the two genes whose products are homologous to the enzyme III^{Man} of *E. coli*.

(iii) *The role of the* levG *gene product in the induction of the levanase operon*

By analyzing *sacL* mutations we have shown that the *levD* and *levE* gene products are involved in the regulation of the levanase operon. Do the enzyme II types play a role in this regulation?

To test this possibility, a *lacZ erm* cassette was inserted into *levG* to give a *levG'-lacZ*$^+$ transcriptional fusion (strain QB5058), as described in Materials and Methods. β-Galactosidase activity was assayed in strain QB5058 grown in CSK medium in the absence or presence of 14 mM-fructose and compared to that observed in strain QB5030, carrying a *sacC'-lacZ*$^+$ fusion (Table 3). The results obtained indicate that in strain QB5058, the β-galactosidase was not induced in the presence of fructose. It appears, therefore, that the inactivation of P30 was responsible for the absence of inducibility of the levanase operon.

To test whether the *levG* gene in *trans* can restore fructose inducibility to a strain carrying an insertion into *levG*, strain QB5067 was constructed (see Materials and Methods and Table 1). This strain contains the *levG'-lacZ*$^+$ fusion and the promoter as well as the four proximal genes of the levanase operon at the *amyE* locus. β-Galactosidase activity was assayed in this strain grown in CSK medium in the presence or absence of 14 mM-fructose (Table 3). An eightfold increase of β-galactosidase synthesis was observed in the presence of fructose. However, the rate of β-galactosidase synthesis remained lower than in the wild-type strain grown in the presence of the inducer. It seems likely that the *levG* gene in *trans* can partially restore the fructose inducibility of the levanase operon in a *B. subtilis* strain containing an insertion into *levG*. The involvement of the *levF* gene product in fructose inducibility remains to be determined.

(iv) *Constitutive expression of the levanase operon in a* ptsI *mutant*

Previous reports suggest that levanase may be synthesized constitutively in a *ptsI* mutant deficient in the enzyme I of the PTS (Gay, 1979). To corroborate this observation, the strain QB5051 containing the *ptsI6* mutation and a transcriptional *sacC'-lacZ*$^+$ fusion was constructed as described in Materials and Methods. Strain QB5051 was grown in CSK medium with or without 14 mM-fructose and β-galactosidase activity was assayed (Table 3). The specific activity of β-galactosidase was 645 units/mg of protein in the absence of fructose and 798 units/mg of protein in the presence of fructose (see Materials and Methods). In a *ptsI6* mutant, the expression of the levanase operon is constitutive and higher than in the wild-type strain. In this strain the fructose can act as an inducer and a catabolite repressor (Martin *et al.*, 1989).

4. Discussion

The levanase gene *sacC* is the distal gene of a 5 kb operon. Four genes, *levD*, *levE*, *levF* and *levG* are present upstream from the *sacC* gene. The deduced polypeptides P16, P18, P28 and P30 are involved in a fructose-PTS. Fructose was transported in a *sacL8* mutant that synthesizes constitutively a functional levanase operon (Fig. 4). Constitutive *sacL* mutants carrying a missense mutation in *levD* (*sacL6*) or a nonsense mutation in *levE* (*sacL5*) do not transport fructose (Fig. 4(a)). These results strongly suggest that P16 (*levD*) and P18 (*levE*) take part in fructose uptake. Moreover, the constitutive uptake measured in the *sacL8* mutant was abolished when a *lacZ-erm* cassette was introduced into *levG*, or when the *levF* gene was partially deleted in the presence of the *levG* gene at the *amyE* locus (see Results, section (b)). The P28 (*levF*) and P30 (*levG*) polypeptides are therefore involved in fructose uptake. As expected, the constitutive transport observed in the *sacL8* mutant is dependent on the general protein of the PTS. Therefore the four proximal genes of the levanase operon form a fructose-PTS that is different from the previously described *fruA* system.

Table 3

Expression of levG'-lacZ$^+$ *and* sacC'-lacZ$^+$ *fusions in mutants carrying a* ptsI6 *mutation or an insertion into* levG

Strain	Relevant genotype	β-Galactosidase specific activity (units/mg protein)	
		CSK medium	CSKFru medium
QB5030	*sacC'-lacZ*$^+$	12	165
QB5051	*ptsI6*, *sacC'-lacZ*$^+$	645	798
QB5058	*levG'-lacZ*$^+$	10	3·5
QB5067	*levG'-lacZ*$^+$ *amyE*::(Pf-*levD-levE-levF-levG*)	7	60

Cultures were grown at 37 °C in CSK medium in the absence or presence of 14 mM-fructose (CSKFru). β-Galactosidase specific activities (expressed in units/mg protein) were determined in extracts prepared from exponentially growing cells (A_{600} = 0·6 to 0·7).

The amino acid sequences of P16, P18, P28 and P30 were compared to other PTS specific proteins. No homology was found with enzyme II^{Fru} of *E. coli* (Prior & Kornberg, 1988). However, the polypeptides P16, P18, P28 and P30 share similarities with the three specific proteins of the mannose-PTS of *E. coli*: III^{Man}, IIP^{Man} and IIM^{Man} (Erni *et al.*, 1987). It is interesting to note that fructose can be taken up *via* the mannose-PTS in *E. coli* (Postma & Lengeler, 1985; Kornberg, 1986).

P28 and P30, which are very similar to the enzymes IIP^{Man} and IIM^{Man}, present the characteristics of membrane proteins (see Results, section (a)). These observations strongly suggest that P28 and P30 correspond to enzyme II types of the PTS.

The P16 and P18 polypeptides are similar to the factor III^{Man}. The *E. coli* enzyme III^{Man} is a single polypeptide with two domains called P13 and P20. These two domains are linked by a 20 residue hinge peptide consisting of Ala-Pro repeats and a few Lys (Erni *et al.*, 1987; Erni, 1989; Fig. 3(a)). These authors have shown that modifications of the hinge (insertion of single amino acid residues, increase or decrease in size), or the synthesis of the two domains P13 and P20 as individual polypeptides, reduced but never completely abolished II^{Man} activity (Erni, 1989). In *B. subtilis*, the *levD* and *levE* genes encode two polypeptides homologous to these two domains and the hinge is absent. Interestingly, the *levD* and *levE* genes overlap at the sequence ATGA. The existence of a putative translational coupling could allow the stoichiometric synthesis of P16 and P18 as proposed for other *B. subtilis* operons (Zalkin & Ebbole, 1988).

In most of the PTS, one or two specific components (an enzyme II or an enzyme II–III pair) are transiently phosphorylated (Postma & Lengeler, 1985). Each of the enzyme II–III pairs and each of the III-independent enzyme II types are believed to be phosphorylated twice: once on each polypeptide for the II–III pairs, or twice on the same polypeptide for the other (Saier *et al.*, 1988). Four polypeptides are involved in the fructose-PTS encoded by the levanase operon, two enzyme II types (P28 and P30), and two factor III types (P16 and P18). This raises the following questions: how does this system function and how many polypeptides are phosphorylated? Interesting data from the mannose-PTS of *E. coli* have been recently described (Erni, 1989). The enzymes IIM^{Man} and IIP^{Man} are not phosphorylated, whereas the enzyme III^{Man} is phosphorylated twice. Both domains of enzyme III^{Man}, P13 and P20, become transiently phosphorylated in this process, P13 at His10 and P20 at His175 (Fig. 3(a)). P13, the N-terminal domain, is phosphorylated by Hpr and P20 is phosphorylated by P13 (Erni, 1989). It seems possible that the fructose-PTS encoded by the levanase operon functions in a similar way to the mannose-PTS of *E. coli*. If so, P16 (homologous to P13) and P18 (homologous to P20) might be phosphorylated successively and, in the presence of P28 and P30, transport and phosphorylate fructose. It is worth noting that

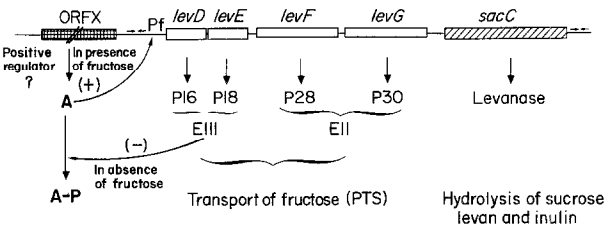


Figure 6. Model of regulation of the levanase operon. The genetic organization of the levanase operon of *B. subtilis* is represented. Convergent arrows correspond to region of dyad symmetry and Pf to the fructose inducible promoter. The *levD*, *levE*, *levF* and *levG* gene products correspond to a fructose-specific PTS. The ORFX gene encodes a putative positive regulator. This activator may exist in 2 forms (A–P), an inactive phosphorylated form or (A), an active non-phosphorylated form. The possible mechanism of regulation of the levanase operon is described in Discussion.

His10 and His175 phosphorylated in III^{Man} are conserved in P16 and P18 (Fig. 3(a)). Further experiments are needed to test these hypotheses.

The polypeptides encoded by the levanase operon have self regulatory roles. Three *sacL* constitutive mutations were localized in structural genes of the levanase operon. The *sacL5* and *sacL7* mutations were nonsense mutations in *levE*. In strains carrying these mutations, the presence of stop codons led to the synthesis of a truncated P18 polypeptide. As those two mutants constitutively synthesized levanase, it was concluded that neither mutation has a polar effect on *sacC* gene expression but that the P18 polypeptide therefore acts as a negative regulator of the levanase operon. The *sacL6* mutation is a missense mutation in *levD*, changing Gly69 to Glu69. In a merodiploid strain carrying the wild-type and the *sacL6* allele the expression of the levanase operon is inducible, suggesting that P16 also acts as a repressor. Therefore, both the *levD* and *levE* gene products that are involved in a fructose-PTS are also negative regulators of the expression of levanase operon.

A specific component of the PTS is also involved in induction of the *bgl* operon of *E. coli* (Mahadevan *et al.*, 1987; Schnetz & Rak, 1988). These authors proposed a model of regulation: the enzyme II^{Bgl}, which is involved in β-glucoside transport, exerts its negative regulator effect by phosphorylating the positive regulator BglG and thereby abolishing its activity (Amster-Choder *et al.*, 1989).

In *B. subtilis*, the expression of *sacA* encoding sucrase and *sacB* encoding levansucrase are also controlled by the PTS (Crutz *et al.*, 1990; Débarbouillé *et al.*, unpublished results).

In the case of the levanase operon, direct interaction of the negative regulators P16 and P18 with the promoter region as for the Lac repressor in *E. coli* (Beckwith, 1987), cannot be excluded. However, levanase induction may be controlled by

a PTS-mediated phosphorylation of a positive regulator like the *bgl* operon (Fig. 6).

An ORF (ORFX) located upstream from the promoter of the operon is involved in the expression of the *sacC* gene. Indeed, its inactivation abolished the expression of the gene (M.D., unpublished results). This result is consistent with ORFX encoding a positive regulator. In addition, the *sacL8* mutation was shown to be located upstream from the operon promoter and may map to the ORFX gene. Work is now in progress to characterize this gene.

The following is a possible model of the regulation of the levanase operon. In the presence of the inducer (fructose), the polypeptides P16, P18, P28 and P30 with the general proteins of the PTS are involved in a phosphotransfer cascade, leading to the transport and phosphorylation of fructose. In the absence of substrate, we postulate that the phosphate group is transferred, probably *via* the polypeptides P16 and P18, to the ORFX gene product thereby abolishing its activity. The disruption of the phosphotransfer cascade at the level of enzyme I, P16 or P18 prevents the inactivation of the activator, leading to a constitutive expression of the levanase operon (Fig. 6). However, polypeptide P30, and maybe P28, seem not to be directly involved in the phosphorylation of the activator. Indeed, when P30 is not functional, the levanase operon is not expressed, even in the presence of fructose. According to this model the consequence of its inactivation, which prevents fructose uptake, may lead to the phosphorylation of the activator by P16 and P18, even in the presence of the inducer. This system differs from the *E. coli bgl* operon in that the factors III of the levanase operon play the part of negative regulators, rather than an enzyme II.

Further studies are required to establish the mechanism of the induction of the levanase operon at the molecular level. In particular the role of the ORFX gene product in this regulation will be analyzed and the phosphorylation of the putative activator by the fructose-PTS will be tested.

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