Protein Phosphorylation Chain of a *Bacillus subtilis* Fructose-Specific Phosphotransferase System and Its Participation in Regulation of the Expression of the *lev* Operon†

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ABSTRACT: The proteins encoded by the fructose-inducible *lev* operon of *Bacillus subtilis* are components of a phosphotransferase system. They transport fructose by a mechanism which couples sugar uptake and phosphoenolpyruvate-dependent sugar phosphorylation. The complex transport system consists of two integral membrane proteins (LevF and LevG) and two soluble, hydrophilic proteins (LevD and LevE). The two soluble proteins form together with the general proteins of the phosphotransferase system, enzyme I and HPr, a protein phosphorylation chain which serves to phosphorylate fructose transported by LevF and LevG. We have synthesized modified LevD and LevE by fusing a His-tag to the N-terminus of each protein allowing rapid and efficient purification of the proteins. We determined His-9 in LevD and His-15 in LevE as the sites of PEP-dependent phosphorylation by isolating single, labeled peptides derived from ^{32}P-labeled LevD, LevD(His)$_6$, and LevE(His)$_6$. The labeled peptides were subsequently analyzed by amino acid sequencing and mass spectroscopy. Mutations replacing the phosphorylatable histidyl residue in LevD with an alanyl residue and in LevE with a glutamate or aspartate were introduced in the *levD* and *levE* genes. These mutations caused strongly reduced fructose uptake via the lev-PTS. The mutant proteins were synthesized with a N-terminal His-tag and purified. Mutant LevD(His)$_6$ was very slowly phosphorylated, whereas mutant LevE(His)$_6$ was not phosphorylated at all. The corresponding *levD* and *levE* alleles were incorporated into the chromosome of a *B. subtilis* strain expressing the *lacZ* gene under control of the *lev* promoter. The mutations affecting the site of phosphorylation in either LevD or LevE were found to cause constitutive expression from the *lev* promoter of *B. subtilis*.

The phosphoenolpyruvate (PEP)[1]:carbohydrate phosphotransferase system (PTS) catalyzes the uptake and concomitant phosphorylation of numerous sugars in both Gram-positive and Gram-negative bacteria (Postma et al., 1993). In addition, the PTS is implicated in regulation of chemotaxis, carbon metabolism, and gene expression (Saier, 1989).

The bacterial phosphotransferase system comprises two cytoplasmic proteins, enzyme I and the histidine-containing phosphocarrier protein (HPr), and several sugar-specific permeases (enzyme II complex) which in some cases can form a single multidomain protein or can be split into several distinct proteins (Postma et al., 1993). During the uptake of a substrate by the PTS, a phosphoryl group is transferred from PEP to the different carbohydrates via several proteins transiently phosphorylated. Enzyme I and HPr are required for the transport and phosphorylation of all PTS sugars and are therefore called the general proteins of the PTS. The sugar-specific PTS proteins which comprise the enzyme II complexes consist of three to four proteins or protein domains termed IIA, IIB, IIC, and IID (Saier & Reizer, 1992). IIC and IID are integral membrane components, whereas IIA and IIB are localized either in the cytoplasm or at the cytoplasmic surface of the inner membrane. IIA and IIB are transiently phosphorylated and act as phosphoryl transfer proteins. The PTS is involved in the vectorial transport of mono- and disaccharides as well as sugar alcohols in *Bacillus subtilis.* These PTS substrates include glucose, fructose, mannose, mannitol, β-glucosides, and sucrose (Gay et al., 1973; Steinmetz, 1993). Enzyme I, HPr, and several enzymes II have been characterized genetically or biochemically in this organism (Fouet et al., 1989). While the HPr protein of Gram-negative bacteria is phosphorylated only once (at His-15) (Weigel et al., 1982), its Gram-positive counterpart is subject to a second, ATP-dependent phosphorylation at Ser-46 (Deutscher & Saier, 1983; Deutscher et al., 1986). Phosphorylation at His-15 requires PEP and enzyme I and is necessary for the phosphoryl transfer to EII and for the sugar uptake and phosphorylation (Reizer et al., 1989). HPr and some EIIBs are phosphorylated at the Nδ1 atom of a histidine, whereas enzyme I and the EIIAs are phosphorylated at the Nϵ2 atom of a histidine (Postma et al., 1993).

On the basis of genetic data, Gay and Delobbe (1977) proposed the existence of three fructose-specific PTS in *B. subtilis*, the expression of which is fructose-inducible. The major fructose uptake system involves the product of the *fruA* gene, the enzyme II specific for fructose. A second fructose-inducible PTS has been identified, the components

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[1] Abbreviations: PTS, phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphocarrier protein; IPTG, isopropyl 1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; PAG(E), polyacrylamide gel (electrophoresis).

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Table 1: Bacterial Strains Used in This Study

strain	genotype	reference
B. subtilis		
168	*trpC2*	laboratory stock
QB5081	*trpC2 amyE*::[PΔB *levD′-′lacZ(cat)*]	Martin-Verstraete et al. (1992)
QB5091	*trpC2 levR8 amyE*::[PΔB *levD′-′lacZ(cat)*]	Martin-Verstraete et al. (1992)
QB5187	*trpC2 amyE*::[PΔB *levD′-′lacZ (aphA3)*]	Stülke et al. (1995)
QB5294	*trpC2 levDH9A amyE*::[PΔB *levD′-′lacZ (aphA3)*]	this work
QB5295	*trpC2 levEH15E amyE*::[PΔB *levD′-′lacZ (aphA3)*]	this work
QB5296	*trpC2 levEH15D amyE*::[PΔB *levD′-′lacZ (aphA3)*]	this work
QB5340	*leuA8 hisAI sacA321 levD6 amyE*::[PΔB *levD′-′lacZ (aphA3)*]	Stülke et al. (1995)
QB5342	*trpC2 levE7 amy*E::[PΔB *levD′-′lacZ (aphA3)*]	Stülke et al. (1995)
E. coli		
TGI	K12Δ(*lac pro*) *supE thi hsd5*/F′ *traD36 proA*$^{+B}$ *lacI*^{q} *lacZ* Δ*M15*	Gibson (1984)
CJ236	*dut ung thi relA*/pJC105[Cm^{R}]	Bio-Rad Laboratories, Richmond, CA
NM522	*supE thi-1* Δ(*lac-proAB*) Δ(*hsdSM-mcrB*) (*rk*$^{-}$ *mk*$^{-}$) (F′ *proAB lacIq lacZ* Δ*M15*)	Gough and Murray (1983)
M15 [pREP4]	*nal*^{S} *str*^{S} *rif*^{S} *lac*$^{-}$ *ara*$^{-}$ *gal*$^{-}$ *mtl*$^{-}$ *F*$^{-r}$ *recA*$^{+}$ *uvr*$^{+}$	Villarejo and Zabin (1974)

of which are encoded by the *lev* operon and resemble the *Escherichia coli* mannose PTS (Martin-Verstraete et al., 1990). The *lev* operon of *B. subtilis* contains five genes. The distal gene, *sacC*, encodes levanase, a β-fructofuranosidase, which hydrolyzes sucrose and fructose polymers such as levan and inulin (Kunst et al., 1977; Martin et al., 1987). The first four genes of the operon, *levD*, *levE*, *levF*, and *levG*, encode a fructose-specific phosphotransferase system (lev-PTS) (Martin-Verstraete, 1990). The expression of the *lev* operon in *B. subtilis* is inducible by fructose and is subject to catabolite repression (Martin et al., 1989). The transcription of the *lev* operon is controlled by (i) the RNA polymerase associated with a specific sigma factor, σ^{L}, which is a homologue of σ^{54} in Gram-negative bacteria (Débarbouillé et al., 1991c); (ii) a transcriptional activator, LevR, which is a member of the NifA/NtrC family of transcriptional regulators (Débarbouillé et al., 1991b); (iii) the LevD and LevE proteins, the IIA and IIB components of the fructose-specific PTS encoded by the *lev* operon, which negatively regulate their own expression, and (iv) the CcpA/P-Ser-HPr complex which regulates the expression of the *lev* operon in response to glucose availability (Martin-Verstraete et al., 1995). The current model for the induction of the *lev* operon predicts that LevR is a positive regulator inactivated by PEP-dependent phosphorylation via LevD and LevE in the absence of fructose in the growth medium. In the presence of fructose, LevD and LevE are dephosphorylated, as they participate in PTS-dependent fructose uptake and phosphorylation, and LevR is consequently present in the cell in the unphosphorylated, active form (Martin-Verstraete et al., 1990).

The mannose transport system of *E. coli* (Kundig & Roseman, 1971; Erni et al., 1987), the sorbose transport system of *Klebsiella pneumoniae* (Wehmeier et al., 1995), and two putative *N*-acetylagalactosamine-specific IIB proteins of *E. coli* (Reizer et al., 1996) are highly homologous to the fructose-specific transport system of *B. subtilis* encoded by the *lev* operon (40−65% identical amino acid residues). These four homologous PTS differ in structure and phylogenetic origin from other known PTS. They possess two membrane components called IIC and IID. The soluble IIA and IIB domains are fused to form IIAB^{Man}. An Ala-Pro rich hinge links the IIA and IIB domain of IIAB^{Man} (Stolz et al., 1993; Markovic-Housley et al., 1994). No IIA protein was found within the *aga* gene cluster of *E. coli*, but a gene presumably encoding IIA^{Aga} has been found far from the *aga* gene cluster between 2.4 and 4.1 min at the *E. coli* genome (Reizer et al., 1996). In the two other systems, the soluble PTS components exist as independent proteins called LevD/SorB and LevE/SorF.

IIAB^{Man} is phosphorylated twice and transfers the phosphoryl group from HPr to the transported substrate in the following order: HPr (His-15) → IIA (His-10) → IIB (His-175) → mannose-6-OH. IIAB^{Man} is presumably phosphorylated at the indicated histidines. Replacement of one of these histidines by other amino acids prevents the phosphorylation of the corresponding domain (Stolz et al., 1993). Phosphoryl group transfer in the IIAB^{Man} dimer occurs within one subunit from domain IIA to domain IIB, but IIA of one subunit can also phosphorylate IIB of the second subunit (Stolz et al., 1993). His-86 of the IIA domain was suggested to play a role in the phosphoryl group transfer between the IIA and IIB domains, since a His-86-Asn mutant protein was not phosphorylated in IIB. This histidine is replaced with an arginine in LevD and with a glutamine in SorF. It is possible that this arginine and glutamine in LevD and SorF, respectively, play a role similar to His-86 of IIA^{Man} in the phosphoryl group transfer. According to the crystal structure of the *E.coli* IIA^{Man} domain, His-86 is located far from His-10 and its role in the phosphoryl group transfer remains unknown (Nunn et al., 1996).

In this paper, we present genetic and biochemical evidence that LevD can be phosphorylated by PEP, enzyme I, and HPr and that phosphorylation of LevE requires LevD in addition to enzyme I, HPr, and PEP. Moreover, we have determined the phosphorylation site of LevD and LevE and replaced the phosphorylatable amino acid in LevD with an alanyl residue and that in LevE with an aspartate, glutamate, or alanyl residue and investigated the effect of the mutations on fructose transport and on the expression of the *lev* operon.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The *B. subtilis* and *E. coli* strains used in this study are listed in Table 1.

B. subtilis was grown in C medium (70 mM K_2HPO_4, 30 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 10 mM $MnSO_4$, 22 mg ferric ammonium citrate per liter, and auxotrophic requirements at a concentration of 100 mg/L). CSK is C medium supplemented with 22 mM potassium succinate and 54 mM potassium glutamate. Solid LB and SP media were used to grow *E. coli* or *B. subtilis* strains, respectively (Martin et al., 1989).

Transformation Procedures. Standard procedures were used to transform *E. coli* cells (Sambrook et al., 1989), and transformants were selected on solid LB medium supplemented with ampicillin (100 μg/mL). *B. subtilis* cells were transformed as previously described using plasmid or chromosomal DNA (Martin-Verstraete et al., 1990), and transformants were selected on solid SP medium containing kanamycin (5 μg/mL) or chloramphenicol (5 μg/mL).

β-Galactosidase Activity. β-Galactosidase activities were measured after growth of *B. subtilis* strains in CSK medium using the method of Miller (1972). One unit of β-galactosidase activity is defined as the amount of enzyme which produces 1 nmol of *o*-nitrophenol per min at 28 °C.

Plasmid Constructions. Standard procedures were used to extract plasmids from *E. coli* (Sambrook et al., 1989). Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the JetSorb kit (Genomed, Bioprobe System).

The *levD* gene and the *levE* gene were cloned into the expression vector pQE-30 (Quiagen) which allowed the fusion of a polyhistidine sequence to the N-terminus of the two proteins. The 438 bp *levD* gene was amplified by PCR using the plasmid pJC6 (Martin-Verstraete et al., 1990) as template and the two following synthetic oligonucleotides, 5′-AAAGGATCCATAGATATGATTTCAGTTATT-3′ and 5′-CATGAATTCTTGCTAACACAAT GTTCATCA-3′, as primers, allowing the creation of a *Bam*HI site at the 5′ end and an *Eco*RI site at the 3′ end of *levD*. The *Bam*HI-*Eco*RI fragment was cloned into the vector pBluescript KS (Stratagene). A *Bam*HI-*Sal*I fragment was cut from the resulting plasmid and cloned into pQE-30, providing plasmid pQE-30D. The 489 bp *levE* gene was amplified by PCR using the plasmid pJC6 (Martin-Verstraete et al., 1990) as template and the two following synthetic oligonucleotides, 5′-GATACAAGAGAGGATCCATGATGAACATTGTG-3′ and 5′-CACTTGC GATTCCCGGGTTGATTGCTGAC-3′, as primers, allowing the creation of a *Bam*HI site at the 5′ end and a *Sma*I site at the 3′ end of *levE*. A *Bam*HI-*Sma*I fragment was cloned into pQE-30, providing plasmid pQE-30E. The correct sequence of the PCR-amplified *levD* and *levE* genes as well as their correct fusion to the vector part encoding the six histidines was confirmed by DNA sequencing. DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (1977), using the Sequenase kit (Amersham).

Site-Directed Mutagenesis. pQE-30E15 is a derivative of pQE-30E in which two bases were exchanged by site-specific oligonucleotide-directed mutagenesis using the following synthetic oligonucleotide: 5′-CACGGATCCATGATGAACATTGTGTTAGCAAGAATTGATGACCGCTTTATTGCCGGC-3′ (57 mer). In this primer, the two nucleotides CA of codon 15 of the wild-type *levE* gene were replaced with GC (underlined) resulting in a His-15 to Ala mutation in LevE. The second oligonucleotide used to amplify the *levE* allele had the following sequence: 5′-TAATTAAAGCTTGGCTGCAGGTCGACCCGGGTTA-3′, containing a *Hin*dIII and a *Sma*I restriction site. After amplification by PCR, a *Bam*HI-*Hin*dIII fragment was cloned into the expression vector pQE-30, giving plasmid pQE-30E15.

The Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad) was used to obtain the mutant proteins LevDH9A, LevEH15D, and LevEH15E based on the procedure described by Kunkel et al. (1987). A 1.1 kb *Pvu*II fragment from pJC6 (Martin-Verstraete et al., 1990) containing the *levD* gene and the 5′ end of the *levE* gene was integrated into M13mp19 cut with *Sma*I to give M13mp19DE. Point mutations replacing His-9 in LevD with Ala or His-15 in LevE with Asp or Glu were introduced in *levD* or *levE* by using this phage DNA and appropriate oligonucleotides according to the manufacturer's description. The presence of the correct mutations was verified by DNA sequencing. M13 replicative forms carrying one of the above mutations were extracted and digested with *Eco*RI and *Sph*I. The corresponding *Eco*RI-*Sph*I DNA fragments were introduced into the integration vector pJH101 (Ferrari et al., 1983) to give pJC33 (*levDH9A*), pJC34 (*levEH15D*), and pJC35 (*levEH15E*).

The *levDH9A* allele was cloned into pQE-30 as follows: the replicative form M13mp19DE containing the *levD* allele encoding His-9-Ala LevD and two appropriate oligonucleotides were used to amplify the *levDH9A* gene by PCR, creating a *Bam*HI site at the 5′ end and a *Sma*I site at the 3′ end of *levD*. The PCR product was cut with *Sma*I-*Bam*HI and integrated into pQE-30 cut with *Sma*I-*Bam*HI, providing plasmid pQE-30D9.

The strains QB5294, QB5295, and QB5296 containing a *levD′-′lacZ* translational fusion at the *amyE* locus and one of the above point mutations in the *levD* or *levE* gene located in the *lev* operon were constructed as follows. Competent cells of *B. subtilis* strain 168 were co-transformed with chromosomal DNA derived from strain QB5187 (*trpC2 amyE*::[PΔB *levD′-′lacZ* (*aphA3*)]) in which the *lacZ* gene was expressed under control of the *lev* promoter and its upstream activating sequence (Stülke et al., 1995) and with the plasmids pJC33, pJC34, or pJC35 linearized with *Pst*I. Kanamycin-resistant transformants carrying the *levD′-′lacZ* translational fusion were found to form white colonies on solid SP medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside when the lev-PTS phosphorylation chain was functional, but blue colonies when the lev-PTS phosphorylation chain was inactivated by replacement of the wild-type *levD* or *levE* gene with one of the mutant genes. The loss of the vector part of pJC33, pJC34, and pJC35 was verified by testing the chloramphenicol sensitivity of the various transformants. The chromosomal copies of the *levD* or *levE* alleles of strains QB5294 (*levDH9A*), QB5295 (*levEH15E*), and QB5296 (*levEH15D*) were amplified by PCR, and the presence of the mutations was verified by DNA sequencing.

Overproduction and Purification of LevD(His)$_6$ and LevE-(His)$_6$ and Their Corresponding Mutant Proteins. E. coli M15 containing the plasmid pREP4 (Quiagen) was transformed with the expression vectors pQE-30D, pQE-30E, pQE-30D9, and pQE-30E15, encoding LevD and LevE as well as the mutant proteins LevDH9A and LevEH15A, respectively. Each of the expressed proteins carried a His-tag fused to the N-terminus. M15[pREP4] cells containing one of the above plasmids were grown overnight and diluted 50-fold into fresh LB medium. When the culture had reached an OD_{600} of about 0.7, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Incubation was continued for 3 h. Cells were collected by centrifugation, resuspended in a buffer (1 mL/g wet cells) containing 100 mM Tris-HCl, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride, and disrupted by two passages through a French Pressure cell at 15 000 psi. The crude

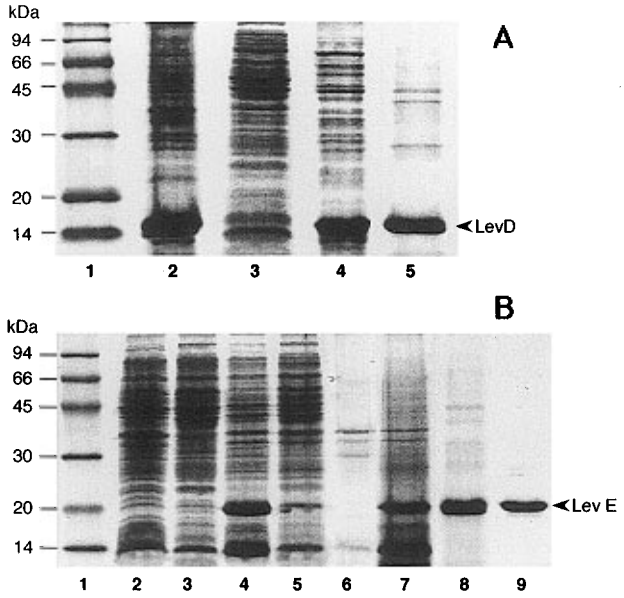

FIGURE 1: 12.5% SDS−PAG on which overexpression, solubilization, and purification of LevD$(His)_6$ and LevE$(His)_6$ is documented. The following samples were applied in panel A: lane 1, molecular weight standards; lanes 2 and 3, pellet and supernatant of a crude extract prepared from LevD$(His)_6$ overexpressing cells; lane 4, solute of the pellet obtained with 1 M guanidinium chloride; lane 5, pool of LevD$(His)_6$ containing fractions after elution from the Ni^{2+}-NTA column. Panel B: lane 1, molecular weight markers; lanes 2 and 3, pellet and supernatant of a crude extract prepared from non-induced cells; lanes 4 and 5, pellet and supernatant of a crude extract prepared from LevE$(His)_6$ expressing cells; lanes 6 and 7, pellet and supernatant after solubilization of precipitated LevE$(His)_6$ with 1.5 M guanidinium chloride; lane 8, pool of LevE-$(His)_6$ containing fractions after elution from the Ni^{2+}-NTA column; lane 9, LevE$(His)_6$ after G10 size exclusion chromatography.

extract was centrifuged for 30 min at 15 000 rpm using a SS34 rotor. The overexpressed LevD or LevE proteins were found mainly in the pellet from which they were recovered by solubilization with 1.5 M guanidinium chloride for 2 h (Figure 1). After centrifugation (15 000 rpm, 10 min), the supernatants containing the overexpressed proteins were applied onto a Ni-NTA column. The column was washed with 3 column volumes of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM Na_2SO_4, and 15% glycerol) and subsequently with 3 column volumes of buffer A containing 30 mM imidazole to elute unspecifically bound proteins before LevD$(His)_6$ or LevE$(His)_6$ or their mutant derivatives were eluted with buffer A containing 300 mM imidazole. The fractions containing LevD, LevE, or their mutant derivatives were pooled and applied on a desalting column (Sephadex G10, Pharmacia) run with 10 mM Tris-HCl, pH 7.4 (Figure 1). Protein concentrations were determined spectrophotometrically (Bio-Rad protein assay). Protein solutions were stored at −20 °C.

Protein Phosphorylation Assay. The two proteins enzyme I and HPr are necessary for the PEP-dependent phosphorylation of LevD$(His)_6$ and LevE$(His)_6$, and they were purified from *Staphylococcus carnosus* (Kohlbrecher et al., 1992) and from *B. subtilis* (Eisermann et al., 1988), respectively. For the phosphorylation of LevD without a His-tag, enzyme I and HPr carrying a His-tag were used. Enzyme I from *B. subtilis* was synthesized with a His-tag fused to its N-terminus after cloning a 1661 bp *Bam*HI-*Sal*I fragment obtained by PCR into the expression vector pQE30 (Quiagen) cut with the same enzymes. Similarly, HPr of *B. subtilis* carrying a His-tag at its N-terminus was synthesized from vector pQE30 containing a *Bam*HI-*Hin*dIII PCR fragment with the *B. subtilis ptsH* gene. The modified enzyme I and HPr carrying 10 additional amino acids at their N-terminus were purified in a one-step procedure on a Ni-NTA column (Quiagen). [^{32}P]PEP used for the protein phosphorylation assays was prepared from γ-[^{32}P]ATP as described by Roosien et al. (1983).

Phosphorylation of LevD$(His)_6$ or LevE$(His)_6$ by enzyme I and HPr was carried out using various protein concentrations. The following composition was found to give optimal results: LevD$(His)_6$ (9 μg) was phosphorylated by incubating a reaction mixture containing enzyme I (0.2 μg), HPr (0.1 μg), $MgCl_2$ (12.5 mM), Tris-HCl (50 mM, pH 7.4), and 1 μM [^{32}P]PEP (0.5 μCi) for 20 min at 37 °C in a total volume of 20 μL. The same reaction mixture containing 0.5 μg of LevD$(His)_6$ was used to phosphorylate 4 μg of LevE$(His)_6$. The phosphorylation reaction was stopped by adding an equal volume of sample buffer (Laemmli, 1970). Proteins were separated by SDS−PAGE (12.5% acrylamide, 0.3% methylenebisacrylamide, Bio-Rad System). Gels were dried for 2 h without prior fixation or coloration and exposed to autoradiography (Biomax MR, Kodak). Identical phosphorylation experiments were attempted with the two mutant proteins LevDH9A$(His)_6$ and LevEH15A$(His)_6$.

Determination of the Phosphorylation Site in LevD$(His)_6$ and in LevE$(His)_6$. LevD$(His)_6$ (400 μg) was phosphorylated as described above and subsequently digested by incubating the phosphorylation mixture with 20 μg of trypsin (in 30 mM Tris-HCl buffer, pH 8.5) for 2 h at 37 °C (protease to protein ratio 1:20). The proteolytic fragments were separated by metal chelate affinity chromatography (Ni-NTA column). A labeled peptide was eluted from the Ni-NTA column with 500 mM imidazole which was further purified by reverse phase high-pressure liquid chromatography on a C18 Delta Pak column (5 μ, 300 Å, 3.9 × 150 mm, Millipore). The following two buffers were used for the elution of peptides from the C18 column: buffer A contained 0.1% trifluoroacetic acid and 10% acetonitrile, and buffer B contained 0.08% trifluoroacetic acid and 90% acetonitrile. Peptides were eluted at a flow rate of 0.8 mL/min using a gradient of 0−60% B in 60 min. The optical density of the effluent was monitored at 220 and 280 nm on a full scale of 0.25 absorption units. Peptides eluting from the column were collected, and radioactivity in the fractions was determined by measuring Cerenkov radiation. The N-terminal amino acid sequence of the labeled peptides obtained from ^{32}P-labeled LevD$(His)_6$ was determined by Edman degradation on an automated amino acid sequencer (Applied Biosystems 473A). In addition, the peptides were analyzed by mass spectroscopy using electrospray ionization. A similar experiment was performed with LevE$(His)_6$. Phosphorylation of LevE$(His)_6$ was carried out in the presence of LevD without a His-tag (gift of B. Erni). Endoproteinase Lys-C was used to digest phosphorylated LevE$(His)_6$ (protease to protein ratio, 1:50) by incubating the phosphorylation mixture in 25 mM Tris-HCl, pH 7.7, containing 1 mM EDTA for 2 h at 37 °C. Two labeled peptides were isolated from [^{32}P]LevE-$(His)_6$ after metal chelate chromatography and reverse phase chromatography, which were carried out as described for phosphorylated LevD$(His)_6$. Amino acid sequence analysis and mass spectroscopy of the labeled peptides were performed as described above. Although the repressor homo-

logue CcpA carrying the same His-tag as LevD(His)$_6$ at its N-terminus (Deutscher et al., 1995) was not phosphorylated by [^{32}P]PEP, enzyme I, and HPr, we carried out control experiments with LevD without a His-tag (LevD was kindly provided by B. Erni) to ensure that the isolated peptide was not phosphorylated at a histidyl residue of the His-tag. LevD was phosphorylated under the same conditions as LevD-(His)$_6$, but enzyme I and HPr of *B. subtilis*, each carrying a His-tag at the N-terminus, were used. The phosphorylation mixture was subsequently passed over a Ni-NTA column. Phosphorylated LevD eluted in the first washing fraction, whereas enzyme I and HPr were bound to the Ni-NTA column. Phosphorylated LevD was subsequently digested with trypsin, and the digest was applied onto a reverse phase column as described above. Amino acid sequence analysis was carried out with the single-labeled peptide eluted from the reverse phase column by applying the same acetonitrile gradient as described for LevD(His)$_6$.

Fructose Uptake Measurements. B. subtilis strains were grown in CSK medium. Sugar uptake assays were performed as described (Delobbe et al., 1971). Medium C containing glycerol (10 g/L) and chloramphenicol (250 mg/L) was used for the fructose uptake experiments. Exponentially growing cells were harvested by centrifugation after they had reached an OD_{600} of 0.6−0.8 and were washed once with the uptake buffer. Cells were resuspended in the uptake buffer and incubated at 37 °C with shaking. [^{14}C]Fructose (37 kBq per mL) and unlabeled fructose (final concentration 0.4 mM) were added. Samples (0.5 mL) were withdrawn at the indicated time points and filtered on membrane filters (0.45 μm pore size, type HA, Millipore). Filters were washed with 15 mL of the uptake solution kept at 37 °C and dried. Radioactivity was measured by scintillation counting.

RESULTS

Overexpression and Purification of LevD(His)$_6$, LevE-(His)$_6$, and Their Mutant Derivatives. Plasmids pQE-30D and pQE-30E containing either the *B. subtilis levD* or *levE* gene were used to produce modified LevD or LevE, carrying 14 or 12 additional amino acids, respectively, (including six histidines) at their N terminus. Expression of LevD(His)$_6$ (Figure 1A, lanes 2 and 3) as well as of LevE(His)$_6$ (Figure 1B, lanes 2−5) was inducible by IPTG. The presence of the His-tag allowed a one-step purification of LevD(His)$_6$, LevE(His)$_6$ (Figure 1) and their mutant derivatives (data not shown). After overexpression, LevD(His)$_6$ and LevE(His)$_6$ were found to be present mainly in inclusion bodies (Figure 1A, lanes 2 and 3, and Figure 1B, lanes 4 and 5). After solubilization with guanidinium chloride, proteins were loaded on a metal chelate affinity chromatography column and proteins bound to the column were eluted with a Tris-HCl buffer, pH 7.4, containing imidazole (300 mM). The purity of LevD(His)$_6$ and LevE(His)$_6$ before and after chromatography was tested by SDS−PAGE (Figure 1A, lanes 4 and 5; Figure 1B, lanes 7 and 8). Purified LevD-(His)$_6$ and LevE(His)$_6$ ran on SDS−PAG according to their expected molecular weight of 18 and 20 kDa, respectively (Figure 1).

In Vitro Phosphorylation of LevD(His)$_6$ and LevE(His)$_6$. LevD(His)$_6$ was phosphorylated by [^{32}P]PEP in the presence of purified enzyme I and HPr (Figure 2, lane 2). LevD-(His)$_6$ could not be phosphorylated if one of these two proteins, enzyme I or HPr, was missing in the reaction mixture (data not shown). LevE(His)$_6$ was phosphorylated only when LevD(His)$_6$ was present in the phosphorylation mixture, indicating that the phosphoryl group is transferred from LevD to LevE (Figure 2, lanes 3 and 4). The overall phosphate group transfer for the protein phosphorylation chain of the fructose-specific phosphotransferase system encoded by the *B. subtilis lev* operon can therefore be written as

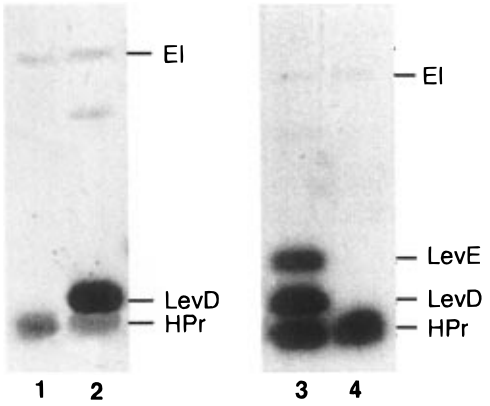

FIGURE 2: In vitro [^{32}P]PEP-dependent phosphorylation of LevD-(His)$_6$ and LevE(His)$_6$. Autoradiogram obtained from a 12.5% SDS−PAG on which samples containing the following proteins have been loaded: lane 1, enzyme I and HPr; lane 2, enzyme I, HPr, and LevD(His)$_6$; lane 3, enzyme I, HPr, LevD(His)$_6$, and LevE(His)$_6$; lane 4, enzyme I, HPr, and LevE(His)$_6$. The detailed phosphorylation conditions are described in Materials and Methods. After 20 min of incubation at 37 °C, sample buffer was added and proteins were separated by SDS−PAGE, followed by autoradiography. The positions of phosphorylated enzyme I, HPr, LevD(His)$_6$, and LevE(His)$_6$ are indicated.

$$\text{PEP} \rightarrow \text{enzyme I} \rightarrow \text{HPr} \rightarrow \text{LevD} \rightarrow \text{LevE} \rightarrow \text{fructose}$$

When carrying out a time dependence of LevD(His)$_6$ phosphorylation, we found that LevD(His)$_6$ was rapidly phosphorylated by enzyme I and HPr using a molar ratio of HPr to LevD(His)$_6$ of 1 to 40 (data not shown). Already after 30 s, the first time point, HPr and LevD(His)$_6$ were found to be completely phosphorylated. Under the employed conditions, LevD(His)$_6$ stayed phosphorylated for over 80 min (last time point), while enzyme I and HPr were almost completely dephosphorylated after 40 and 60 min, respectively, probably due to slow inactivation of enzyme I which is assumed to form inactive monomers when present at low concentrations (Chauvin et al., 1994). Interestingly, there seems to be only a slow phosphate group transfer from phosphorylated LevD(His)$_6$ to HPr, as HPr remained dephosphorylated despite the presence of phosphorylated LevD-(His)$_6$ in the reaction mixture. The almost complete disappearance of phosphorylated HPr can be due neither to inactivation of HPr, as HPr is very stable and resists even heat treatment, nor to digestion with a protease contaminating one of the purified proteins, as the band corresponding to HPr was not diminished during incubation as judged by PAGE. By contrast, phosphorylation of LevE(His)$_6$ was much slower using a molar ratio of LevD(His)$_6$ to LevE-(His)$_6$ of 1 to 8. Maximal phosphorylation was reached between 5 and 15 min of incubation at 37 °C, and phosphorylated LevE(His)$_6$ was subsequently slowly de-

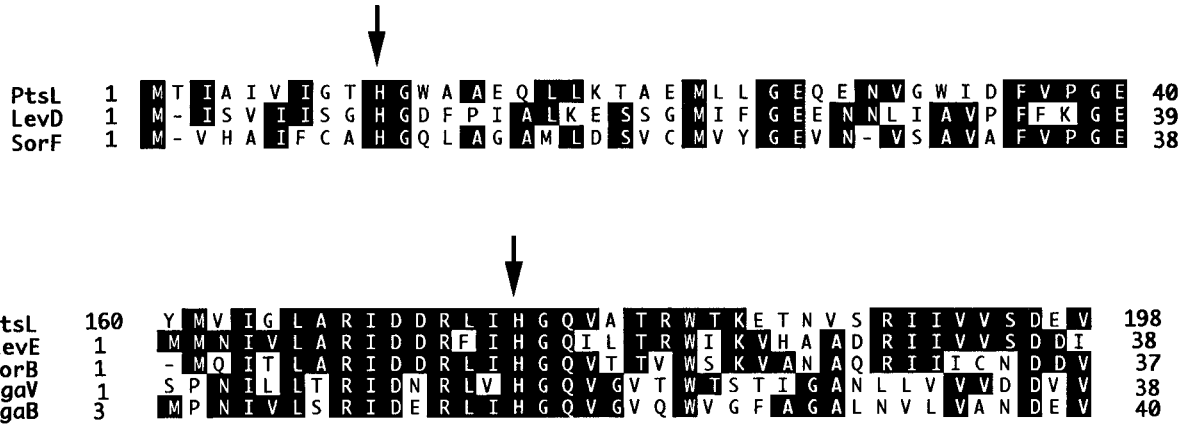

FIGURE 3: Multiple alignment of the regions around the conserved phosphorylation site of the N-terminal domain of PtsL (IIA^{Man}), LevD, and SorF (upper panel) and of the C-terminal domain of PtsL (IIB^{Man}), LevE, SorB, AgaV, and AgaB (lower panel). Amino acids conserved in at least two of the three (panel A) or five (panel B) aligned proteins are boxed. The sites of phosphorylation are indicated by arrows. The *E. coli* PtsL sequence was taken from Erni et al. (1989), the sequences of LevD and LevE of *B. subtilis* were taken from Martin-Verstraete et al. (1990), the *K. pneumoniae* SorF and SorB sequences were taken from Wehmeier et al. (1995), and the *E. coli* AgaB and AgaV sequences were taken from Reizer et al. (1996).

phosphorylated with a small amount still being phosphorylated after 80 min.

The phosphate bond in phosphorylated LevD$(His)_6$ or LevE$(His)_6$ was rapidly hydrolyzed under acidic conditions, whereas it was found to be fairly stable at alkaline conditions (pH higher than 10) suggesting that the phosphoryl group in these two proteins is bound to a histidyl or cysteyl residue forming either a phosphoamidate or thiophosphate bond (Mattoo et al., 1984; Pas & Robillard, 1988; Postma et al., 1993).

Identification of the Phosphorylation Site in LevD and LevE. Only His-9 in LevD and His-15 in LevE are conserved within the proteins belonging to the mannose class PTS, $IIAB^{Man}$ (also called PtsL) of *E. coli*, SorF and SorB of *K. pneumoniae*, and AgaV and AgaB of *E. coli* (Figure 3), although both *B. subtilis* proteins contain several histidyl residues. When one of the conserved histidyl residues of $IIAB^{Man}$ was replaced with another amino acid, no phosphorylation of the corresponding domain of $IIAB^{Man}$ was observed (Stolz et al., 1993).

In order to identify the phosphorylated histidine within the amino acid sequence of LevD and LevE, 400 μg of [^{32}P]-LevD$(His)_6$ and 300 μg of [^{32}P]LevE$(His)_6$ were prepared and subsequently digested with trypsin or endoproteinase Lys-C, respectively. The peptides carrying the N-terminal His-tag of either LevD$(His)_6$ or LevE$(His)_6$ were expected to contain the presumed phosphorylation site, His-9 in LevD and His-15 in LevE. The proteolytic fragments were therefore first passed over a Ni-NTA column, and for LevD-$(His)_6$ as well as for LevE$(His)_6$ a strongly labeled peptide could be eluted with 500 mM imidazole. These peptides were further purified by reverse phase chromatography on a C18 column. A major peptide and a strong peak of radioactivity coeluted at 36% buffer B in the case of LevE-$(His)_6$ (Figure 4, panel C), whereas in the case of LevD-$(His)_6$ a major peptide and a strong peak of radioactivity coeluted at 41% buffer B (data not shown). Radioactivity was measured in all fractions eluted from the reverse phase column (Figure 4, panel D). In addition to the radioactive peak eluting in the first fractions and corresponding to hydrolyzed phosphate, one major and one minor radioactive peak were detected for LevE$(His)_6$. The minor peak eluted at 28% buffer B and contained about 4% of the radioactivity found in the major peak (Figure 4, panel D). No peptide could be detected in the fraction corresponding to the minor peak observed in the Lys-C digest of LevE$(His)_6$, as was shown by amino acid sequence analysis and mass spectroscopy. In the case of LevD$(His)_6$, only one radioactive peak eluting at 41% buffer B was observed. All other fractions collected during the separation of digests of ^{32}P-labeled LevD$(His)_6$ or LevE$(His)_6$ on a reverse phase column contained less than 2% of the radioactivity found in the major radioactive peak. A digest of LevE$(His)_6$ with LysC and the peptides not bound to the Ni-NTA column were also separated on the C18 reverse phase column (Figure 4, panels A and B). Compared to Figure 4A, the signal of the peptide eluting at 36% buffer B was diminished in Figure 4B. In addition, a few peptides present in Figure 4A were found to be absent in Figure 4B. These peptides were probably unspecifically retarded on the Ni-NTA column. Amino acid sequence analysis of the major radioactive peptides obtained by reverse phase chromatography revealed the sequence GSHHHHHH for the peptide isolated from LevD$(His)_6$ and MRGSHHHHHHGSMMNIVLA for the peptide isolated from LevE$(His)_6$, confirming that the labeled peptides are derived from the N-terminal part of the corresponding protein. In order to determine the size of the whole peptides, mass spectroscopy was carried out using electrospray ionization. The mass of the labeled fragment derived from LevD-$(His)_6$ and LevE$(His)_6$ was found to be 3137.5 and 4356.9 Da, respectively. The value determined for the labeled peptide derived from LevD$(His)_6$ was in agreement with the value calculated for the second N-terminal tryptic peptide ranging from amino acid 3 to 27 (3138 Da). However, the mass determined for the labeled Lys-C peptide derived from LevE$(His)_6$ differed from the value calculated for the N-terminal peptide ranging from amino acid 1 to 36 (4340 Da) by 16.9, suggesting that one of the methionyl residues present in this peptide was oxidized to the sulfoxide. In addition to the histidines present in the His-tag, each peptide contained only one histidyl residue which corresponds to the histidyl residue conserved within the members of the mannose class PTS, His-9 in LevD and His-15 in LevE (Figure 3). These two histidyl residues therefore most likely represent the site of phosphorylation in LevD and LevE. To make sure that LevD$(His)_6$ is indeed phosphorylated at His-9

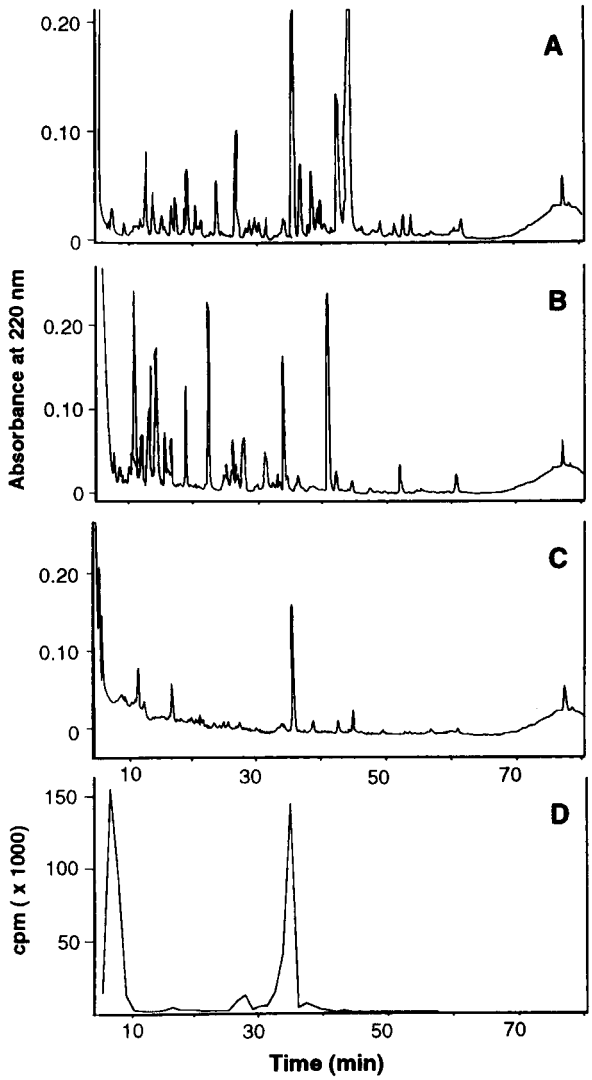

FIGURE 4: Reverse phase chromatography on a C18 column of Lys-C digests of phosphorylated LevE(His)$_6$. (A) Reverse phase chromatography on a C18 column of 17 nmol of purified, phosphorylated LevE(His)$_6$ digested with LysC and not passed over a Ni-NTA column. Phosphorylated, digested LevE(His)$_6$ was passed over a Ni-NTA column, and the washing fraction (flow-through) (B) or the peptides eluted with imidazole (C) were applied onto a C18 column. After a 5 min wash with 10% acetonitrile in 0.1% trifluoroacetic acid, peptides were eluted with a linear gradient from 10% to 60% buffer B (see Materials and Methods) in 60 min (the flow rate was 0.8 mL/min). A gradient from 60% to 100% buffer B in 10 min was subsequently applied. The optical density at 220 nm was measured in the effluent, and eluting peptides were collected manually. (D) Distribution of radioactivity was determined in the various fractions by measuring Cerenkov radiation.

and not at one of the histidyl residues of the His-tag, peptides were prepared from 400 μg of [^{32}P]LevD without a His-tag by cleavage with trypsin. Phosphorylation was carried out with enzyme I and HPr from *B. subtilis*, each carrying a His-tag at the N-terminus. After 20 min of incubation, the assay mixture was loaded on a Ni-NTA column to remove phosphorylated enzyme I and HPr. Phosphorylated LevD was collected, digested with trypsin, and loaded on a reverse phase column. A single-labeled peak was observed which was found to coelute with a peptide of the following N-terminal sequence: MISVIISGHGDF. This peptide represents the N-terminus of LevD and it contains His-9, thus suggesting that His-9 is indeed the site of phosphorylation in LevD.

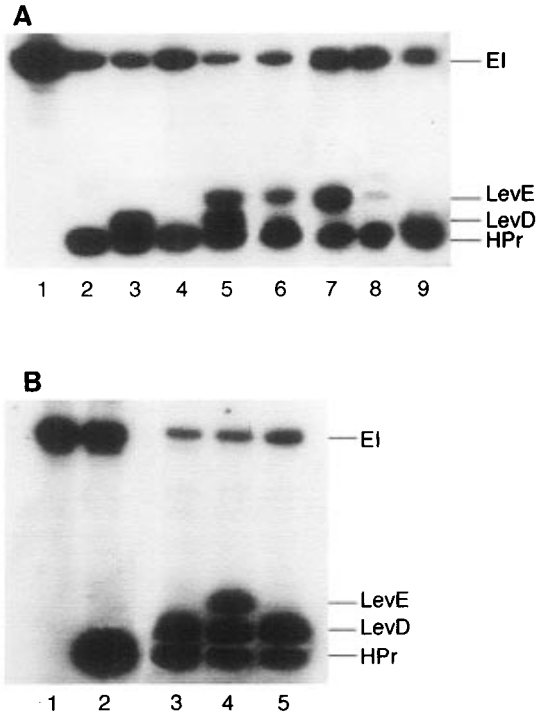

FIGURE 5: In vitro [^{32}P]PEP-dependent phosphorylation of (A) LevDH9A(His)$_6$ and (B) of LevEH15A(His)$_6$ mutant proteins. Experimental conditions for LevDH9A(His)$_6$ and LevEH15A(His)$_6$ phosphorylation were the same as described in Figure 2 for the corresponding wild-type proteins. An autoradiogram obtained from a 12.5% polyacrylamide−SDS gel is shown on which samples containing the following proteins had been loaded. Panel A: lane 1, enzyme I; lane 2, enzyme I and HPr; lane 3, enzyme I, HPr, and LevD(His)$_6$; lane 4, enzyme I, HPr, and LevDH9A(His)$_6$; lanes 5 and 7, enzyme I, HPr, LevD(His)$_6$ (9 μg in lane 5; 0.5 μg in lane 7), and LevE(His)$_6$; lanes 6 and 8, enzyme I, HPr, LevDH9A(His)$_6$ (9 μg in lane 6; 0.5 μg in lane 8), and LevE(His)$_6$; lane 9, enzyme I, HPr, LevDH9A(His)$_6$ (0.5 μg), and LevEH15A(His)$_6$. Panel B: lane 1, enzyme I; lane 2, enzyme I and HPr; lane 3, enzyme I, HPr, and LevD(His)$_6$; lane 4, enzyme I, HPr, LevD(His)$_6$, and LevE(His)$_6$; lane 5, enzyme I, HPr, LevD(His)$_6$, and LevEH15A-(His)$_6$. The positions of phosphorylated enzyme I, HPr, LevD(His)$_6$, and LevE(His)$_6$ are indicated.

In Vitro Phosphorylation Assays with the Mutant Proteins LevDH9A(His)$_6$ and LevEH15A(His)$_6$. Each of the conserved and presumably phosphorylated histidyl residues, His-9 of LevD and His-15 of LevE, was replaced by site-directed mutagenesis with an alanine (see Materials and Methods), a non-phosphorylatable amino acid. The mutant proteins were expressed from the vector pQE-30 and carried a His-tag at the N-terminus as described for the wild-type proteins. The two mutant proteins, LevDH9A(His)$_6$ and LevEH15A(His)$_6$, were purified and tested for potential PEP-dependent phosphorylation. Under the conditions employed for the experiments shown in Figure 5A, LevDH9A(His)$_6$ was not phosphorylated (Figure 5A, lane 4). However, when LevDH9A(His)$_6$ was stored in a buffer composed of 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 100 mM KCl, and 50% glycerol, slight phosphorylation of the mutant protein could be observed (Figure 5A, lane 9). Phosphorylation of LevDH9A(His)$_6$ was only slightly increased when carrying out a time dependence up to 170 min. This result confirms that His-9 represents the major site of phosphorylation in LevD. As shown in Figure 5B, lane 5, LevEH15A(His)$_6$ could not be phosphorylated *in vitro* under conditions in which the corresponding wild-type LevE(His)$_6$ was strongly

Table 2: Effect of Point Mutations in *levD* or *levE* on the Expression of the *levD′-′lacZ* Translational Fusion

strain	relevant genotype	β-galactosidase activity (units/mg of protein)[a]
QB5187	*levD*$^+$*levE*$^+$	20 (350)[b]
QB5340	*levD6*	4160
QB5294	*levDH9A*	3600
QB5342	*levE7*	4290
QB5295	*levEH15E*	3585
QB5296	*levEH15D*	3250

[a] Bacteria were grown at 37 °C in CSK medium in the absence of fructose. β-Galactosidase activities, expressed in units/mg of protein, were determined in extracts prepared from exponentially growing cells (A_{600} = 0.6−0.8). [b] Activity after induction with 0.2% fructose is given in parentheses.

phosphorylated (Figure 5B, lane 4). Even after storage of LevEH15A(His)$_6$ in the buffer composed of 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 100 mM KCl, and 50% glycerol, extending the incubation (up to 80 min) or increasing the amount of LevE in the reaction mixture (up to 10 μg), no phosphorylation of the mutant protein could be detected (data not shown), suggesting that His-15 is the only site of phosphorylation in LevE.

Interestingly, wild-type LevE(His)$_6$ was found to be slowly phosphorylated by the mutant protein LevDH9A(His)$_6$ (Figure 5A, lanes 6 and 8). Two conditions of phosphorylation were tested. In lane 6 of Figure 5A, 9 μg of LevDH9A(His)$_6$ was incubated together with 4 μg of LevE-(His)$_6$, whereas in lane 8 a 10-fold lower amount of enzyme I and HPr and only 0.5 μg of LevDH9A(His)$_6$ was used together with 4 μg of LevE(His)$_6$. All other conditions were identical. In the presence of the higher amount of LevDH9A-(His)$_6$, LevE(His)$_6$ was phosphorylated much more strongly compared to the experiment carried out in the presence of the lower amount of LevDH9A(His)$_6$. If the mutant protein LevEH15A(His)$_6$ was incubated with 9 μg of LevDH9A-(His)$_6$, no phosphorylation of the mutant LevE could be observed (Figure 5A, lane 9).

Construction and Characterization of Strains Carrying Point Mutations in the levD or levE Gene. To study the mechanism by which LevD and LevE regulate the expression of the *B. subtilis lev* operon, we constructed three *B. subtilis* mutant strains in which the amino acid of the phosphorylation site of either LevD (His-9) or LevE (His-15) was replaced with other amino acids. *B. subtilis* mutants synthesizing one of the following mutant proteins, LevDH9A, LevEH15E, or LevEH15D, were obtained by co-transformation using chromosomal DNA of strain QB5187, carrying a *levD′-′lacZ* fusion at the *amyE* locus, and one of the following plasmids, pJC33, pJC34, or pJC35, and subsequent screening of the transformants for the presence of mutations (see Materials and Methods).

The effect of point mutations in *levD* (*levD6* or *levDH9A*) or in *levE* (*levE7*, *levEH15D* or *levEH15E*) on the expression of the *levD′-′lacZ* translational fusion present at the *amyE* locus was tested. The strains were grown in CSK medium and their β-galactosidase activities were measured (Table 2). As previously described for the *levD6* (*levDG59E*) and the *levE7* mutations (stop codon at amino acid 22 in LevE) (Martin-Verstraete et al., 1990), the *levDH9A*, *levEH15D*, and *levEH15E* mutations caused also constitutive expression of the *lacZ* gene being under control of the *lev* promoter (Table 2). β-Galactosidase activity in the mutant strains was about 150-fold elevated compared to a non-induced wild-type strain and 10-fold higher than in the induced wild-type strain. These results suggest that modification of LevD or LevE at the site of PEP-dependent phosphorylation abolishes the negative effect exerted by LevD and LevE on the activity of the transcriptional activator LevR.

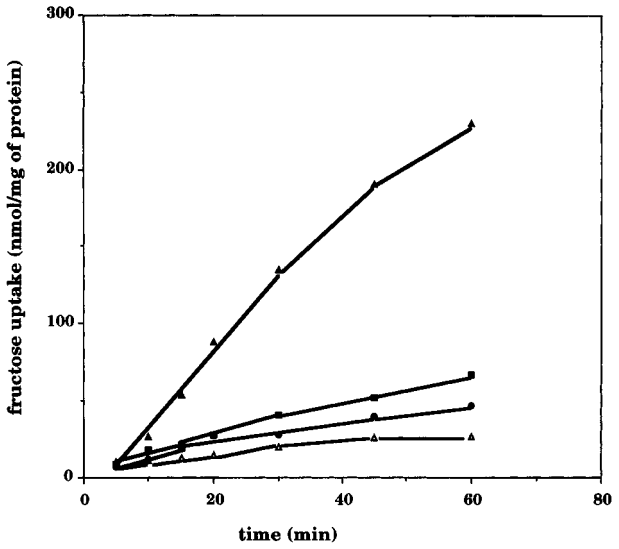

FIGURE 6: Fructose uptake in various *B. subtilis* strains. The strains were grown in CSK medium in the absence of fructose. The uptake experiments were conducted as described in Materials and Methods with the *levR8* mutant strain QB5091 (filled triangles), with the wild-type QB5081 (open triangles), with the *levEH15D* mutant strain QB5296 (circles), and with the *levDH9A* mutant strain QB5294 (squares).

Fructose Uptake in Strains Carrying a levDH9A, levEH15E, or levEH15D Allele. B. subtilis strains contain three fructose-specific PTS, the expression of which is inducible by fructose (Gay & Delobbe 1977; Martin-Verstraete et al., 1990). To distinguish fructose uptake via the PTS encoded by the *lev* operon from fructose uptake via other systems, mutants were studied which expressed the *lev* operon constitutively. In these mutants, the *lev* operon is expressed even in the absence of fructose, whereas other fructose uptake systems are not expressed since they require induction (Gay & Delobbe, 1977).

Strains QB5294 (*levDH9A)*, QB5296 (*levEH15D*), QB5091 (*levR8*), and QB5081 (wild-type) were grown in CSK medium in the absence of fructose. Assays of [^{14}C]fructose uptake were performed in these strains as indicated in Materials and Methods. The results obtained are presented in Figure 6. Constitutive expression of the *lev* operon resulting in rapid fructose uptake was observed in strain QB5091 (*levR8*). In strains QB5294 (*levDH9A*) and QB5296 (*levEH15D*), fructose uptake was much lower compared to strain QB5091 but was about 1.5−2-fold higher compared to the uninduced wild-type strain QB5081. These results confirm that LevD and LevE are indispensable components for fructose uptake via the lev-PTS.

DISCUSSION

The *lev* operon of *B. subtilis* encodes the enzyme levanase and four proteins forming a fructose-specific PTS. This fructose-specific PTS is induced at low fructose concentrations but repressed at high fructose concentrations (Martin et al., 1989). Expression of the *lev* operon is regulated by the complex transcriptional activator LevR (Débarbouillé et

al., 1991b). LevR contains a helix−turn−helix motif, a DNA binding domain homologous to the NtrC/NifA family of transcriptional activators, and a phosphorylation domain homologous to the phosphorylation domain of PTS-regulated antiterminators such as BglG, SacT, SacY, and LicT (Débarbouillé et al., 1991a,b; Postma et al., 1993; Steinmetz, 1993; Martin-Verstraete et al., 1994). BglG has been suggested to be phosphorylated via enzyme I, HPr, and the enzyme II specific for β-glucosides (Schnetz & Rak, 1990). By contrast, LevR has been shown to be phosphorylated by PEP, enzyme I, and HPr, and its PEP-dependent phosphorylation was proposed to play a role in catabolite repression (Stülke et al., 1995). However, genetic data suggested that similar to BglG, the function of LevR is also regulated by the sugar-specific PTS components LevD and LevE. Mutations in *levD* or *levE* which abolished fructose uptake via the lev-PTS caused constitutive expression of the *lev* operon. On the basis of the genetic data, phosphorylation of LevR via LevD and LevE has been proposed (Débarbouillé et al., 1991a).

To investigate the suggested role of LevD and LevE in the regulation of expression of the *lev* operon at the biochemical level, we studied the phosphorylation of the IIA (LevD) and IIB (LevE) components of the lev-PTS. For this purpose, we first synthesized and purified modified LevD and LevE with both proteins bearing a His-tag at the N-terminus. We demonstrated that LevD is phosphorylated by PEP in the presence of enzyme I and HPr whereas phosphorylation of LevE required the presence of LevD in addition to enzyme I and HPr. We determined the site of PEP-dependent phosphorylation as His-9 in LevD and as His-15 in LevE by purifying and sequencing a ^{32}P-labeled peptide derived from each protein. His-10 in IIA^{Man} which is homologous to His-9 of LevD was found to be located at the end of the subunit interface of the dimer (Nunn et al., 1996). Mutant proteins were synthesized and purified in which the phosphorylatable histidine in LevD and LevE was replaced with an alanine (Figure 5). The mutant protein LevDH9A(His)$_6$ was found to be very slowly phosphorylated, whereas LevEH15A(His)$_6$ was not phosphorylated at all. Phosphorylation of LevE(His)$_6$ was strictly dependent on the presence of LevD(His)$_6$ (see Figure 2, lane 4). Nevertheless, weak phosphorylation of LevE(His)$_6$ was observed in the presence of the mutant protein LevDH9A(His)$_6$. The phosphorylation rate of LevE(His)$_6$ increased with the amount of LevDH9A(His)$_6$ present in the phosphorylation assay. However, the mutant protein LevEH15A(His)$_6$ was not phosphorylated in the presence of LevDH9A(His)$_6$. These results suggest that LevDH9A(His)$_6$ can slowly be phosphorylated at a second site of phosphorylation from where the phosphate group can also be transferred to His-15 in LevE. A similar result has been reported for IIAB^{Man} of *E. coli* (Stolz et al., 1993). The IIA^{Man} domain is homologous to LevD, and the IIB^{Man} domain is homologous to LevE (see Figure 3). A IIAB^{Man} mutant protein in which the phosphorylatable histidine of the IIA domain was replaced with a non-phosphorylatable amino acid could still be slowly phosphorylated at the IIB domain. It is not clear whether this additional phosphorylation pathway is of physiological significance or whether it is an artifact caused by the mutation affecting the site of PEP-dependent phosphorylation in the IIA domain of the mannose-specific PTS of *E. coli* and in LevD of the fructose-specific PTS of *B. subtilis*. It is interesting to note that neither with LevD nor with LevD-(His)$_6$ could a second phosphorylated peptide be detected.

B. subtilis levD and *levE* mutant strains have previously been isolated which exhibited constitutive expression of the *lev* operon. In the mutant strain *levD6*, Gly-69 of LevD was replaced with a Glu, whereas in the mutant strains *levE5* and *levE7*, stop codons were introduced at position 126 or 22 of LevE, respectively (Kunst et al., 1977; Martin-Verstraete et al., 1990). As mutations in enzyme I (*ptsI*) also caused constitutive expression of the *lev* operon (Martin-Verstraete et al., 1990), it has been proposed that LevE inhibits LevR activity by PEP-dependent phosphorylation (Débarbouillé et al., 1991a). It was assumed that in the presence of fructose, the phosphate group of phosphorylated LevE would mainly be used to phosphorylate fructose and LevR would be present in the dephosphorylated, active form. One problem with this model was that due to the assumed rapid reversible transfer of the phosphoryl group between the various PTS proteins (Postma et al., 1993), the rapid uptake of glucose or mannitol for example should also lead to dephosphorylation of histidyl-phosphorylated HPr, as has been shown for glucose-metabolizing *Streptococcus mutans* cells (Vadeboncoeur et al., 1991), and hence to dephosphorylation of LevD and LevE and consequently to the induction of the *lev* operon, which in fact is not the case. The observation that the phosphoryl group transfer from phosphorylated LevD to HPr occurs very slowly, although LevD is rapidly phosphorylated by histidyl-phosphorylated HPr, might provide an answer to this problem. These studies will have to be carried out in more detail using purified, phosphorylated LevD. In addition, it will be interesting to see whether other PTS, the induction of which is regulated by the sugar-specific PTS components and by antiterminators, behave similarly with respect to phosphorylation of their sugar-specific components.

To obtain further evidence that the interruption of the lev-PTS phosphorylation chain is responsible for the constitutive expression of the *lev* operon in the *ptsI*, *levD*, and *levE* mutant strains, we constructed *B. subtilis* mutants in which His-9 of LevD was replaced with an alanine and His-15 of LevE was replaced with a glutamate or an aspartate. We observed that all three mutations caused strong reduction of fructose transport via the lev-PTS and constitutive expression of the *lacZ* gene being under control of the *lev* promoter and its upstream activating sequence. β-Galactosidase activity in the *levD* or *levE* mutant strains was elevated about 150-fold compared to a non-induced wild-type strain and was similar to the β-galactosidase activity measured in cells bearing the previously described *levD6* or *levE7* allele. According to these and previous data, phosphorylation of LevE at His-15 seems to inhibit *lev* operon expression.

The exact mechanism by which P-LevE regulates LevR is unknown. It is possible that P-LevE phosphorylates LevR or that P-LevE interacts allosterically with LevR, thus regulating LevR activity either directly or influencing its enzyme I- and HPr-dependent phosphorylation (Stülke et al., 1995). We had expected that, by introducing negatively charged amino acids at the site of phosphorylation in LevE, one of the two mutant proteins (H15E or H15D LevE) might structurally resemble phosphorylated LevE, as has been reported for HPr (S46D mutant HPr resembles seryl-phosphorylated HPr; Wittekind et al., 1989). If P-LevE regulated LevR activity by protein−protein interaction, we

anticipated to see an inhibitory effect by one of the LevE mutant proteins on *lev* operon expression. Since expression of both *levE* alleles caused constitutive expression of the *levD′-′lacZ* fusion, the detailed mechanism of regulation of LevR by LevE remains unknown and will be subject of further studies.

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