Novel phosphotransferase system genes revealed by genome analysis – the complete complement of PTS proteins encoded within the genome of *Bacillus subtilis*

Jonathan Reizer,¹ Steffi Bachem,² Aiala Reizer,¹ Maryvonne Arnaud,³ Milton H. Saier Jr¹ and Jörg Stülke²

Author for correspondence: Jörg Stülke. Tel: +49 9131 8528818. Fax: +49 9131 8528082.
e-mail: jstuelke@biologie.uni-erlangen.de

¹ Department of Biology,
University of California at
San Diego, La Jolla,
CA 92093-0116, USA

² Lehrstuhl für
Mikrobiologie, Institut für
Mikrobiologie, Biochemie
und Genetik der Friedrich-
Alexander-Universität
Erlangen-Nürnberg,
Staudtstr. 5, D-91058
Erlangen, Germany

³ Unité de Biochimie
Microbienne, Département
des Biotechnologies,
Institut Pasteur, 25 rue du
Dr Roux, F-75724 Paris
Cedex 15, France

***Bacillus subtilis* can utilize several sugars as single sources of carbon and energy. Many of these sugars are transported and concomitantly phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). In addition to its role in sugar uptake, the PTS is one of the major signal transduction systems in *B. subtilis*. In this study, an analysis of the complete set of PTS proteins encoded within the *B. subtilis* genome is presented. Fifteen sugar-specific PTS permeases were found to be present and the functions of novel PTS permeases were studied based on homology to previously characterized permeases, analysis of the structure of the gene clusters in which the permease encoding genes are located and biochemical analysis of relevant mutants. Members of the glucose, sucrose, lactose, mannose and fructose/mannitol families of PTS permeases were identified. Interestingly, nine pairs of IIB and IIC domains belonging to the glucose and sucrose permease families are present in *B. subtilis*; by contrast only five Enzyme IIA^{Glc}-like proteins or domains are encoded within the *B. subtilis* genome. Consequently, some of the EIIA^{Glc}-like proteins must function in phosphoryl transfer to more than one IIB domain of the glucose and sucrose families. In addition, 13 PTS-associated proteins are encoded within the *B. subtilis* genome. These proteins include metabolic enzymes, a bifunctional protein kinase/phosphatase, a transcriptional cofactor and transcriptional regulators that are involved in PTS-dependent signal transduction. The PTS proteins and the auxiliary PTS proteins represent a highly integrated network that catalyses and simultaneously modulates carbohydrate utilization in this bacterium.**

Keywords: sugar transport, PTS, phosphorylation, gene regulation, *Bacillus subtilis*

INTRODUCTION

Bacteria have evolved a highly sophisticated multi-protein sugar transport and phosphorylation system, the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS is composed of two general energy-coupling proteins, Enzyme I (EI), a histidine-containing phosphoprotein (HPr) and several sugar-specific Enzymes II (EII). The phosphorylation chain from

phosphoenolpyruvate to the incoming sugar proceeds via EI, HPr and EII. EIIs are multidomain proteins, the domains of which may be organized in a single protein or as several individual polypeptides. Two of the EII domains (EIIA and EIIB) are hydrophilic and involved in phosphoryl transfer, while domain IIC (and IID, if present) is membrane-bound and catalyses sugar transport. In the PTS, transport of the sugar occurs only if the substrate is concomitantly phosphorylated (Postma *et al.*, 1993; Saier & Reizer, 1992).

In addition to its function in sugar transport, the PTS is one of the major signal transduction systems of the

Abbreviation: EI and EII, Enzymes I and II; HPr, histidine-containing phosphoprotein; PTS, phosphotransferase system; PRD, PTS regulation domain.

bacterial cell, as suggested by the multitude of regulatory functions exerted by PTS components. In both Gram-positive and Gram-negative bacteria proteins of the PTS are involved in carbon catabolite repression. They also control the induced expression of several catabolic operons in response to inducer availability by modifying the activities of transcriptional regulators, transport proteins and enzymes. Moreover, the PTS is involved in the regulation of nitrogen metabolism, chemotaxis towards carbohydrates and genetic competence (Postma *et al.*, 1993; Powell *et al.*, 1995; Saier & Reizer, 1994; Stülke & Hillen, 1998). Many, but not all of these regulatory processes involve phosphotransfer between PTS proteins and the proteins whose activities are modulated by the PTS. These phosphorylation reactions link the activities of the target proteins to PTS activity and thus to the availability of PTS substrates. Glycerol kinase from low-GC Gram-positive bacteria is phosphorylated by HPr in the absence of repressing carbon sources. This phosphorylation stimulates the catalytic activity of the enzyme (Charrier *et al.*, 1997). Similarly, lactose transport activity in *Streptococcus thermophilus* is controlled by HPr-dependent phosphorylation of a IIA-like regulatory domain fused to the C-terminal end of the membrane-bound lactose:H⁺ symporter (Gunnewijk *et al.*, 1999). Moreover, several positively acting transcriptional regulators have been shown to possess conserved PTS regulation domains (PRDs) (Tortosa *et al.*, 1997; Stülke *et al.*, 1998). The activator and antiterminator proteins that contain PRDs are often subject to dual control by the PTS: in the absence of the inducer of the controlled operon, they are phosphorylated and thus inactivated, but their activity may also depend on an additional HPr-dependent phosphorylation. This latter phosphorylation occurs only in the absence of a repressing carbon source, thus providing a means for hierarchical expression of genes required for the utilization of secondary carbon sources (for review see Stülke *et al.*, 1998).

The PTSs of enteric bacteria and of low-GC Gram-positive bacteria have been the subject of intensive investigation (see Saier & Reizer, 1994). More recently, the potential regulatory roles of PTS components of *Mycoplasma* spp., non-enteric Gram-negative bacteria such as *Haemophilus influenzae* and *Alcaligenes eutrophus*, and the high-GC Gram-positive bacterium *Streptomyces coelicolor* have been investigated (Zhu *et al.*, 1993; Macfayden *et al.*, 1996; Pries *et al.*, 1991; Titgemeyer *et al.*, 1995). The advent of complete genome sequence analysis of the complete set of PTS proteins encoded within a given genome has resulted in the identification of several novel genes and proteins (see Reizer *et al.*, 1996; Reizer & Reizer, 1996).

Here, we present an analysis of the complete set of genes encoding PTS and PTS-associated proteins in the model Gram-positive bacterium *Bacillus subtilis*. We show that *B. subtilis* encodes 15 complete PTS permeases of which only seven have been characterized previously. Sequence and functional analyses have allowed us to propose

substrates for seven of the eight novel permeases. Moreover, 13 PTS-associated proteins are also encoded in the *B. subtilis* genome. The significance of these findings is discussed.

METHODS

Bacterial strains, plasmids and culture media. The *Bacillus subtilis* strains used in this work are listed in Table 1. *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) and TGI (Gibson, 1984) were used for cloning experiments.

Plasmid pGP109, used to disrupt *malP*, was constructed as follows. A 1024 bp fragment internal to *malP* was amplified by PCR using the primers SB17 (5'-TATGAATTCTTTG-GAAGCGCGATGTTTGTGCC) and SB18 (5'-TATGGA-TCCCATTGTGGCGGCAAGCACGGCGTG). The resulting PCR product was cut with *EcoRI* and *BamHI* (these sites were generated by PCR). The PCR product was then cloned into pBluescript SK- (Stratagene) linearized with the same enzymes to give pGP108. A 1.4 kb *EcoRI* fragment from pSpec (P. Trieu-Cuot, Institut Pasteur, France) containing the *spc* gene, which confers resistance to spectinomycin, was inserted into the unique *MfeI* site of pGP108. The resulting plasmid was pGP109.

gamP (*ybfS*) was disrupted as follows. A fragment of 977 bp internal to *gamP* was amplified by PCR using the oligonucleotides X1 (5'-GGGCGAGGGAATTCCGATTAT) and X2 (5'-CTGGTTCAGAAGCTTTGCCG). The PCR product was cut with *EcoRI* (generated by PCR) and *HindIII* and cloned into the integrative plasmid pHT181 (Lereclus & Arantes, 1992) to give pX1. *B. subtilis* 168 was transformed with pX1 and the plasmid was integrated into the chromosome. The DNA of the *B. subtilis* strain harbouring pX1 was extracted, hydrolysed by *EcoRI* and religated. *E. coli* TGI was transformed with the ligation mixture, yielding plasmid pHT181::X1 carrying a 3 kb *EcoRI* fragment. This fragment contained the PCR-amplified region and about 2 kb upstream of that region. A *StuI* digestion of pHT181::X1 provided a fragment of 947 bp corresponding to the last 677 bp of *gamP*, the first 93 bp of *gltP* and 147 bp of the *gamP/gltP* intergenic region. This *StuI* fragment was then replaced by an *aphA3* cassette (Trieu-Cuot & Courvalin, 1983) located on a 1.5 kb *Clal* fragment, leading to plasmid pHT181::X1::aphA3.

ypqE was disrupted by the following approach. A fragment of 594 bp, carrying *ypqE* and 90 bp downstream of the stop codon of this gene was amplified by PCR using the primers *ypq-1* (5'-GAAGAATTCATATGCTGAAAAATTATTC-GGAAT) and *ypq-2* (5'-CCCCCGGGTTCGACTACAGT TTACCGAATATTTG). The amplified fragment was cloned into pUC19 at the *EcoRI* and *SalI* sites (sites were introduced with the PCR primers), to yield plasmid pXX1. A 1.5 kb *Clal* fragment carrying *aphA3* was inserted into pXX1 at the unique *AvaI* site, yielding plasmid pXX1::aphA3. The 2.1 kb *EcoRI-SalI* fragment carrying *ypqE* interrupted by *aphA3* from pXX1::aphA3 was inserted into plasmid pHT181 between the *EcoRI* and *SalI* sites, giving pHT181::XX1::aphA3.

E. coli was grown in LB medium (Sambrook *et al.*, 1989) whereas *B. subtilis* was grown in SP medium or C minimal medium (Martin-Verstraete *et al.*, 1990). The minimal media were supplemented with auxotrophic requirements (at 50 mg l⁻¹) and carbon sources as indicated. CSE is C medium supplemented with potassium succinate (6 g l⁻¹) and potassium glutamate (8 g l⁻¹). Doubling times were determined by growing the bacteria in C minimal medium supplemented with 0.2% (w/v) of the carbon source indicated. CSE

Table 1. *B. subtilis* strains used in this work

Strain	Genotype or description	Source or reference*
168	<i>trpC2</i>	Laboratory stock
GP110	<i>trpC2 malP::spc</i>	pGP109 → 168
GP113	<i>trpC2 amyE::(ΔLA ptsG'-lacZ aphA3) ptsG::ΔEIIA</i>	Bachem & Stülke (1998)
QB5350	<i>trpC2 ptsH-H15A amyE::(levD'-lacZ aphA3)</i>	Stülke <i>et al.</i> (1995)
QB5448	<i>trpC2 amyE::(ΔLA ptsG'-lacZ aphA3)</i>	Stülke <i>et al.</i> (1997)
QB6098	<i>trpC2 gamP::aphA3</i>	pHT181::X1: <i>aphA3</i> → 168
QB6099	<i>trpC2 ypqE::aphA3</i>	pHT181::XX1: <i>aphA3</i> → 168

* Arrows indicate construction by transformation.

containing 0.2% (w/v) glucitol was used for precultures. Agar plates were prepared by the addition of 17 g Bacto agar 1⁻¹ (Difco) to LB, SP or C medium.

DNA manipulation, transformation and phenotypic characterization. DNA extraction and manipulation were performed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturers.

Standard procedures were used to transform *E. coli* and transformants were selected on LB plates containing ampicillin (100 µg ml⁻¹) or spectinomycin (100 µg ml⁻¹) (Sambrook *et al.*, 1989). *B. subtilis* was transformed with chromosomal or plasmid DNA according to the two-step protocol described by Kunst & Rapoport (1995). Transformants were selected on SP plates containing spectinomycin (100 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹) or kanamycin (5 µg ml⁻¹).

Computer-aided analyses. Database searches were performed using the BLAST 2.0 program (Altschul *et al.*, 1990) on the NCBI server (<http://www.ncbi.nlm.nih.gov/blast/>). The organization of *B. subtilis* gene clusters was determined using the *SubtiList* database (Moszer *et al.*, 1995).

RESULTS AND DISCUSSION

Genes encoding general energy coupling proteins of the PTS

Tables 2 and 3 list all recognized PTS proteins and PTS-associated proteins encoded within the *B. subtilis* genome. Interestingly, only one complete EI-like protein and just two HPr-like proteins, i.e. HPr and Crh, are present (see Tables 2 and 3). By contrast, the *E. coli* chromosome encodes five EI paralogues (EI, EI^{Ani}, EI^{Ntr} and the two EI domains in the multidomain proteins ADI and TTP) and five paralogues of HPr (HPr, FPr, NPr, DPr, TPr) (Blattner *et al.*, 1997; Reizer & Saier, 1997). Domains similar to the phosphohistidine domain of phosphoenolpyruvate synthase (similar to EI) are attached to the C termini of pyruvate kinases of several Gram-positive bacteria, including *B. subtilis* (Table 3), *Bacillus stearothermophilus* and *Lactobacillus delbrueckii* (Sakai & Ohta, 1993; Nguyen & Saier, 1995; Branny *et al.*, 1996). The presence of multiple paralogues of EI and HPr in *E. coli* and the scarcity of these paralogues in *B. subtilis* suggests that the latter

organism evolved alternative mechanisms to satisfy the specific regulatory and/or functional roles of these proteins.

Genetic organization of gene clusters encoding sugar-specific PTS permeases

The second group of genes listed in Table 2 encodes proteins that fall into the Glc family. Two of these permeases (PtsG and GamP) exhibit the IICBA domain structure, whereas the remaining two permeases (MalP and NagP) lack a IIA domain and exhibit the IICB domain order. Two genes (*yyzE* and *ypqE*) encode two distinct IIA^{Glc}-like proteins. YpqE is a full length paralogue (168 residues) that presumably functions with the IICB permease(s) of this family or the IIBC permease(s) of the Scr family. YyzE, the second IIA^{Glc}-like protein, is a truncated IIA homologue (76 residues) possessing the two active site histidyl residues but lacking about 70 and 30 residues corresponding, respectively, to the N and C termini of the orthologous *crr* gene product of enteric bacteria (Nelson *et al.*, 1984). It is not known if YyzE is functional in the PTS phosphorylation cascade. *nagP*, encoding a putative permease for *N*-acetylglucosamine, forms a monocistronic transcriptional unit and is not linked to other genes involved in the utilization of *N*-acetylglucosamine (see Fig. 1a). *malP* is the distal gene of a presumptive three-gene operon encoding also a phospho- α -glucosidase (MalA) that has the capacity to hydrolyse phosphorylated maltose generated by MalP (Fig. 1b). *gamP* required for the utilization of glucosamine (see below) is the second gene of a two-gene operon. The proximal gene of this operon, *gamA* (*ybfT*), encodes a putative glucosamine-6-phosphate-deaminating isomerase. Divergently transcribed relative to the putative *gamAP* operon is a gene encoding a transcriptional repressor of the GntR family (see Fig. 1c). *ypqE* seems to form a monocistronic unit (Fig. 1d) while *yyzE* is followed by *bglA* encoding a phospho- β -glucosidase (Fig. 1e) (Zhang & Aronson, 1994).

Five PTS permeases are included within the Scr family (Table 2, section 3). Four of these (SacP, SacX, TreP and YbbF) have the IIBC domain structure. The fifth member of this family, BglP, has the IIBCA domain order.

Table 2. Genes encoding PTS proteins in *B. subtilis*

Gene*	Map position (degrees)†	Encoded protein	Domain structure‡	No. of residues	Accession no.§	Reference¶
1. General energy-coupling proteins						
<i>ptsH</i>	125	HPr	H	87	P08877 (BG10200)	1
<i>ptsI</i>	125	EI	I	570	P08838 (BG10201)	1
2. Glucose permease (Glc) family		Sugar substrate				
<i>gamP</i> (<i>ybfS</i>)	22	Glucosamine	IICBA	631	gi2632521 (BG11014)	2, Fig. 1(c)
<i>nagP</i> (<i>yflF</i>)**	72	N-Acetylglucosamine**	IICB	452	gi2443228 (BG12941)	2, Fig. 1(a)
<i>malP</i> (<i>glvC</i>)	76	Maltose	IICB	527	P54715 (BG11848)	3, Fig. 1(b)
<i>ptsG</i>	125	Glucose	IICBA	699	P20166, P08875 (BG10198)	4
<i>ypqE</i>	200	Unknown	IIA	168	P50829 (BG11450)	2, Fig. 1(d)
<i>yyzE</i>	352	Unknown	IIA (truncated)	76	gi2636559 (BG14172)	2, Fig. 1(e)
3. Sucrose permease (Scr) family						
<i>ybbF</i> **	16	Unknown β -glucoside**	IIBC	446	gi1256135 (BG11567)	2, Fig. 2
<i>treP</i>	73	Trehalose	IIBC	470	P39794 (BG11009)	5
<i>sacP</i>	333	Sucrose	IIBC	460	P05306 (BG10595)	6
<i>sacX</i> ††	337	Sucrose	IIBC	459	P15400 (BG10560)	7
<i>bglP</i> (<i>sytA</i>)	345	Aryl- β -glucosides	IIBCA	609	P40739 (BG10934)	8
4. Lactose permease (Lac) family						
<i>ywbA</i>	337	Unknown	IIC	444	P39584 (BG10562)	9, Fig. 3(b)
<i>licA</i> (<i>celC</i>)**‡‡	338	Oligo- β -glucoside**‡‡	IIA	110	P46319 (BG11349)	10
<i>licB</i> (<i>celA</i>)**‡‡	338	Oligo- β -glucoside**‡‡	IIB	102	P46318 (BG11347)	10
<i>licC</i> (<i>celB</i>)**‡‡	338	Oligo- β -glucoside**‡‡	IIC	452	P46317 (BG11348)	10
<i>ydhM</i> **	54	Oligo- β -mannoside**	IIB	103	gi1945097 (BG12190)	2, Fig. 3(a)
<i>ydhN</i> **	54	Oligo- β -mannoside**	IIA	110	gi1945098 (BG12191)	2, Fig. 3(a)
<i>ydhO</i> **	54	Oligo- β -mannoside**	IIC	442	gi1945099 (BG12192)	2, Fig. 3(a)
5. Mannose permease (Man) family						
<i>levD</i> §§	236	Fructose§§	IIA	146	P26379 (BG10316)	11
<i>levE</i> §§	236	Fructose§§	IIB	163	P26380 (BG10317)	11
<i>levF</i> §§	236	Fructose§§	IIC	269	P26381 (BG10318)	11
<i>levG</i> §§	236	Fructose§§	IID	275	P26382 (BG10319)	11
6. Fructose/mannitol permease (Fru) family						
<i>mtlA</i>	38	Mannitol	IICBA	610	P42956 (BG11215)	2, Fig. 4(a)
<i>manP</i> (<i>yjdD</i>)	109	Mannose	IIBCA	589	gi2633555 (BG13176)	2, Fig. 4(b)
<i>fruA</i> §§	129	Fructose§§	IIABC	635	gi2633811 (BG11938)	2, Fig. 4(c)

*Synonymous designations and y names are shown in parentheses.

† The calculated map position was derived from the *SubtiList* database (Moszer *et al.*, 1995).

‡ Protein domains include: I, EI; H, HPr; IIA, the domain bearing the first permease-specific phosphorylation site; IIB, the domain bearing the second permease-specific phosphorylation site; IIC, the hydrophobic transmembrane permease domain; IID, a partially hydrophobic transmembrane domain present in members of the mannose permease family.

§ Accession numbers with the prefix P, gi and BG denote identifiers from SWISS-PROT, NCBI and the *SubtiList* database, respectively.

¶ References: 1, Gonzy-Tréboul *et al.* (1989); 2, Kunst *et al.* (1997); 3, Yamamoto *et al.* (1996); 4, Zagorec & Postma (1992); 5, Schöck & Dahl (1996); 6, Fouet *et al.* (1987); 7, Zukowski *et al.* (1990); 8, Le Coq *et al.* (1995); 9, Glaser *et al.* (1993); 10, Tobisch *et al.* (1997); 11, Martin-Verstraete *et al.* (1990). Reference to the Figures is given where appropriate.

|| The main sugar substrate of the corresponding permease is indicated.

** The proposed designations of these genes and the proposed functional assignment of their protein products are based on homology of proteins encoded in the corresponding operon to previously characterized proteins.

†† The proposed regulatory role of *SacX*, a putative sucrose permease, in transcriptional antitermination of *sacB* is yet to be established (Tortosa *et al.*, 1997).

‡‡ It is yet to be established whether penta-, tetra- or trisaccharides, the LicS-catalysed degradation products of lichenan (β -1,3-1,4-glucan), is the main sugar substrate of the Lic permease (Tobisch *et al.*, 1997; Schnetz *et al.*, 1996).

§§ While the Lev permease is composed of four distinct polypeptides chains that collectively catalyse the formation of fructose 6-phosphate, the non-homologous FruA permease is composed of a single polypeptide chain catalysing the formation of fructose 1-phosphate.

Altogether, the *B. subtilis* chromosome possesses nine genes encoding nine pairs of IIB and IIC domains that belong to the glucose and sucrose families. By contrast, only five IIA^{Glc}-like proteins or domains are encoded within the *B. subtilis* genome. Consequently, some of the IIA^{Glc}-like proteins or domains must function in transfer of the phosphoryl group to more than one IIB^{Glc}- and/or IIB^{Scr}-like proteins as was previously suggested with the

IIA^{Glc} domain of the glucose-specific EII complex (Sutrina *et al.*, 1990). The operons containing the genes encoding EIIs for sucrose, trehalose and aryl- β -glucosides have all been described (Fouet *et al.*, 1987; Zukowski *et al.*, 1990; Schöck & Dahl, 1996; Le Coq *et al.*, 1995). *ybbF*, encoding a novel EII of the Scr family, is the fifth gene in a putative eight-gene operon (Fig. 2). Based on the fact that one of the genes (*ybbD*) of this

Table 3. Genes encoding PTS-associated proteins in *B. subtilis*

Gene*	Map position (degrees)*		No. of residues	Accession no.*	Reference†
1. Metabolic enzymes		Encoded protein			
<i>glpK</i>	86	Glycerol kinase	496	P18157 (BG10187)	1
<i>pykA</i>	255	Pyruvate kinase	585	P80885 (BG12661)	2
2. Protein kinases/phosphatases					
<i>prkA</i>	83	Serine protein kinase	631	P39134 (BG10804)	3
<i>ptsK</i> (<i>bprK</i> , <i>yvoB</i>)	307	HPr(Ser) kinase/phosphatase	310	gi3122218 (BG14125)	4, 5, 6
3. Transcriptional regulators					
(a) Transcriptional antiterminators		Regulated operon			
<i>glcT</i> (<i>ykwA</i>)	124	<i>ptsGHI</i>	285	gi2633759 (BG12593)	7
<i>sacT</i>	334	<i>sacPA</i>	276	P26212 (BG10593)	8
<i>sacY</i> (<i>sacS</i>)	337	<i>sacB</i>	280	P15401 (BG10559)	9
<i>licT</i>	343	<i>bglPH</i> ; <i>licS</i> (<i>bglS</i>)§	277	P39805 (BG10474)	10
(b) Transcriptional activators					
<i>mtlR</i> (<i>ydaA</i>)‡	40	<i>mtlAD</i>	694	gi1881227 (BG12049)	11
<i>manR</i> (<i>yjdC</i>)‡	108	<i>yjdDEF</i>	648	gi2633554 (BG13175)	11, Fig. 4(b)
<i>levR</i>	236	<i>levDEFG sacC</i>	938	P23914 (BG10677)	12
<i>licR</i> (<i>celR</i>)	338	<i>licBCAH</i> (<i>celABCD</i>)§	641	P46321 (BG11346)	13
(c) Transcriptional cofactors					
<i>crh</i> (<i>yvcM</i>)	305	Carbon catabolite-repressive operons	85	gi1945653 (BG12403)	14

* See footnotes *, † and ‡ of Table 2.

† References: 1, Holmberg *et al.* (1990); 2, Lapidus *et al.* (1997); 3, Fischer *et al.* (1996); 4, Galinier *et al.* (1998); 5, Reizer *et al.* (1998); 6, Kravanja *et al.* (1999); 7, Stülke *et al.* (1997); 8, Débarbouillé *et al.* (1990); 9, Zukowski *et al.* (1990); 10, Schnetz *et al.* (1996); 11, Kunst *et al.* (1997); 12, Débarbouillé *et al.* (1991); 13, Tobisch *et al.* (1997); 14, Galinier *et al.* (1997).

‡ The proposed designations of these genes and the proposed regulatory assignments of their protein products is based on homology of proteins encoded in the corresponding operon to previously characterized proteins.

§ Preliminary data cited in Tobisch *et al.* (1997) suggest that LicT is also involved in the regulation of the *licBCAH* operon.

gene cluster encodes a β -glycosidase homologue, we propose that YbbF is involved in the utilization of unknown β -glucosides.

The Lac family (Table 2, section 4) includes two EII complexes each encoded by three distinct genes (*licBCA* and *ydhMNO*). Thus, YdhMNO and LicBAC are homologous to the IIB, IIA and IIC proteins that constitute the lactose-specific PTS permeases of Gram-positive bacteria (Lengeler *et al.*, 1994). The Lic-PTS is encoded within the *licBCAH* operon that also includes a phospho- β -glucosidase encoding gene, *licH* (Tobisch *et al.*, 1997). The *ydhMNO* genes are proximal in a putative eight-gene operon that also encodes a β -1,4-mannosidase, a β -glycosidase, a mannose-6-phosphate isomerase and a fructokinase (Fig. 3a). Based on these observations we propose that the Ydh-PTS is involved in the transport of oligo- β -mannosides. This would be analogous to the Lic-PTS which transports oligomeric β -glucosides. Indeed, the *ydhMNO* operon was recently shown to be involved in mannan utilization and to be induced by konjac glucomannan (Sadaie & Yata, 1998). A paralogous IIC domain (YwbA), the function of which is yet to be determined, is an additional member of the *B. subtilis* lactose permease family. *ywbA* is linked to a gene of unknown function and it is not clustered to any other gene involved in carbohydrate utilization (Fig. 3b).

The LevDEFG EII complex (Table 2, section 5) is a fructose-6-phosphate-forming fructose permease of the Man family. It is the only member of the Man family present in *B. subtilis* (Martin-Verstraete *et al.*, 1990).

The Fru family (Table 2, section 6) includes the following three permeases, each containing three domains (IIA, IIB and IIC) that reside on a single polypeptide chain: (i) MtlA with a CBA domain order homologous to the *E. coli* mannitol permease; (ii) FruA with an ABC domain sequence homologous to the fructose-1-phosphate-forming fructose-specific PTS permease of mycoplasmas (Reizer *et al.*, 1996); and (iii) ManP (YjdD) with a BCA domain order. All three domains of FruA and the putative mannose permease (ManP) exhibit significant sequence similarity with protein domains of the fructose-specific permease (IIABC^{Fru}; fructose-1-phosphate-forming) of mycoplasmas (Reizer *et al.*, 1996). *mtlA*, encoding EII^{Mtl}, is the first gene in a bicistronic operon which also encodes mannitol-1-phosphate dehydrogenase (Fig. 4a). *manP* is the first gene in a putative tricistronic operon. The second gene encodes a mannose-6-phosphate isomerase, whereas the function of the third gene is unknown (Fig. 4b). *fruA*, encoding the fructose permease, is the distal gene in a tricistronic operon. The first gene of this putative operon, *fruR*, encodes a repressor of the DeoR family and the second gene, *fruB*, encodes an orthologue of the *E. coli* fructose-1-phosphate kinase (Fig. 4c).

In contrast to *E. coli*, glucitol and galactitol PTS permeases (Lengeler *et al.*, 1994; Nobelmann & Lengeler, 1995) are not present in *B. subtilis*. Furthermore, none of the sugar-specific EII complexes of *B. subtilis* exhibit a mosaic structure like that observed for the Sgc permease of *E. coli* (Reizer *et al.*, 1996). We also note that PTS permeases of the Scr family have the

Fig. 1. Genetic arrangement of genes encoding PTS proteins of the Glc family. Functionally equivalent genes are shown by the same types of arrows. The numbers below the names of the proteins indicate their length. TR denotes transcriptional regulator. (a) Genetic map of the *nagP* region. This gene seems to form a monocistronic transcriptional unit. The other genes tentatively involved in *N*-acetylglucosamine utilization are located in the 307° region of the *B. subtilis* chromosome (Kunst *et al.*, 1997). (b) Genetic map of the *mal* gene cluster. The first gene, *malA*, is preceded by a potential *cre* sequence mediating CcpA-dependent carbon catabolite repression. A potential ρ -independent terminator is located downstream of *malP*. (c) Genetic map of the *gam* gene cluster. A potential ρ -independent terminator is located downstream of *gamP*. *gamA* is preceded by a potential σ^A -dependent promoter. *YbgA* shares sequence similarity with transcriptional repressors of the GntR family. (d) Genetic map of the *ypqE* region. *ypqA* which is oriented divergently relative to *ypqE* encodes a protein of unknown function. Both genes are followed by potential ρ -independent transcriptional terminators. (e) Genetic map of the *bglA* region. *yydK* encodes a potential repressor of the GntR family. The two divergent transcription units are followed by transcriptional terminators.

Fig. 2. Genetic map of the *ybbF* gene cluster. The convention of presentation is as described in the legend to Fig. 1. A putative ρ -independent terminator is located downstream of *ybbC*. *YbbH* shares sequence similarity with transcriptional repressors of the RpiR family. The *ybbI* gene product is similar to an *E. coli* protein encoded in a locus that also contains a gene encoding a PTS permease homologous to *YbbF*.

BC(A) domain order, members of the Glc family exhibit the CB(A) domain order and members of the Fru family have variable domain orders (IICBA, IIBCA and IIABC). Finally, the *B. subtilis* EII complexes of the Lac and Man families consist of single domain proteins.

PTS-associated proteins

Expression of several catabolic operons in *B. subtilis* is controlled by PTS-dependent phosphorylation of inducer-generating enzymes or transcriptional regulators (Deutscher *et al.*, 1997; Stülke *et al.*, 1998).

Glycerol utilization in *B. subtilis* depends on a functional PTS, although glycerol is transported into cells by a non-PTS permease (Reizer *et al.*, 1984; Beijer & Rutberg, 1992). Mutations that bypass this dependency were isolated and found to be in *glpK*, the gene encoding glycerol kinase which generates glycerol 3-phosphate, the internal inducer of the *glp* regulon in *B. subtilis* (Wehtje *et al.*, 1995). Subsequently, direct phosphorylation of glycerol kinase by EI and HPr was demonstrated (Charrier *et al.*, 1997).

A protein domain, PRD, is found in transcriptional regulators such as RNA-binding antiterminators and DNA-binding activators that serves as a target of PTS-dependent phosphorylation (Tortosa *et al.*, 1997; Reizer & Saier, 1997; Stülke *et al.*, 1998). PRD-containing regulators are found primarily in Gram-positive bacteria. Eight PRD-containing regulators are encoded within the *B. subtilis* chromosome (see Table 3). All eight proteins regulate the expression of genes encoding PTS permeases and associated catabolic enzymes. PRD-containing transcriptional antiterminators and the activator protein LevR have been studied extensively (Deutscher *et al.*, 1997; Martin-Verstraete *et al.*, 1998; Stülke *et al.*, 1998). Four antiterminators of this type are present in *B. subtilis* (see Table 3). They control the expression of genes required for glucose, sucrose and β -glucoside utilization (Stülke *et al.*, 1997; Steinmetz *et al.*, 1989; Schnetz *et al.*, 1996). In addition, PRD-containing transcriptional activators, LevR and LicR, control

Fig. 4. Genetic arrangement of genes encoding PTS proteins of the Fru family. The convention of presentation is as described in the legend to Fig. 1. (a) Genetic map of the *mtl* gene cluster. *mtlD* is followed by a potential ρ -independent terminator. (b) Genetic map of the *man* gene cluster. Potential ρ -independent terminators are located downstream of *manR* and *yjdF*. ManR is homologous to PRD-containing transcriptional activators. (c) Genetic map of the *fru* gene cluster. A potential ρ -independent terminator is located downstream of *fruA*. FruR shares sequence similarity with transcriptional repressors of the DeoR family.

expression of the levanase and *licBCAH* operons, respectively (Débarbouillé *et al.*, 1991; Tobisch *et al.*, 1997). The LevR activator contains two PRDs. LicR contains two PRDs and a C-terminal domain homologous to EIIA^{Fru}. The phosphorylation site in this domain is conserved and a mutation of this site was shown to result in constitutive LicR activity (Tobisch *et al.*, 1999). The complete nucleotide sequence of the *B. subtilis* genome revealed the presence of two novel

putative transcriptional activators that are similar to LicR. Since *yjdC* is located just upstream of the operon involved in mannose utilization (see Fig. 4b), the designation ManR was proposed (Stülke *et al.*, 1998). YdaA is homologous to the regulators of mannitol catabolism of *B. stearothermophilus* (Henstra *et al.*, 1999) and *Clostridium acetobutylicum* (accession no. U53868). The designation MtlR was thus proposed for YdaA (Stülke *et al.*, 1998).

Table 4. Utilization of various carbon sources by mutants affected in PTS constituents

Strain	Mutation	Doubling time (min) on:*			
		Glucose	Maltose	Glucosamine	N-Acetylglucosamine
168		60	85	85	180
QB5350	<i>ptsH</i>	360	NG	> 360	NG
GP113	<i>ptsG</i>	180	230	90	180
GP110	<i>malP (glvC)</i>	70	630	90	240
QB6098	<i>gamP (ybfS)</i>	60	110	220	240
QB6099	<i>ypqE</i>	60	90	80	240

* NG, No growth.

Phenotypes of *B. subtilis* strains defective in EII components

The functional role of the GlvC and YbfS permeases was determined. *glvC* was identified in the framework of the *B. subtilis* sequencing project and proposed to encode a β -glucoside-specific EII (Yamamoto *et al.*, 1996). However, the functional characterization of genes encoding a maltose-specific EII and a phospho- α -glucosidase from *Fusobacterium mortiferum* revealed that the homologous GlvA of *B. subtilis*, encoded in the same operon as GlvC, is in fact a phospho- α -glucosidase rather than a phospho- β -glucosidase (Bouma *et al.*, 1997; Thompson *et al.*, 1998). In addition, GlvC exhibits higher sequence similarity to PTS permeases of the glucose/maltose family than to the sucrose/ β -glucoside-specific PTS permeases. To study the functional role of *glvC* we constructed a *glvC* mutant of *B. subtilis*. We additionally constructed strains containing *ypqE* and *ybfS* mutations (Kunst *et al.*, 1997; see Table 2). To obtain a comprehensive set of mutants we used strain GP113 in which the part of *ptsG* encoding the IIB and IIA domains of the glucose permease was deleted. The IIA domain of the glucose permease has been shown to phosphorylate IIB domains of the sucrose- and trehalose-specific PTS permeases that lack a IIA domain (Sutrina *et al.*, 1990; Dahl, 1997).

B. subtilis 168 and the mutant strains were grown in C minimal medium with either glucose, maltose, glucosamine or N-acetylglucosamine as a single source of carbon and energy and their doubling times were determined (Table 4). All four sugars were readily utilized by the wild-type strain, although the generation time of cultures grown on N-acetylglucosamine was longer than the generation time of cells grown on either glucose, maltose or glucosamine. The PTS is required for efficient catabolism of all the carbon sources tested as is evident from the slow growth rate (or the lack of growth) of the *ptsH* mutant strain. As observed previously, the *ptsH* mutant strain grew slowly on glucose, suggesting the presence of a non-PTS system for glucose transport and phosphorylation (Stülke *et al.*, 1995). The recently identified glucose transporter, GlcP, is presumably responsible for this residual growth (Paulsen *et al.*, 1998). Similarly, slow growth of the *ptsH* mutant

was observed with glucosamine, whereas maltose and N-acetylglucosamine were not utilized by this strain. The IIA and IIB domains of the glucose permease were required for efficient growth on glucose and maltose while the *ptsG* mutation did not affect growth on glucosamine or N-acetylglucosamine. Four conclusions can be drawn from these results. (i) PTS permeases distinct from PtsG may transport and phosphorylate glucose, albeit with low efficiency. (ii) Phosphotransfer via the IIA^{Glc} domain appears to be necessary for efficient utilization of maltose. (iii) The *glvC* mutant strain GP110 grew normally on all the carbon sources tested with the exception of maltose. We propose, therefore, that *glvC* encodes a maltose-specific PTS permease and we redesignate the gene *malP*. This finding is in agreement with the presence of a phospho- α -glucosidase-encoding gene within the *malP* operon (see Fig. 1b) and with the strong sequence conservation of MalP with other maltose- and glucose-specific PTS permeases. (iv) The *ybfS* mutation resulted in impaired growth in the presence of glucosamine, suggesting that the EII encoded by *ybfS* catalyses the transport and phosphorylation of this sugar. This observation is reinforced by the genetic arrangement. *ybfT*, which precedes *ybfS*, exhibits a high degree of sequence similarity to the characterized (deaminating) glucosamine-6-phosphate isomerase of *E. coli* (Fig. 1c). Therefore, we propose to designate the *ybfT* and *ybfS* genes as *gamA* and *gamP*, respectively. The *ypqE* mutation did not yield a recognizable phenotype under the conditions tested here and the function of the encoded protein therefore remains unknown.

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