

Sugar Uptake and Carbon Catabolite Repression in *Bacillus megaterium* Strains With Inactivated *ptsHI*

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Abstract

We have determined the role played by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in carbon catabolite repression (CCR) of xylose utilization in *Bacillus megaterium*. For that purpose we have cloned, sequenced and inactivated the genes *ptsH* and *ptsI* of *B. megaterium*, encoding HPr and EI of the PTS, respectively. While glucose uptake of a *ptsHI* mutant is not affected at 12.5 mM of glucose, CCR of the *xyl* operon is reduced in this mutant from 16-fold to 3-fold. This may be attributed to the loss of the corepressor of CcpA, HPr(Ser-P), or could result from the slower growth rate of the mutant. In contrast, CCR exerted by fructose or mannitol is completely abolished. We conclude that glucose triggers additional mechanisms of CCR than fructose or mannitol. The remaining 3-fold glucose repression is relieved in a strain in which *ptsHI* and *glk*, encoding glucokinase, are inactivated. This result indicates that glucose metabolism is necessary for CCR. The ability of the *ptsHI* mutant to take up glucose suggests the existence of a second, non-PTS glucose uptake system. The K_m and v_{max} values of this transporter ranged between 2 and 5 mM and 154 to 219 nmol/[(mg protein)*min], respectively.

Introduction

Sugars are generally transported into bacterial cells against a concentration gradient. The phosphoenolpyruvate:sugar phosphotransferase system (PTS), in which sugar transport is coupled to phosphate transfer from phosphoenolpyruvate to the sugar via enzyme I (EI), HPr and an enzyme II (EII) complex, is commonly employed in bacteria for preferred sugars like glucose. HPr and EI are non-specific enzymes, and EII are membrane-bound, sugar-specific multi-domain enzymes consisting of a single or up to three polypeptides. They catalyze the transport and phosphorylation of their cognate sugars (reviewed by Meadow *et al.*, 1990; Saier and Reizer, 1992; Postma *et al.*, 1993). Sugar transport may also be driven by preexisting concentration gradients, e.g. in proton symporters (Paulsen *et al.*, 1998) or by ATP hydrolysis (Quentin *et al.*, 1999).

Glucose, the preferred carbon source of a number of bacteria, is mostly taken up by the PTS. However, recently obtained results indicate the existence of PTS-independent glucose uptake systems in, e.g., *B. subtilis* (Paulsen *et al.*, 1998), *B. licheniformis* (Tangney *et al.*, 1993), *Staphylococcus xylosus* (Fiegler *et al.*, 1999), *Streptococcus bovis* (Russell, 1990), *S. mutans* (Keevil *et al.*, 1986; Buckley and Hamilton, 1994; Cvitkovitch *et al.*, 1995), *Lactobacillus brevis* (Romano *et al.*, 1987; Ye *et al.*, 1994; Ye and Saier, 1995), *Zymomonas mobilis* (Barnell *et al.*, 1990; 1992; Parker *et al.*, 1995; Weisser *et al.*, 1995), *Brucella abortus* (Rest and Robertson, 1974; Essenberg *et al.*, 1997) and *Vibrio parahaemolyticus* (Sarker *et al.*, 1994). These transport systems allow the respective strains to grow on high glucose concentrations when their PTS has been inactivated.

In addition to taking up sugars, the PTS also delivers the signals leading to catabolite repression in Gram positive bacteria of low GC content (reviewed in Stülke and Hillen, 1999). CCR of the *B. megaterium xyl* operon exerted by glucose utilizes multiple mechanisms (Gösseringer *et al.*, 1997; Späth *et al.*, 1997). We describe here the cloning and inactivation of *ptsHI* encoding HPr and enzyme I from *B. megaterium*, and investigate the influence of their inactivation on glucose uptake and CCR.

Results

Cloning of *B. megaterium ptsHI*

We transformed the *ptsH* mutant *B. subtilis* MD177 (Arnaud *et al.*, 1992) with pWH1509KGBI, an *AluI* derived gene library of *B. megaterium* (Hueck *et al.*, 1995). Two out of 14,000 transformants grew to larger colonies on MOPSO minimal medium supplemented with 12.5 mM glucose and 4 mg/l neomycin. After restreaking, the plasmids were prepared from the candidates, transformed into *E. coli* and retransformed into *B. subtilis* MD177 to verify the link between phenotype and plasmid. One such plasmid, called pWH1523, contains a continuous chromosomal fragment of about 6 kbp. pWH1523 was partially restricted with *Sau3A* and the fragments were recloned into the *EcoRV* site of pWH1509K, transformed into *B. subtilis* MD177 and screened for *ptsH* complementation as described above. About one in 18 colonies showed the desired phenotype. The smallest complementing plasmid contained a 4.4 kbp insert and was called pWH1524.

Nucleotide sequence of *B. megaterium ptsHI*

2,300 bps were sequenced on both strands (accession number AJ 005075) and compared to the *pts* sequence of *B. subtilis* to locate *ptsH*. The deduced amino acid sequence exhibits the expected high identities to HPr proteins of other bacteria: 82% to *B. subtilis*, 76% to *B. stearothermophilus*, 66% to *S. mutans*, and *S. salivarius*, 67% to *S. aureus* and 42% to *E. coli*. The sequence downstream from *ptsH* encodes *ptsI*. The deduced amino

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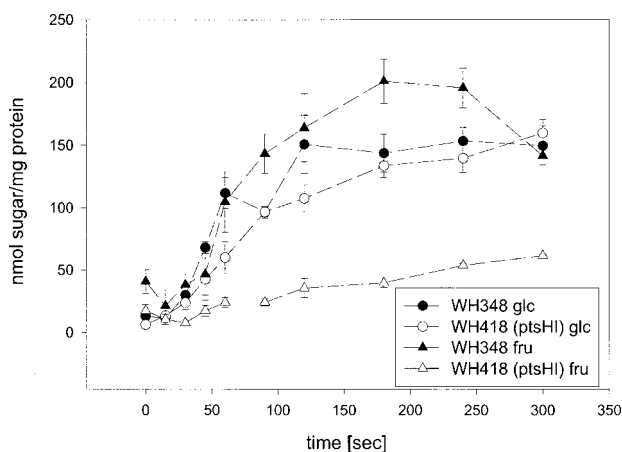


Figure 1. Glucose and fructose uptake in *B. megaterium* WH348 and WH418 (*ptsHI*). Sugar uptake into the wildtype *B. megaterium* WH348 is represented by filled symbols, the uptake into the *ptsHI* mutant WH418 is shown as open symbols. Uptake of glucose is shown using circles, uptake of fructose is shown as triangles.

acid sequence shows 79% identity to EI of *B. subtilis*, 77% to *B. stearothermophilus*, 67% to *S. aureus*, 63% to *S. mutans* and 49% to *E. coli*.

As in *B. subtilis* (Gonzy-Tréboul *et al.*, 1989), the *ptsH* and *ptsI* reading frames overlap by one nucleotide. Potential ribosome binding sites are located eleven nucleotides upstream from the *ptsH* and the *ptsI* start codons, respectively. 149 (TTGAAA at -35 and TAAAA at -10, spacing 16 nucleotides) and 79 (TTTACA at -35 and TATCTT at -10, spacing 18 nucleotides) nucleotides upstream of the *B. megaterium ptsH* start codon we located two potential promoters, each showing three mismatches from the consensus sequence of σ^A promoters in *B. subtilis* (Haldenwang, 1995). Both contain a -16 region with one mismatch to the proposed consensus sequence TGTG (Voskuil *et al.*, 1995). Since the *ptsH* promoter of *B. subtilis* is in a similar position, 50 bps upstream from the start codon, both genes may be cotranscribed from a promoter upstream of *ptsH*, as in *B. subtilis* (Gonzy-Tréboul *et al.*,

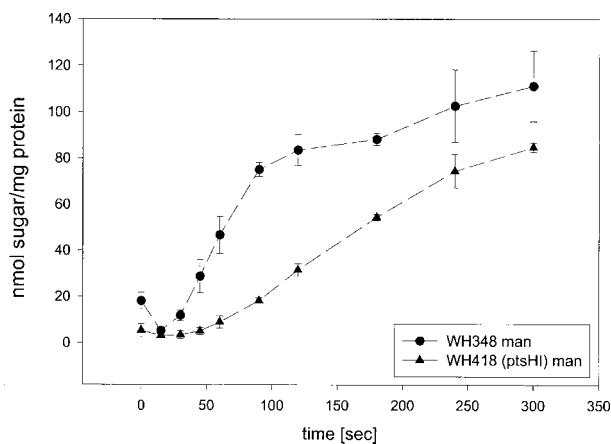


Figure 2. Mannitol uptake in *B. megaterium* WH348 and WH418. Mannitol uptake into WH348 is represented by the circles, that into WH418 by the triangles.

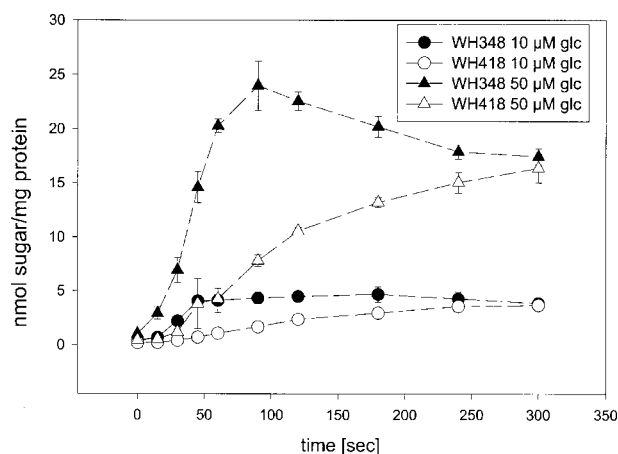


Figure 3. Glucose uptake in WH348 and WH418 at concentrations of 10 and 50 μ M of glucose. Glucose uptake into WH348 is represented by circles, the uptake into the *ptsHI* mutant WH418 is represented by open symbols. Uptake of glucose at 10 μ M is shown as circles, that at 50 μ M of glucose as triangles.

1989) and *E. coli* (De Reuse and Danchin, 1988).

Construction of *B. megaterium ptsHI* Strains

To inactivate *ptsH*, pWH150Ke was constructed for integration into the chromosome of *B. megaterium* WH348. This vector contains the chromosomal fragment of pWH1524 in which *ptsH* is inactivated by an inserted erythromycin resistance cassette as described in Experimental procedures. The double crossover replacing *ptsH* by *ptsH::erm* was isolated by selecting for erythromycin resistance, followed by screening for neomycin sensitive, small colonies on LB. The resulting *B. megaterium* strain was called WH418. The construction was confirmed by Southern Blot analysis (data not shown). WH428 (inactivated *ptsHI* and *glk*) was similarly constructed.

Growth and Sugar Transport of *B. megaterium ptsHI*

The *ptsHI* deficient strain *B. megaterium* WH418 should be unable to transport sugars via the PTS. We have determined uptake of glucose, fructose and mannitol in WH348 (parent of WH418) and WH418 in the presence of 1 mM of the respective sugar. The results are depicted in Figures 1 and 2. The *ptsHI* mutant WH418 takes up glucose almost as efficiently as the wildtype WH348 at this concentration but hardly transports any fructose, whereas WH348 takes up fructose as well as glucose. This result correlates with the lack of growth on plates with fructose as the sole carbon source where only very small colonies of WH418 can be detected after incubation for 72 h at 37°C. In contrast, WH348 as well as WH418 grow on plates with glucose as the sole carbon source after incubation for 36 h at 37°C, but the colonies of WH348 are about 4-fold larger than those of WH418. We also measured growth of the two strains in liquid minimal medium with glucose as the sole carbon source. The wild-type WH348 shows a doubling time of 110 min under these conditions. In contrast, WH418 shows an elevated generation time of about 180 min.

Uptake of mannitol in the wild-type WH348 is much less efficient than uptake of glucose or fructose, and

Table 1. CCR of *xyl* Expression Exerted by Glucose

Strain	relevant genotype	β -galactosidase activity [miller-units]		
		M9-S ^a	M9-SX ^b	M9-SXG ^c RF
WH348	Wt	19 ± 1	1790 ± 90	15.7
WH419	$\Delta ccpA$	21 ± 1	2050 ± 120	1.2
WH417	<i>Glk</i>	22 ± 1	1800 ± 140	7.0
WH418	<i>PtsHI</i>	21 ± 1	1600 ± 50	2.6
WH428	<i>glk ptsHI</i>	20 ± 1	1690 ± 40	1.2

^a β -galactosidase activities in minimal medium M9 containing 0.5% succinate.

^b β -galactosidase activities in minimal medium M9 containing 0.5% succinate and 0.25% xylose

^cfactors of repression in minimal medium M9 containing 0.5% succinate, 0.25% xylose and 0.25% glucose (β -galactosidase activity in the presence of xylose divided by activity in the presence of xylose and glucose).

Table 2. CCR of *xyl* Expression Exerted by Fructose and Mannitol

strain	relevant genotype	β -galactosidase activity [miller-units]		
		M9-SX ^a	M9-SXF ^b RF	M9-SXM ^c RF
WH348	wt	1210 ± 75	3.4	3.3
WH419	$\Delta ccpA$	1170 ± 65	1.1	1.0
WH417	<i>glk</i>	1040 ± 60	3.2	3.2
WH418	<i>ptsHI</i>	1030 ± 30	1.2	0.9
WH428	<i>glk ptsHI</i>	1780 ± 80	1.5	1.0

^a β -galactosidase activities in minimal medium M9 containing 0.5% succinate and 0.25% xylose.

^bfactors of repression in minimal medium M9 containing 0.5% succinate, 0.25% xylose and 0.25% fructose (β -galactosidase activity in the presence of xylose divided by activity in the presence of xylose and fructose).

^cfactors of repression in minimal medium M9 containing 0.5% succinate, 0.25% xylose and 0.25% mannitol (β -galactosidase activity in the presence of xylose divided by activity in the presence of xylose and mannitol).

WH418 takes up mannitol even less efficiently, which correlates also with the growth phenotype on plates: WH348 grows within 36-48 hours to smaller colonies on mannitol than it does on glucose or fructose while WH418 grows similarly slow on mannitol or fructose.

Uptake of glucose at concentrations of 10 and 50 μ M is shown in Figure 3. Glucose uptake at 10 μ M is less efficient in WH348 and WH418 than at 50 μ M, but it accumulates faster in WH348 than in WH418. This difference is clearer in the presence of 50 μ M glucose, where the uptake is much faster in WH348 than in WH418. Thus, the PTS is the more efficient glucose transporter at 50 μ M. However, at 1 mM glucose, another uptake system is able to transport glucose as efficiently as the PTS.

We determined the K_m and v_{max} values of the second glucose transporter. For that purpose, glucose uptake in WH418 was measured for 1 min at various glucose concentrations ranging from 50 μ M to 10 mM. The K_m and v_{max} values, 2.5 to 5 mM and 154 to 219 nmol/[(mg protein)*min], respectively, were deduced from the corresponding Lineweaver Burke plots (not shown).

Influence of *ptsHI* on Carbon Catabolite Repression of the *xyl* Operon

We have quantitated *in vivo* the roles played by *ptsHI* and *glk* in CCR of xylose utilization in *B. megaterium*. The *xylA-lacZ* transcriptional fusion used for that purpose contains *xyIO* and *cre* mediating xylose induction and carbon catabolite repression by glucose, fructose or mannitol, as shown in Tables 2 and 3.

Expression of the *xylA-lacZ* fusion is 68-fold inducible in WH348 by xylose and 16-fold repressed by glucose on top of xylose. A 2.6-fold glucose repression is observed in WH418, in which *ptsH* and *ptsI* are not expressed. WH428 contains an additional inactivation of *glk* encoding glucokinase which by itself leads to a two-fold reduction of CCR in the presence of glucose as shown in Table 1 strain WH417 (Späth et al., 1997). CCR of the *xyl* operon in WH428 is reduced to the level seen in the *ccpA* strain WH419. CCR of the *xyl* operon exerted by fructose or mannitol is 3.4-fold. Knocking out the glucokinase in strain WH417 (*glk*) has no influence on CR exerted by these two sugars as shown in Table 2, but knocking out *ptsHI* in addition totally abolishes CR exerted by fructose or mannitol.

Discussion

A Non-PTS Glucose Uptake System in *B. megaterium*

Glucose uptake in *B. megaterium* WH418(*ptsH*) is only slightly reduced at 1 mM as compared to the parent WH348. A *B. subtilis ptsH* mutant does not take up glucose (Bachem et al., 1997) or is at least strongly impaired in glucose uptake (Paulsen et al., 1998). The fact that an insertional inactivation of *ptsH* results in the lack of fructose uptake shows that *B. megaterium* does not contain another active copy of *ptsH*. We conclude that an efficient non-PTS glucose uptake system must exist in *B. megaterium*. Similar systems have previously been described for *B. licheniformis* (Tangney et al., 1993) and recently for *B. subtilis* (Paulsen et al., 1998). The *B. megaterium* system differs from that in *B. subtilis* in its low affinity for glucose and its high activity in the absence of a PTS (Paulsen et al., 1998). The one found here in *B. megaterium* is quite efficient down to concentrations of 50 μ M of glucose, but the difference to the wild-type is clearly visible as uptake of glucose in WH418 is slower than in WH348 at 50 μ M. The non-PTS uptake mechanism is not active at 10 μ M of glucose. Thus, the PTS is required for growth at lower glucose concentrations. *Streptococcus mutans* also possess a glucose specific PTS and an alternative glucose transport system. The latter is only functional under conditions of high growth rates, low pH or excess glucose, conditions which repress the synthesis of EII^{Glc} and, therefore, uptake via the PTS (Ellwood and Hamilton, 1982; Ellwood et al., 1979). The non-PTS system has an apparent K_m of 125 μ M for glucose and an apparent v_{max} of 0.87 nmol/ [μ mg cells]*min] (Cvitkovitch et al., 1995). It is suggested that this glucose transporter is linked to the proton motive force and probably requires internal glucose phosphorylation as there is a reciprocal relationship between the activities of the glucose PTS and glucokinase. In contrast, expression of *B. subtilis* EII^{Glc} is not repressed by high glucose concentrations (Stülke et al., 1997). Glucose:H⁺ symporters have been reported for *Lactobacillus brevis* (Romano et al., 1987; Ye et al., 1994) and *Bacillus subtilis* (Paulsen et al., 1998). They show a high affinity for glucose with K_m values of 4 μ M and 20 μ M, respectively. The glucose:H⁺ symporter of *Lactobacillus brevis* is allosterically inhibited by binding of HPr(Ser-P) to

Table 3. Strains and Plasmids Used in This Study

Strain/Plasmid	Genetic Marker	Source of reference
Strains		
<i>B. megaterium</i> WH348	<i>lac, gdh2</i> (xylA1-spoVG-lacZ), (containing <i>O2</i> , <i>O1_L</i> und <i>O1_R</i>)	Schmiedel D., unpublished
<i>B. megaterium</i> WH417	<i>glk'-luxAB'-glk</i>	Späth <i>et al.</i> , 1997
<i>B. megaterium</i> WH419	$\Delta ccpA$	this study
<i>B. megaterium</i> WH418	<i>ptsH'-erm'-ptsH, ptsI</i>	this study
<i>B. subtilis</i> 168		BGSC, Ohio State University
<i>B. subtilis</i> MD177	<i>ptsH::cat</i>	Arnaud <i>et al.</i> (1992)
Plasmids		
pSKII ⁺ pE194	Ap ^R , <i>lacZ'</i> Em ^R	Stratagene Horinouchi and Weisblum (1982)
pWH1509K	Ap ^R , Tc ^R , Kn ^R	Rygun and Hillen (1992)
pWH1509KGBI	Ap ^R , Kn ^R	Hueck <i>et al.</i> (1994)
pWH1509Ke	Ap ^R , Kn ^R	this study
pWH1523	Ap ^R , Kn ^R , <i>ptsHI</i>	this study
pWH1524	Ap ^R , Kn ^R , <i>ptsHI</i>	this study
pWH1525	Ap ^R , <i>ptsHI</i>	this study
pWH1525e	Ap ^R , Em ^R , <i>ptsH::em</i>	this study

Kn^R, kanamycin resistance; Ap^R, ampicillin resistance; Em^R, erythromycin resistance; Tc^R, tetracycline resistance

the permease, thereby regulating glucose uptake (Ye and Saier, 1995). GlcP, the glucose:H⁺ symporter of *B. subtilis*, is a member of the major facilitator family, and forms a subfamily together with the fucose:H⁺ symporter of *Escherichia coli* (Gunn *et al.*, 1994) and the glucose/galactose:H⁺ symporter of *Brucella abortus* (Rest and Robertson, 1974; Essenberg *et al.*, 1997). Since even a *ptsGHI glcP* double mutant shows some glucose uptake, a third system may exist. In *Zymomonas mobilis*, an organism living in habitats with high sugar concentrations, the potential facilitator Glf with a K_m for glucose of 10-15 mM takes up that sugar (Parker *et al.*, 1995; Weisser *et al.*, 1995). It shows similarities to several eucaryotic glucose facilitators. Glucose uptake by facilitated diffusion in addition to a PTS has also been suggested for *Staphylococcus xylosus* (Fiegler *et al.*, 1999). Glucose transport in this organism is mediated by GlcU and depends on a functional glucose kinase (Glc). The high K_m of the second glucose uptake system in *B. megaterium* suggests that it operates by facilitated diffusion. As in *Staphylococcus xylosus* and *Streptococcus mutans*, glucose would then be phosphorylated internally by glucokinase (Späth *et al.*, 1997).

Carbon Catabolite Repression of the *B. megaterium* xyl Operon

Expression of the *B. megaterium* xyl operon is induced by xylose and subject to CCR mediated by CcpA, cre, HPr(Ser-P), PtsK and Glk (Hueck *et al.*, 1995; Gösseringer *et al.*, 1997; Schmiedel *et al.*, 1997; Späth *et al.*, 1997; Reizer *et al.* 1998). The reduced CCR in WH418 (16- to 2.6-fold) is not due to the lack of a metabolic signal since glucose uptake is not affected. Thus, the importance of HPr(Ser-P) for CCR of xyl is underlined by this result. The residual glucose repression indicated that HPr(Ser-P) is not the only contributor to CCR. Inactivation of *glk* in strain WH417 (Späth *et al.*, 1997) reduced glucose repression from 16- to 7-fold. Inactivation of *ptsHI* and *glk* in WH428

results in the same 1.2-fold glucose repression as the *ccpA* mutation in strain WH419. Two different mechanisms are conceivable by which glucokinase could trigger CcpA activity. Since the synthesis of glucose-6-phosphate depends on glucokinase in the absence of the PTS, metabolic activation of PtsK may lead to phosphorylation of an alternative but as of yet in *B. megaterium* unknown Crh-like cofactor for CcpA. This possibility has been shown in *B. subtilis* (Galiniere *et al.*, 1997). Alternatively, Glc-6-P itself may trigger CcpA binding to cre of the xyl operon of *B. megaterium* (Gösseringer *et al.*, 1997). The influence of Glc-6-P as an effector of CCR had also been shown for CCR of the *gnt* operon in *B. subtilis* (Miwa *et al.*, 1997). Thus, glucose mediated CCR in *B. megaterium* differs from fructose mediated CCR in that it triggers multiple mechanisms. This probably reflects the greater importance of glucose as a signal for favourable growth conditions in natural habitats of *B. megaterium*.

Experimental Procedures

General Methods

Plasmid DNA from *E. coli* was prepared using the Nucleobond Kit (Macherey and Nagel, Dueren, FRG). Preparation of total DNA from *Bacilli* (Gärtner *et al.*, 1988) and Southern Blot analysis (Southern, 1975) were done as described before. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977), using [³²P]dATP (Amersham, Braunschweig, FRG), sequenase (USB, Cleveland, Ohio, USA) and synthetic primers. Recombinant *B. megaterium* strains were constructed using temperature sensitive plasmids as described earlier (Rygun and Hillen, 1992). If needed, erythromycin was used in a final concentration of 2.5 mg/l and neomycin in a final concentration of 4 mg/l for selection in *Bacillus*. For selection in *E. coli*, ampicillin was used at a final concentration of 100 mg/l. All other general methods were done as described before (Sambrook *et al.* 1989, Rygun and Hillen 1992).

Bacterial Strains and Plasmids

All bacterial strains and plasmids used and constructed in this study are listed in Table 3. *Escherichia coli* DH5 α (Sambrook *et al.* 1989) was generally used for cloning. *Bacillus megaterium* WH348 (kindly provided by D. Schmiedel) was the parental strain of strains WH417 (*glk*), WH418 (*ptsHI*), WH419 ($\Delta ccpA$) and WH428 (*ptsHI glk*).

Plasmid pWH2041 was used as described earlier for the construction of $\Delta ccpA$ strains (Hueck *et al.* 1995). This plasmid carries the *B. megaterium ccpA* gene with an in-frame deletion. The double crossover replacing *ccpA* by $\Delta ccpA$ was isolated by screening for blue colonies on M9/SXG medium, indicating a lack of CR. The mutant *ccpA* was integrated in the wildtype strain WH348. The resulting strain was WH419 ($\Delta ccpA$).

Strain WH417 (WH348 *glk*) was kindly provided by C. Späth. For construction of WH428 (*ptsHI glk*), plasmid pWH668 was used as described earlier (Späth *et al.* 1997). This plasmid carries the *B. megaterium glk* gene destroyed by insertion of a *spoVG-luxAB*-fusion. The intact *glk* gene of strain WH418 (*ptsHI*) was replaced by the destroyed gene, yielding strain WH428 (*ptsHI glk*).

Culture and Growth Conditions

B. megaterium and *E. coli* were grown in LB medium. M9 medium supplemented with 0.05% yeast extract and 0.05% casamino acids was used as a minimal medium for *B. megaterium*. 0.5% succinate was added to the M9 medium as a general carbon source. MOPSO minimal medium (Neidhardt *et al.*, 1974) with 10 mM glucose as sole carbon source was used for screening of complemented *B. subtilis ptsH* mutants.

Glucose Uptake Measurements

Cells were grown in M9 minimal medium (Hueck *et al.*, 1995) with 0.5% succinate and glucose, fructose or mannitol as indicated above. Cells were harvested by centrifugation at an OD_{600} of 0.4, washed three times in transport buffer (50 mM Tris-HCl, pH 7.2, 20 mM $MgCl_2$) and resuspended in the same buffer. Aliquots of this cell suspension were used for the uptake assay. ^{14}C glucose (specific activity 74 $\mu Ci/\mu mol$; Amersham) were added to adjust the suspension to 1 mM glucose and 4×10^6 counts per minute unless otherwise indicated. At times indicated in the respective figures, samples were taken, filtered through nitrocellulose (0.45 μm pores; Satorius, Goettingen, FRG) and washed immediately three times with ice-cold 0.9% NaCl. Filters were dried and the amount of radioactivity was determined. The amount of protein in the cell suspension was determined using a Bradford protein assay kit (BioRad, München, FRG). For the kinetic analysis glucose concentrations between 50 μM and 10 mM were used. Glucose uptake was measured for 1 minute and samples were taken every 10 seconds.

β -Galactosidase Assays

Cells were grown in M9 medium supplemented with 0.05% casamino acids, 0.05% yeast extract, and the respective regulatory carbon sources described in the results and discussion sections to an optical density of 0.4 to 0.6 at 600 nm. β -galactosidase activities were assayed on the same day by the method of Miller (Miller, 1972).

Acknowledgements

We thank D. Schmiedel for strain WH348. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 473 "Schaltvorgänge der Transkription" and the Fonds der Chemischen Industrie.

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