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., 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 Alul derived gene library of B. megaterium (Hueck et al., 1995). Two out of 14,000 transformants grew to larger colonies on MOPS 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 AJ005075) 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. steaothermophilus, 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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acid sequence shows 79% identity to El 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 *σA* 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*., 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 *ptsH* 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 *ptsH* 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
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 μM is shown in Figure 3. Glucose uptake at 10 μM is less efficient in WH348 and WH418 than at 50 μM, but it accumulates faster in WH348 than in WH418. This difference is clearer in the presence of 50 μM glucose, where the uptake is much faster in WH348 than in WH418. Thus, the PTS is the more efficient glucose transporter at 50 μM. 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 μM to 10 mM. The K_m and V_max values, 2.5 to 5 mM and 154 to 219 nmol/[g 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 xylO 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 μM 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 μM. The non-PTS uptake mechanism is not active at 10 μM 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^BC 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 μM for glucose and an apparent V_max of 0.87 nmol/[μg cells]^{min} (Cvitkovich 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^BC 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 μM and 20 μM, respectively. The glucose:H^+ symporter of Lactobacillus brevis is allosterically inhibited by binding of HPN(Ser-P) to

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>relevant genotype</th>
<th>M9-SX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M9-SX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>M9-SXM&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>WH348</td>
<td>WT</td>
<td>19 ± 1</td>
<td>1790 ± 90</td>
<td>15.7</td>
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<tr>
<td>WH419</td>
<td>ΔccpA</td>
<td>21 ± 1</td>
<td>2050 ± 120</td>
<td>1.2</td>
</tr>
<tr>
<td>WH417</td>
<td>Glk</td>
<td>22 ± 1</td>
<td>1800 ± 140</td>
<td>7.0</td>
</tr>
<tr>
<td>WH418</td>
<td>ptsI</td>
<td>21 ± 1</td>
<td>1600 ± 50</td>
<td>2.6</td>
</tr>
<tr>
<td>WH428</td>
<td>glk ptsI</td>
<td>20 ± 1</td>
<td>1800 ± 40</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>-β-galactosidase activities in minimal medium M9 containing 0.5% succinate.

<sup>b</sup>-β-galactosidase activities in minimal medium M9 containing 0.5% succinate and 0.25% xylose.

<sup>c</sup>-β-galactosidase activity in the presence of xylose divided by activity in the presence of xylose and glucose.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>relevant genotype</th>
<th>M9-SX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M9-SX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>M9-SXM&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WH348</td>
<td>wt</td>
<td>1210 ± 75</td>
<td>3.4</td>
<td>3.3</td>
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<tr>
<td>WH419</td>
<td>ΔccpA</td>
<td>1170 ± 65</td>
<td>1.1</td>
<td>1.0</td>
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<tr>
<td>WH417</td>
<td>Glk</td>
<td>1040 ± 60</td>
<td>3.2</td>
<td>3.2</td>
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<tr>
<td>WH418</td>
<td>ptsI</td>
<td>1030 ± 30</td>
<td>1.2</td>
<td>0.9</td>
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<tr>
<td>WH428</td>
<td>glk ptsI</td>
<td>1780 ± 80</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>-β-galactosidase activities in minimal medium M9 containing 0.5% succinate and 0.25% xylose.

<sup>b</sup>-β-galactosidase activities 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).

<sup>c</sup>-β-galactosidase activity in the presence of xylose divided by activity in the presence of xylose and mannitol.
the permease, thereby regulating glucose uptake (Ye and Saier, 1995). GlcP, the glucose:H\(^+\) symporter of \textit{B. subtilis}, is a member of the major facilitator family, and forms a subfamily together with the fucose:H\(^+\) symporter of \textit{Escherichia coli} (Gunn et al., 1994) and the glucose/galactose:H\(^+\) symporter of \textit{Brucella abortus} (Rest and Robertson, 1974; Essenberger et al., 1997). Since even a \textit{ptsGHI} glc\textit{P} double mutant shows some glucose uptake, a third system may exist. In \textit{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; Weissert et al., 1995). It shows similarities to several eucaryotic glucose facilitators. Glucose uptake by facilitated diffusion in \textit{Staphylococcus xylosus} (Fiegler et al., 1999). Glucose transport in this organism is mediated by GlcU and depends on a functional glucose kinase (Glk). The high \(K_m\) of the second glucose uptake system in \textit{B. megaterium} suggests that it operates by facilitated diffusion. As in \textit{Staphylococcus xylosus} and \textit{Streptococcus mutans}, glucose would then be phosphorylated internally by glucokinase (Späth et al., 1997).

### Carbon Catabolite Repression of the \textit{B. megaterium} \textit{xyl} Operon

Expression of the \textit{B. megaterium} \textit{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 \textit{xyl} is underlined by this result. The residual glucose repression indicated that HPr(Ser-P) is not the only contributor to CCR. Inactivation of \textit{glk} in strain WH417 (Späth et al., 1997) reduced glucose repression from 16- to 7-fold. Inactivation of \textit{ptsHI} and \textit{glk} in WH428 results in the same 1.2-fold glucose repression as the \textit{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 \textit{B. megaterium} unknown Crh-like cofactor for CcpA. This possibility has been shown in \textit{B. subtilis} (Galiner et al., 1997). Alternatively, Glc-6-P itself may trigger CcpA binding to cre of the \textit{xyl} operon of \textit{B. megaterium} (Gösseringer et al., 1997). The influence of Glc-6-P as an effector of CCR has also been shown for the \textit{gnt} operon in \textit{B. subtilis} (Miwa et al., 1997). Thus, glucose mediated CCR in \textit{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 \textit{B. megaterium}.

### Experimental Procedures

#### General Methods

Plasmid DNA from \textit{E. coli} was prepared using the Nucleobond Kit (Macherey and Nagel, Dueren, FRG). Preparation of total DNA from \textit{Bacillus} (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 [\(\alpha\-\text{P}\)]dATP (Amersham, Braunschweig, FRG), sequenase (USB, Cleveland, Ohio, USA) and synthetic primers. Recombinant \textit{B. megaterium} strains were constructed using temperature sensitive plasmids as described earlier (Rygus and Hillen, 1992). If needed, erythromycin was used in a final concentration of 2.5 mg/l. All other general methods were done as described before (Sambrook et al., 1989, Rygus and Hillen 1992).

#### Bacterial Strains and Plasmids

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

### Table 3. Strains and Plasmids Used in This Study

<table>
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<th>Strain/Plasmid</th>
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<td>\textit{\textit{lacP}\textit{A}}\textit{R}</td>
<td>this study</td>
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<td>\textit{ptsHI\textit{mer}-ptsH}, \textit{ptsI}\textit{R}</td>
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<td>\textit{Em}\textit{R}</td>
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<td>this study</td>
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</table>

\(\textit{Kn}\textit{R}\), kanamycin resistance; \(\textit{Ap}\textit{R}\), ampicillin resistance; \(\textit{Em}\textit{R}\), erythromycin resistance; \(\textit{Tc}\textit{R}\), tetracycline resistance
Plasmid pHWH2041 was used as described earlier for the construction of JccpA strains (Hueck et al. 1995). This plasmid carries the B. megaterium ccpA gene with an in-frame deletion. The double crossover replacing ccpA by JccpA 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 (JccpA).

Strain WH417 (WH348 gik) was kindly provided by C. Späth. For construction of WH4428 (ptsHglm gik), plasmid pHWH68 was used as described earlier (Späth et al. 1997). This plasmid carries the B. megaterium gik gene destroyed by insertion of a spoVG-luxAB-fusion. The intact gik gene of strain WH418 (ptsH) was replaced by the destroyed gene, yielding strain WH4428 (ptsHglm gik).

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% succinat was added to the M9 medium as a general carbon source. MOPS 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 OD600 of 0.4, washed three times in transport buffer (50 mM Tris-HCl, pH7.2, 20 mM MgCl₂) and resuspended in the same buffer. Aliquots of this cell suspension were used for the uptake assay. ³⁵C glucose (specific activity 74 μCi/mmol; Amersham) were added to adjust the suspension to 1mM glucose and 4 x 10⁵ 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 protein CcpA 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).

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References


