Two Glucose Transport Systems in *Bacillus licheniformis*

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Received 7 December 1992/Accepted 26 January 1993

*Bacillus licheniformis* NCIB 6346 showed active accumulation of glucose which was inhibited by agents which affect the transmembrane proton gradient. Phosphotransferase (PTS) activity, identified as phosphoenolpyruvate-dependent phosphorylation of glucose, was found in cell extracts but could not be demonstrated in cells permeabilized with toluene when assays were conducted at pH 6.6. The same was true for mannitol and fructose phosphotransferase activities. Cells grown on fructose accumulated glucose at a slower rate than glucose-grown cells, and extracts prepared from them did not contain glucose PTS activity. Examination of the effects of analogs on glucose uptake and phosphorylation showed that 2-deoxyglucose was not a PTS substrate, but did markedly inhibit glucose uptake, with stronger inhibition in cells grown on fructose. Glucose accumulation by whole cells grown on glucose became less sensitive to the uncoupler tetrachlorosalicylanilide (TCS) as the pH was raised from 6.6 to 8.0, while in fructose-grown cells TCS was equally effective across this pH range. PTS activity was exhibited by toluene-treated cells at pH 7.5 and above, although the system itself in extracts was not affected by pH in the range of 5.0 to 8.0. The results are consistent with the presence of two glucose transport systems, one a PTS and the other operating by an alternative mechanism, and suggest that the PTS in *B. licheniformis* may be regulated in a pH-dependent manner.

Transport systems in bacterial cells have two important functions. First, they are responsible for the accumulation of nutrients and as such are an integral part of metabolism. Second, they form part of the cell’s apparatus for sensing, and reacting to, the environment and in this respect are essential in regulating the response of the organism to small molecules in its environment. Recognition of these functions has stimulated much recent research in this area (20, 22).

Sugars are generally transported into the cell through some active mechanism which uses energy to accumulate the molecules against a concentration gradient. A large number of bacterial transport processes operate by proton symport, in which protons serve as coupling ions and accumulation of the substrate is driven by respiration or ATP hydrolysis via the intermediary of a transmembrane electrochemical proton gradient or proton motive force (22). A characteristic of these systems is that substrate accumulation is prevented in the presence of uncouplers such as tetrachlorosalicylanilide (TCS) or carbonyl cyanide chlorophenylhydrazone (CCCP) which increase the proton conductance of the membrane and so collapse the electrochemical gradient. Other transport systems are coupled to phosphate-bond energy. The best-studied example of this type is the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system, or PTS. This system is responsible for the transport and phosphorylation of several sugars in *Escherichia coli*, *Bacillus subtilis*, and many other obligately and facultatively anaerobic bacteria (13, 21, 22). The system consists of several proteins, and its presence is indicated by PEP-dependent phosphorylation of sugar in permeabilized cells or cell extracts. In gram-positive bacteria, phosphorylation of the low-molecular-weight component HPr by an ATP-dependent kinase appears to be a central feature of regulation of sugar uptake and efflux (5, 17).

*Bacillus licheniformis* is a close relative of *B. subtilis* and is a major industrial organism being used for most of the world’s production of α-amylase and alkaline protease as well as bacitracin (16). In these circumstances, starch is almost invariably used as the major carbon source for economic reasons and to avoid catabolite repression of enzyme synthesis. Little attention has been paid to the control of metabolism of starch breakdown products, and sugar transport mechanisms have not been characterized. We have recently shown that maltose transport in *B. licheniformis* is via H⁺ symport (25). In this report, we present results which indicate the presence of two glucose transport systems in this commercially important bacterium.

**MATERIALS AND METHODS**

Organism and growth conditions. *B. licheniformis* NCIB 6346 was maintained and grown as described previously (25).

**Measurement of sugar uptake by whole cells.** A suspension containing 1 mg of cells in 100 mM potassium phosphate buffer (pH 6.6) was allowed to equilibrate at 37°C for 3 min, and radiolabeled sugar (9.5 mM, 1.05 Ci mol⁻¹) was added to give a final concentration of 0.2 mM. The total assay volume was 1.0 ml. At the times indicated, samples (0.15 ml) were removed, filtered through glass fiber discs (Whatman GF/F), and washed with 10 ml buffer. Discs were dried under a heat lamp, and their radioactivity was counted in scintillation cocktail 0 (4 ml; BDH Scintran). When the effects of energy inhibitors and sugar analogs on sugar uptake were examined, these were added at the start of the temperature equilibration period. The accumulation of the nonmetabolizable analog 2-deoxyglucose was dependent on the addition of an exogenous energy source, and assays of its uptake were done in the presence of 1 mM glutamine.

**Permeabilization of cells.** Cell suspensions were adjusted to a density of 2 mg ml⁻¹ with 100 mM potassium phosphate buffer (pH 6.6) and permeabilized by treatment with toluene (1%, wt/vol). The mixture was vortexed for 30 s and then incubated for 30 min at 37°C. Permeabilized cells were

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diluted with phosphate buffer before assays of sugar phosphorylation.

**Preparation of cell extracts.** Extracts were prepared by the method of Mitchell and Booth (14), as described by Tangney et al. (25).

**Assay of sugar phosphorylation.** Phosphorylation of sugars by tolenuened cells and cell extracts was observed by the method of Gachelin (8) as described by Tangney et al. (25). The assay volume in all cases was 1.0 ml. Permeabilized cells (1 mg in potassium phosphate buffer) or extracts (0.4 ml in potassium phosphate buffer) were equilibrated at 37°C for 3 min in the presence of 5 mM MgCl₂ and, when appropriate, either 1 mM PEP or 1 mM ATP. Radiolabeled sugar (9.5 mM; 1.05 Ci mol⁻¹) was added to 0.2 mM, and samples (0.15 ml) were taken and added to 2 ml of 1% (wt/vol) barium bromide in 80% (vol/vol) ethanol. Precipitates were removed by filtration on glass fiber discs (Whatman GF/F) and washed with 5 ml of 80% (vol/vol) ethanol, and radioactivity was determined as described above.

**Measurement of transmembrane pH gradient.** The pH gradient was estimated from the distribution of the weak acid salicylic acid, essentially by procedures described previously (1). Cells were harvested as described above and resuspended in buffer of the desired pH to a density of 1 mg (dry weight) ml⁻¹. An aliquot of the suspension (4 ml) was incubated at 37°C for 3 min, and [¹⁴C]salicylic acid (5 μl; 57 Ci mol⁻¹; final concentration, 1.1 μM) and ²H²O (20 μl, 10 nCi) were added. In experiments determining the effect of TCS, the uncoupler (10 μl; 5 μg ml⁻¹ final concentration) was present during the preincubation, and control incubations received the same volume of ethanol. After 10 min of incubation at 37°C, triplicate 1-ml samples were removed into Eppendorf tubes and centrifuged for 30 s. Aliquots (100 μl) of the supernatants were removed and added to control pellets which had been prepared in exactly the same way but without exposure to the labeled compounds. The remainder of the supernatant fractions were discarded, and the pellets were resuspended in 600 μl of potassium phosphate buffer (pH 6.6). The control pellets were resuspended in the labeled supernatant (100 μl) plus 500 μl of phosphate buffer. Finally, the contents of each tube were fully dissolved in 4 ml of toluene-Triton X-100-based liquid scintillation fluid. The ²H and ¹⁴C contents of each sample were determined by double-isotope liquid scintillation counting.

The intracellular concentration of salicylic acid was calculated by reference to the measured intracellular volume. The procedure for determining the cell volume was exactly as described above, except that hydroxy[methyl-¹⁴C]inulin (20 μl, 18.2 Ci mol⁻¹; final concentration, 0.7 μM) replaced salicylic acid. The intracellular volume was calculated from the distribution of the two isotopes as described previously (23).

Errors of estimation of the pH gradient can arise through binding of the probe to the cells or cellular components. The extent of binding of [¹⁴C]salicylic acid was estimated by repeating measurements with cells which had been permeabilized by toluene treatment. The apparent accumulation of salicylic acid by these cells was subtracted from whole-cell values.

**Materials.** PEP (tri(cyclohexylammonium) salt), valinomycin, and sugar analogs were purchased from Sigma, ATP (disodium salt) was from Boehringer, and tetraphenylphosphonium (TPP) bromide was from Aldrich. D-[¹⁴C]glucose, D-[¹³C]fructose, D-[¹⁴C]mannitol, 2-deoxy-D-[¹³C]glucose, hydroxy[methyl-¹⁴C]inulin, [carboxyl-¹⁴C]salicylic acid, and tritiated water were obtained from American.

sham. TCS was the kind gift of I. R. Booth, University of Aberdeen. All other chemicals were of the highest purity available.

**RESULTS**

Sugar uptake by whole cells. Glucose-grown cells of *B. licheniformis* were incubated in potassium phosphate buffer at pH 6.6 accumulated [¹⁴C]glucose without the addition of an exogenous energy source. The uptake of glucose was abolished by the uncoupler TCS, indicating that it occurred by an energy-dependent mechanism. Collapse of the membrane potential by treatment with either valinomycin or TPP⁺ also significantly inhibited glucose uptake (Fig. 1). The simplest explanation of these results was the direct involvement of a transmembrane electrochemical proton gradient in energization of sugar uptake.

Resting cells of *B. licheniformis* were also shown to accumulate fructose and mannitol following growth on the respective sugar. Each of these processes was also energy dependent as shown by the effect of TCS, which totally inhibited uptake of both substrates (data not shown). Cells grown on fructose, mannitol, or malate (25) accumulated glucose, indicating that glucose transport was constitutive.

Sugar phosphorylation in permeabilized cells and extracts. We observed hybridization of a plasmid carrying the *B. subtilis* ptsH (HP) gene and a portion of the ptsI (enzyme I) gene to chromosomal DNA of *B. licheniformis* NCIB 6346, suggesting the presence of the PTS in this strain (data not shown). In tolune-treated cells, glucose was phosphorylated in the presence of ATP, indicating the presence of a kinase, but no phosphorylation was supported by PEP (Fig. 2). However, extracts prepared from *B. licheniformis* NCIB 6346 showed PEP-dependent glucose phosphorylation, confirming the presence of a functional PTS (Fig. 3A). Glucose was also phosphorylated by ATP in extracts, consistent with the phosphorylation observed in tolune-treated cells.

The existence of PTs for fructose and mannitol was also investigated in extracts prepared from cells grown on the appropriate substrate. For fructose, the pattern was similar to that found for glucose (Fig. 3B). Mannitol, on the other hand, was phosphorylated only in the presence of PEP,
showing that a mannitol kinase was not present and eliminating the possibility that apparent PTS activity for all sugars was due to conversion of PEP to ATP in the assay mixture (Fig. 3C). Thus, extracts of B. licheniformis were shown to contain phosphotransferases for glucose, fructose, and mannitol, although none of these activities was detectable in permeabilized cells.

Since glucose transport activity was constitutive, the presence of a glucose PTS was examined in extracts prepared from cells grown on other carbon sources. Extracts prepared from maltose-grown cells were previously shown to exhibit glucose PTS activity (25). Similarly, extracts of mannitol-grown cells showed glucose PTS activity, but extracts of fructose-grown cells did not (Fig. 4). In the absence of PTS activity, glucose uptake in fructose-grown cells must therefore have occurred by an alternative, non-PTS system.

_Effect of analogs on glucose uptake and phosphorylation._ When added at 100-fold excess concentration, several glucose analogs caused virtually complete inhibition (>95%) of glucose PTS activity in extracts, while 2-deoxyglucose and 6-deoxyglucose did not inhibit the activity significantly (<5%; Table 1). None of the analogs tested inhibited ATP-dependent glucose phosphorylation (data not shown). These results indicate that the PTS also abolished uptake of glucose into whole cells. On the other hand, 6-deoxyglucose did not inhibit glucose uptake at all, while 2-deoxyglucose inhibited glucose uptake in glucose-grown cells by about 50%. Therefore, of the analogs tested, only 2-deoxyglucose could apparently be used to discriminate between the two glucose uptake systems.

Radiolabelled 2-deoxyglucose was not phosphorylated by PEP in cell extracts, confirming that it is not a PTS substrate (data not shown). However, this analog was accumulated by whole cells in the presence of glutamine as an energy source, and its effect on glucose uptake by fructose-grown cells lacking the glucose PTS was severe (Table 1). These results confirm that in glucose-grown cells, 2-deoxyglucose inhibited only the non-PTS transport system, and indicate that both transport systems contribute to the uninhibited uptake of glucose.
TABLE 1. Effects of glucose analogs on glucose transport activities in *B. licheniformis*<sup>a</sup>

<table>
<thead>
<tr>
<th>Glucose analog</th>
<th>Glucose uptake in glucose-grown cells</th>
<th>% Inhibition of:</th>
<th>Glucose uptake in fructose-grown cells</th>
<th>Glucose PTS in glucose extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Methyl-glucoside</td>
<td>&gt;95</td>
<td></td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>3-Methyl-glucoside</td>
<td>&gt;95</td>
<td></td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>6-Deoxy-d-glucose</td>
<td>&lt;5</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5-Thioglucose</td>
<td>&gt;95</td>
<td></td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>β-D-Thioglucose</td>
<td>&gt;95</td>
<td></td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>45-55</td>
<td></td>
<td>85-95</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Glucose transport and phosphorylation were assayed as described in Materials and Methods. Analogs were added at 100 times the concentration of <sup>14</sup>C-labelled glucose.

**Effect of pH on glucose transport and phosphorylation.** The rate and extent of glucose uptake by glucose-grown cells was not markedly affected by increasing the external pH from 6.6 to 8.0, but the sensitivity of uptake to TCS gradually decreased (Fig. 5A). Nevertheless, the effects of 2-deoxyglucose on glucose uptake at pH 8.0 were identical to its effects at pH 6.6 (data not shown), demonstrating that each system was responsible for around 50% of glucose uptake irrespective of the assay pH. The internal pH, however, remained almost constant as the external pH varied (Table 2). At an external pH of 6.6, TCS caused a significant lowering of the cytoplasmic pH from 7.88 to 7.14. On the other hand, at pH 8.0 the uncoupler did not alter the cytoplasmic pH (Table 2). PTS activity was assayed over a range of pH in both permeabilized cells and extracts. In cells permeabilized by toluene, alteration of the external pH had a marked effect, with activity found at pH 7.5 and above, but not at lower pH values (Fig. 6). However, in extracts there was little variation in activity within the pH range of 5.0 to 8.0. Therefore, although pH appeared to be an important determinant of PTS activity in whole cells and permeabilized cells, the system itself was not inherently sensitive to pH within the range studied.

Fructose-grown cells, which lack a glucose PTS, accumulated glucose at a significantly slower rate than cells grown on glucose, but this accumulation was still totally abolished by TCS at an external pH of 6.6. In these cells, glucose uptake remained highly sensitive to the uncoupler at pH 8.0 (Fig. 5B). The simplest explanation of these results is that uptake of glucose which occurs in glucose-grown cells in the presence of TCS at pH 8.0 is due to the PTS and that inhibition of this system by the uncoupler at an external pH of 6.6 is due to acidification of the cytoplasm. Consistent with this interpretation, treatment of the cells with valinomycin or TPP<sup>+</sup> would not perturb the cytoplasmic pH, and so residual PTS-mediated glucose uptake is observed (Fig. 1), despite inhibition of uptake via the second, non-PTS system.

**DISCUSSION**

There are many instances of bacteria producing more than one transport system for the same substrate (13, 21, 22), and it is generally assumed that the metabolic versatility provided is advantageous to the organism. The activity of different transport systems may vary depending on the growth conditions or phase, while the individual systems may have very different affinities for the substrate, allowing cellular transport and metabolism to respond to conditions of substrate starvation or excess. For example, *Streptococcus*
TABLE 2. Effect of TCS on internal pH of B. licheniformis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH (external)</th>
<th>ΔpH</th>
<th>pH (internal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6.6</td>
<td>1.28</td>
<td>7.88</td>
</tr>
<tr>
<td>+TCS</td>
<td>6.6</td>
<td>0.54</td>
<td>7.14</td>
</tr>
<tr>
<td>Untreated</td>
<td>8.0</td>
<td>0</td>
<td>8.0</td>
</tr>
<tr>
<td>+TCS</td>
<td>8.0</td>
<td>0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* Measurements of the transmembrane pH gradient were performed as described in Materials and Methods.

mutans has been reported to have two transport systems for both glucose and sucrose, with each sugar being transported by a PTS and an additional, non-PTS system (6, 10, 11). In chemostat-grown cells, PTS activity was shown to be dependent on growth rate and pH. Thus, the lower-affinity, non-PTS system apparently made a more significant contribution to total sugar accumulation during growth at high rates in the presence excess sugar, and at low pH (6, 7, 10).

In the present investigation of B. licheniformis, two transport activities were observed for glucose. PTS activity was observed in cell extracts prepared from cells grown on glucose. The second system, which was also energy dependent, was operational in fructose-grown cells which lacked PTS activity and was inhibited by uncouplers and treatments expected to lead to a collapse of the membrane potential. However, while these observations are consistent with a proton motive force-dependent mechanism, this cannot be concluded unequivocally, since the uncoupler TCS has also been shown to cause a large decrease in the ATP pool in B. licheniformis cells (25). Whether the uptake of glucose is driven by the proton motive force or ATP, glucose will be delivered into the cytoplasm without chemical modification and then phosphorylated by glucokinase.

Based on the growth phenotype of pts mutants, the existence of a second transport system for glucose, additional to the well-characterized PTS, has been also described in B. subtilis (9). However, the mechanism of this system, which could be either a second PTS or a system which is regulated by the PTS, was not investigated. Transport of glyceral by a non-PTS mechanism in B. subtilis has been shown to be regulated by the PTS (19), while we have recently reached a similar conclusion for maltose transport (24).

PTS activity could not be observed in cells permeabilized with toluene at pH 6.6, but the system was active at this pH in extracts. The concentration of toluene used (1%) was the minimum required to decrypify the cells, and parallel experiments showed that the glucose PTS was active in cells of B. subtilis treated in the same way. The possibility that PTS activity was dependent on the phase of growth, as for the fructose PTS of Arthrobacter pyridinolysis, is induced at a late stage of growth (15), was considered. However, when both toluenized cells and extracts from different growth stages were assayed, the results were identical. Changes in growth conditions (media, pH, and anaerobiosis) also had no effect on PTS activity in permeabilized cells. The previous demonstration of PTS activity in B. licheniformis (12) involved assays on cells which had been frozen and thawed and thus would probably have been effectively disrupted. Our finding of activity in extracts is therefore consistent with the earlier data.

The variation in degree of inhibition of glucose uptake by an uncoupler at different external pH values suggested that the PTS may be sensitive to intracellular pH, becoming inactive when this was lowered to around 7.0. Consistent with this proposal, PTS activity in permeabilized cells was observed only at pH 7.5 and above, but nevertheless the PTS itself, assayed in extracts, showed no such sensitivity. One possible explanation of these findings is that the PTS may be regulated in a pH-dependent manner, with the regulation operating in permeabilized cells, and presumably whole cells also, but not in extracts. The nature of any such regulation remains to be established, although since PTS activity for several substrates responded in the same manner, it seems reasonable to suggest that it would be exerted at the level of one or both of the general PTS proteins, enzyme I and HPr. One possible target is the association of these soluble proteins with the membrane; this may be disrupted at low pH in intact or permeabilized cells, while protein aggregates may be dispersed during extract preparation, thus eliminating pH sensitivity. Alternatively, a mechanism based on a regulatory phosphorylation of PTS proteins may be involved. Phosphorylation of the phosphocarrier protein HPr by an ATP-dependent protein kinase and hydrolysis of the phosphoryl bond by a phosphatase has been demonstrated in streptococci and B. subtilis (2, 4, 18, 21). This phosphorylation of HPr at residue Ser-46 causes a drastic inhibition of phosphorylation of the protein by PEP and enzyme I (3) and the wide spectrum of HPr proteins phosphorylated by the streptococcal kinases (2, 18) suggests that this may be a widespread mechanism for regulation of the system in gram-positive bacteria. A difference in the phosphorylation state of HPr in permeabilized cells and extracts might conceivably contribute to the different patterns of activity observed.

In conclusion, evidence has been presented for the presence of two distinct glucose transport systems in B. licheniformis NCIB 6346. While both systems appear to be func-
tional in whole cells, a more complete description of these systems, their interactions, and their control is essential for an understanding of glucose metabolism in this organism.

ACKNOWLEDGMENTS

This work was supported by a grant to M.T. from the Commission of European Communities under the BRIDGE program. We are grateful to Jonathan Reizer for useful comments and suggestions.

REFERENCES