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Glucose Transport by a Mutant of *Streptococcus mutans* Unable To Accumulate Sugars via the Phosphoenolpyruvate Phosphotransferase System

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Streptococcus mutans **transports glucose via the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS). Earlier studies indicated that an alternate glucose transport system functions in this organism under conditions of high growth rates, low pH, or excess glucose. To identify this system, *S. mutans* BM71 was transformed with integration vector pDC-5 to generate a mutant, DC10, defective in the general PTS protein enzyme I (EI). This mutant expressed a defective EI that had been truncated by approximately 150 amino acids at the carboxyl terminus as revealed by Western blot (immunoblot) analysis with anti-EI antibody and Southern hybridizations with a fragment of the wild-type EI gene as a probe. Phosphotransfer assays utilizing ^{32}P-PEP indicated that DC10 was incapable of phosphorylating HPr and EIIA^{Man}, indicating a nonfunctional PTS. This was confirmed by the fact that DC10 was able to ferment glucose but not a variety of other PTS substrates and phosphorylated glucose with ATP and not PEP. Kinetic assays indicated that the non-PTS system exhibited an apparent K_s of 125 μM for glucose and a V_{max} of 0.87 nmol mg (dry weight) of cells^{-1} min^{-1}. Sugar competition experiments with DC10 indicated that the non-PTS transport system had high specificity for glucose since glucose transport was not significantly inhibited by a 100-fold molar excess of several competing sugar substrates, including 2-deoxyglucose and α-methylglucoside. These results demonstrate that *S. mutans* possesses a glucose transport system that can function independently of the PEP PTS.**

Carbohydrate metabolism by *Streptococcus mutans* results in the formation of acid end products that can contribute to the demineralization of tooth enamel, leading to dental caries (11, 42). The phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) (29) is the principal transport process in oral streptococci for glucose and a variety of sugars, including mannose, fructose, sucrose, lactose, and maltose (2, 17, 37). In the PTS, phosphate is transferred from PEP via the general PTS proteins HPr and EI to the sugar-specific, membrane-bound protein EII and then to the incoming sugar. Much of the current information on the structure of the PTS has come from work with *Escherichia coli* and *Salmonella typhimurium*, particularly with respect to the various sugar-specific EIIs (29). The arrangement of the domains that make up the EIIs can vary depending on the organism and the sugar to be transported, appearing either (i) as a single membrane-bound protein consisting of three domains (A, B, and C); (ii) as two or more proteins, one of which is membrane bound (B and C) and one of which is soluble (IIA or EIII); (iii) with domains A and B fused into a single soluble protein and associated with two membrane components (C and D); or (iv) with domains IIA and IIB existing as separate soluble proteins (29, 34). While other variations in the domain organization are known, phosphoryl transfer generally occurs sequentially via EI, HPr, EIIA, and EIIB, with the EIIC and EIID components probably forming a translocation channel in the membrane.

With respect to *S. mutans*, information is available on the genetic arrangement of some, but not all, of the PTS and associated components responsible for the transport of sucrose (*scrA*) (35) and mannitol (20). More complete information is available on the lactose operon in this organism, including the nucleotide and deduced amino acid sequences of the repressor, the tagatose-6-phosphate pathway, and IIA^{Lac} (*lacF*) and IICB^{Lac} (*lacE*) of the PTS (30). A more recent report (21) has indicated that the gene for phospho-β-galactosidase (*lacG*), the enzyme that cleaves lactose phosphate that is generated by the lactose PTS, is also located in this operon. We have cloned and sequenced the genes for the general PTS proteins HPr and EI in *S. mutans* and shown that they are not associated with other PTS genes (3), a result shown earlier for *S. salivarius* (10).

Several lines of evidence support the notion of a second, non-PTS transport system for glucose in *S. mutans*. Early chemostat studies (8, 9, 15) with *S. mutans* Ingbritt suggested the existence of such a system in cells grown at high growth rates and low pH and with sucrose and excess glucose, conditions repressing the glucose PTS. Furthermore, a reciprocal relationship was shown to exist between the activity of the glucose PTS and glucokinase, suggesting an uptake system requiring the phosphorylation of free intracellular glucose (12). Later kinetic studies with *S. mutans* DR0001 grown in continuous culture with glucose limitation at growth rates of 0.04 to 0.6 h^{-1} indicated two transport processes, a high-affinity system with K_s values of 6.8 to 8.0 μM, shown to be the PTS, and a lower-affinity system with K_s values of 57 to 125 μM (18). A PTS-defective strain, *S. mutans* DR0001/6, on the other hand, possessed only one transport system with K_s values of 62 to 133 μM. Subsequent continuous culture studies with *S. mutans* Ingbritt (16, 40, 41) demonstrated that the repression of the glucose PTS was associated with reduced synthesis of membrane EII^{Glc}; in fact, cells grown at pH 5.0 were completely devoid of EII^{Glc} activity (40), confirming earlier results obtained with decryptified cells (15). More recently, membrane

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vesicles prepared from chemostat-grown cells of *S. mutans* Ingbritt and devoid of cytoplasmic components, including EI and HPr, demonstrated glucose counterflow, indicating a carrier capable of transporting glucose (4). Early models suggested that the non-PTS glucose transport system was linked to proton motive force in *S. mutans* (18, 22); however, this hypothesis was questioned by more recent research (6).

The discovery by genetic means of a novel binding protein-dependent sugar uptake system (33) in *S. mutans* has also suggested a possible route for glucose uptake. This multiple sugar metabolism (MSM) system is encoded on an 11-kb multigene region of the *S. mutans* chromosome and is primarily responsible for the transport of raffinose, melibiose, and isomaltosaccharides. One essential gene in this operon is *msmK*, which codes for an ATP-binding protein, the first such protein identified in gram-positive bacteria. A recent report (36) has indicated that unlabelled glucose could effectively disrupt transport of radiolabelled melibiose by *S. mutans*, although this is not conclusive proof of glucose transport by the MSM system.

With the exception of the MSM studies, PTS-independent transport studies with intact cells of *S. mutans* have been hampered by the presence of the PTS and it has not been possible to characterize alternative transport processes. The objective of the current work was to study glucose transport in *S. mutans* in the absence of PTS activity. The preferred mutants would be those defective in the gene for either general PTS protein EI or HPr, since they are required for the transport of all PTS sugars. We have generated a mutant, *S. mutans* DC10, defective in EI from information obtained during a previous study (3) on the sequence and expression of the genes for these proteins from *S. mutans* NG-5. The mutant contains a truncated *ptsI* gene missing approximately 150 amino acids from the C-terminal end and cannot be phosphorylated by ^{32}P-PEP or support glucose phosphorylation via PEP. We have used this mutant to study the characteristics and properties of non-PTS glucose transport in this organism.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *E. coli* DH5α [ϕ80d Δ*lacZ* M15 *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ$^-$ *gyrA relA1* F$^-$ Δ(*lacZYA-argF*) *U169*], used as a host strain for the isolation and maintenance of plasmids in this study, was maintained in LB broth and on plates (28) containing ampicillin (50 μg ml^{-1}) or erythromycin (750 μg ml^{-1}). *S. mutans* BM71 was utilized as a parent strain in the construction of *ptsI* mutant strain DC10. Streptococci were grown and maintained in tryptone-yeast extract (TYE) broth or plates (1.0% tryptone, 0.5% yeast extract, 0.35% K_2HPO_4, 0.2% glucose or raffinose). The selective media, used to isolate PTS-defective streptococcal transformants, included (i) TYE containing 1.6% agar, 1% raffinose, and 8 μg of erythromycin ml^{-1} and (ii) blood agar (Oxoid blood base agar no. 2, 5% sheep blood, 0.0005% hemin) containing 8 μg of erythromycin ml^{-1}. Mutant DC10 was maintained on TYE-raffinose (0.3%) plates with 8 μg of erythromycin ml^{-1}. Sugar fermentation was determined by anaerobic incubation of the test organism on streptococcal sugar agar plates (2.0% Proteose Peptone, 0.5% yeast extract 0.5% NaCl, 0.1% Na_2HPO_4, 1.5% agar, 0.002% bromocresol purple) containing the various sugars at a concentration of 1%. The rate of acid production was measured by autotitration of metabolizing cells with standardized KOH as described previously (19).

Recombinant DNA methodology and bacterial transformations. Agarose gel electrophoresis was carried out in Tris-acetate-EDTA buffer, while Southern hybridizations were carried out in aqueous solutions as described by Maniatis et al. (25). DNA was transferred to Hybond N$^+$ (Amersham) for detection by radiolabelled probes and to Photogene Nylon (Gibco/BRL, Mississauga, Ontario, Canada) for detection by biotin-labelled probes. Radiolabelled DNA probes were prepared by using the nick translation or random primer labelling kit, and biotin-labelled DNA probes were prepared with the Bio-Nick labelling kit (Gibco/BRL). Recombinant plasmids used for sequencing were produced by subcloning specific restriction fragments after purification from agarose gels with the GENECLEAN kit (Bio 101, La Jolla, Calif.). Rapid screening of plasmids was done from 1.5-ml *E. coli* cultures by alkaline lysis (1). *E. coli* was transformed by electroporation with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) by the method of Dower et al. (7).

Streptococcal integration vector pDC-5 was constructed by first subcloning a 450-bp blunt-ended fragment from an internal region of the *ptsI* gene into pUC9. This fragment contained an internal *Eco*RV site into which a 1.8-kb *Hin*cII-*Sma*I fragment containing the erythromycin resistance gene from pDP3 (generously supplied by R. Burne, University of Rochester) was inserted. The vector was linearized with *Nde*I and used to transform BM71. Electrocompetent cells were prepared for transformation by growing an overnight culture of *S. mutans* BM71 in Todd-Hewitt broth supplemented with 10% heat-inactivated horse serum (Sigma Chemical Co., St. Louis, Mo.); 125 μl of this culture was used to inoculate 5 ml of prewarmed Todd-Hewitt broth–10% heat-inactivated horse serum. The culture was incubated anaerobically until it reached an optical density at 600 nm of 0.25, when the cells were cooled on ice, centrifuged at 15,000 × *g* for 5 min, washed three times in ice-cold 300 mM sucrose, and finally resuspended in 100 μl of 300 mM sucrose. The DNA was added to the cell suspension, transferred to a pre-chilled 0.1-cm cuvette after 1 min on ice, and pulsed at 2.5 kV, 200 Ω, and 25 μF in a Bio-Rad Gene Pulser. Immediately after pulsing, 1 ml of Todd-Hewitt broth was added to the cuvettes and the contents were transferred to a 1.5-ml microcentrifuge tube and incubated at 37°C for 90 min. The cells were centrifuged for 5 min at 15,000 × *g*, resuspended in 100 μl of Todd-Hewitt broth, and plated on the appropriate selective media.

Protein electrophoresis and Western immunoblotting. Cell extracts of *S. mutans* containing HPr and EI for use in Western immunoblotting were prepared as previously described (41). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (23) and transferred to Immobilon P membranes (Millipore) as described by Towbin et al. (38). After incubation of the membranes with the anti-EI primary antibody (1:500 dilution in Tris-buffered saline), EI- and HPr-specific bands were detected by incubation with goat anti-rabbit immunoglobulin G alkaline phosphate conjugate followed by detection with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (BCIP).

^{32}P-PEP phosphorylation of PTS proteins. Phosphorylation of PTS protein with ^{32}P-PEP and subsequent autoradiography were done by the method of Bourassa et al. (2) with a slight modification. ^{32}P-PEP was synthesized by the method of Mattoo and Waygood (27) by using purified carboxykinase from *E. coli*, kindly provided by H. Goldie, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The positive control contained 35 μg of protein from an *S. mutans* BM71 membrane-free cell extract. Samples were incubated at room temperature in a 25-μl reaction mixture containing 0.1 mM ^{32}P-PEP (180 Ci mol^{-1}), 5 mM $MgCl_2$, 12.5 mM NaF, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5). The reactions were stopped after 5 min by addition of 10 μl of 188 mM Tris-HCl (pH 8.0), which contained 6% (wt/vol) SDS, 30% (vol/vol) glycerol, 6% (vol/vol) 2-mercaptoethanol, and 0.005% bromophenol blue. Samples were loaded onto a 1.5-mm-thick SDS–12.5% polyacrylamide gel which was electrophoresed for 1 h at 200 V in a Bio-Rad Mini Protean II apparatus. The dried gel was placed on X-ray film with an intensifying screen at −70°C for 22 h.

Detection of the various forms of HPr. Four separate 500-ml samples (200 mg of cells sample^{-1}) were rapidly removed during the exponential phase to a stirred solution containing 100 mM Tris-citrate buffer (pH 4.0) with chloramphenicol (50 mg ml^{-1}) and gramicidin D (1 mM), and the pH of the sample was immediately lowered to 4.5 with 5 N HCl. The cell suspension was centrifuged at 16,000 × *g* for 10 min, resuspended in a minimal amount of the supernatant, recentrifuged at 27,000 × *g* for 20 min, and frozen at −70°C. The frozen cell pellet was used within 2 to 3 h to prepare the membrane-free cell extract by a slight modification of the alumina grinding method of Vadeboncoeur et al. (39). After grinding, a small volume (1.5 ml) of buffer (10 mM HEPES buffer [pH 7.0] containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 μM pepstatin A) was added and the alumina was removed by centrifugation at 3,000 × *g* for 5 min. The supernatant was then centrifuged at 16,000 × *g* for 20 min to remove intact cells and cell debris and then subjected to centrifugation at 100,000 × *g* for 18 h before being assayed for HPr.

The various forms of HPr were separated by crossed immunoelectrophoresis as described by Vadeboncoeur et al. (39) with minor alterations to the protocol. Agarose (1%) was dissolved in Tris-barbitone buffer containing (in grams liter^{-1}) sodium barbitone (5.01), Tris base (8.86), calcium lactate (0.11), and sodium azide (0.13). The samples were diluted to 2 mg of protein ml^{-1} with 10 mM HEPES buffer (pH 7.0), and 5 μl was deposited at the cathodic end of the gel. Electrophoresis in the first dimension was conducted for 75 min at 10 V/cm and 10°C on an LKB Bromma 2117 Multiphor apparatus. Electrophoresis in the second dimension, conducted for 19 h at 2 V/cm and 4°C, involved electrophoresis against a polyclonal rabbit antibody (8 mg per plate) directed against the purified HPr protein of *S. mutans* DR0001. Following electrophoresis, the gels were dried at room temperature overnight and stained with Coomassie blue.

Quantitation of HPr under each precipitin peak involved scanning each gel in a 300 DPI Apple Scanner (Apple Computer, Inc., Cupertino, Calif.), transferring the image to the Image 1.36 program (National Institutes of Health, Bethesda, Md.), and determining the number of pixels under each peak. These values were then compared to a standard curve (0 to 2,000 ng of HPr protein) to determine the number of nanograms of the various forms of HPr protein that existed in the

TABLE 1. Sugars and sugar alcohols transported by the PEP PTS and non-PTS systems capable of supporting growth and metabolism of wild-type *S. mutans* BM71 and *ptsI* mutant DC10

S. mutans strain	Fermentation substrate(s)[a]	
	PTS	Non-PTS
BM71	Glucose, maltose, sucrose, mannitol, lactose, sucrose, fructose	Raffinose, melibiose
DC-10	Glucose	Raffinose, melibiose

[a] Tests were conducted on carbohydrate-indicator agar plates with anaerobic incubation for 3 days and included all of the substrates listed for BM71.

sample. The final values were expressed as micrograms of HPr milligram of cellular protein^{-1}.

Sugar transport and kinetics. Glucose transport was measured on mid-log-phase, glucose-grown cells washed three times in 50 mM Na-K phosphate buffer (pH 7.0). The cells (0.5 mg [dry weight] ml^{-1}) were incubated at 37°C in a reaction mixture containing 1 mM [^{14}C]glucose (9 Ci mol^{-1}) in 50 mM Na-K phosphate buffer (pH 7.0) with a final volume of 5.0 ml. Samples (0.5 ml) were removed at 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 min, filtered through 0.45-μm-pore-size HA filters (Millipore, Bedford, Mass.), and washed three times with 1 ml of 50 mM Na-K phosphate buffer (pH 7.0) equilibrated at 37°C. The dried filters were then counted in 5.0 ml of Aquasol (NEN Research Products, Montreal, Quebec, Canada) in a liquid scintillation counter. Kinetics of glucose transport were determined essentially as previously described (19) with the concentrations of glucose ranging from 0.01 to 10 mM and the reactions being terminated at 0.1 min. Rates were expressed as nanomoles of sugar transported milligram (dry weight) of cell material^{-1} minute^{-1}.

Sugar competition. The specificity of non-PTS glucose transport activity was tested by sugar competition experiments in which possible competing sugars were added to glucose transport reactions at a 100-fold excess. Assays were identical to those used for glucose transport with reactions containing 1 mM [^{14}C]glucose (9 Ci mol^{-1}) and the competing carbohydrate at 100 mM. The reaction mixtures were incubated for 2 min and then filtered, washed, and counted. Samples were taken in quadruplicate, and the mean values were used to determine the relative activities.

Glucose phosphorylation. Phosphorylation of glucose via ATP and PEP was carried out as described previously (4). Intact cells were permeabilized with toluene, diluted in buffer to 1 mg ml^{-1}, and assayed in a reaction mixture containing 4 mM PEP or ATP, 50 μg (dry weight) of cells in PTS buffer (50 mM K phosphate buffer [pH 7.0] containing 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 20 mM NaF) in a final volume of 500 μl. The reaction was initiated by addition of 2 mM [^{14}C]glucose (9 Ci mol^{-1}), and after 30 min of incubation at 37°C, the phosphorylated sugar was separated by precipitation with 10 volumes of 30 mM $BaBr_2$ in 90% (vol/vol) ethanol. After 30 min of incubation on ice, the suspensions were filtered through 0.45-μm-pore-size HA Millipore filters, rinsed twice with 2 ml of cold 80% ethanol, and counted. Activity was expressed as nanomoles of glucose phosphorylated milligram of dry cells^{-1} minute^{-1}.

Chemicals and enzymes. Restriction enzymes, T4 DNA ligase, goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate, and DNA and protein molecular weight standards were from Gibco/BRL and were used as directed by the supplier. Radiolabelled nucleotides [γ-^{32}P]dATP, [α-^{32}P]dCTP, and [^{14}C]glucose were obtained from Dupont New England Nuclear. All other chemicals were obtained from Sigma Chemical Company, Fisher Scientific Limited, or Difco. Rabbit anti-EI (*S. salivarius*) and anti-HPr (*S. mutans*) polyclonal antibodies were a generous gift from Christian Vadeboncoeur, Laval University, Sainte Foy, Quebec, Canada.

RESULTS

Construction and characterization of *ptsI* mutant DC10. *S. mutans* BM71 was transformed with linearized pDC-5, and two erythromycin-resistant transformants were picked from blood agar plates after 6 days of anaerobic incubation at 37°C. One transformant, DC10, selected for all subsequent experiments, was compared to BM71 for the ability to metabolize and grow on a variety of sugar substrates. As illustrated in Table 1, wild-type strain BM71 was able to grow and ferment a variety of PTS sugars and the non-PTS substrates melibiose and raffinose on streptococcal sugar agar plates. However, growth of DC10 was only apparent after 3 days of incubation on plates containing melibiose, raffinose, or glucose. The failure to ferment or grow on the other PTS substrates suggested that the PTS was nonfunctional. Growth curves of DC10 in TYE–0.3% glucose broth exhibited a doubling time of 180 min compared with a doubling time of 40 min for the parent strain BM71 (Fig. 1). With glucose as the substrate, the rates of acid production by glucose-grown BM71 and mutant DC10 cells were 212 ± 14 and 56 ± 8 nmol of acid neutralized mg of dry cells^{-1} min^{-1}, respectively, indicating a decrease in acid production of about 75%. Transport assays with [^{14}C]glucose revealed that the rate of glucose uptake by DC10 (1.4 ± 0.3 nmol of glucose mg of dry cells^{-1} min^{-1}) was only 1.2% of that exhibited by wild-type strain BM71 (115 ± 12 nmol mg of dry cells^{-1} min^{-1}).

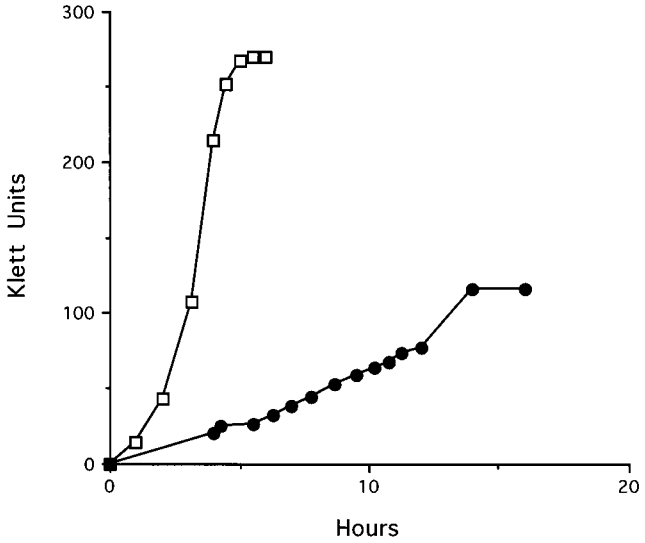

FIG. 1. Growth of wild-type *S. mutans* BM71 (□) and *ptsI* mutant DC10 (●) in TYE–0.3% glucose broth.

To confirm integration of pDC-5 into the *ptsI* gene of BM71, chromosomal DNAs from the parent and transformant DC10 were subjected to Southern blot analyses with radiolabelled probes that contained either the erythromycin resistance gene from pDP-3 or a 3.5-kb *Eco*RI-*Sst*I restriction fragment containing the cloned *ptsI* gene from *S. mutans*. Figure 2 shows the results of the latter, with the *ptsI* probe hybridizing to a 4.8-kb *Sst*I fragment from BM71 (lane F) and a 6.6-kb *Sst*I fragment from DC10 (lane C). This increase in size corresponds to the size of the erythromycin resistance gene (1.8 kb). Probing of the same blot with the erythromycin resistance gene also revealed hybridization to a 6.6-kb *Sst*I fragment in DC10, with no hybridization to the parent strain (data not shown). The same blot was also probed with the ampicillin resistance gene from pUC9 to ensure that ampicillin resistance had not been transferred to DC10 by integration vector pDC-5. No apparent hybridization was observed.

Analysis of the cytoplasmic extracts of BM71 and transformant DC10 by Western blotting with anti-EI antibody from *S. salivarius* revealed that DC10 expressed a truncated protein with an apparent size of 56 kDa. The truncated EI showed less apparent reactivity with the anti-EI antibody than the parent EI as demonstrated by the lower intensity of the 56-kDa band (Fig. 3). This is likely due either to an alteration of the antigenic sites or, possibly, to rapid degradation of the truncated protein so that its steady-state level was lower than that of the wild-type protein. In comparison with the EI of the parent strain, BM71, which has an apparent size of 67 kDa, along with the predicted site of integration, we determined that the DC10

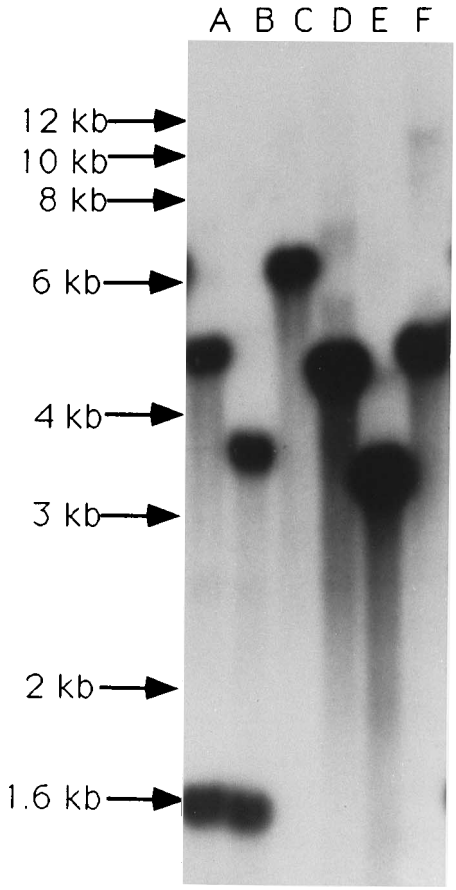

FIG. 2. Southern hybridization of DC10 and BM71 restriction-digested chromosomal DNAs probed with a radiolabelled 3.5-kb *Eco*RI-*Sst*I fragment containing the *ptsI* gene from *S. mutans*. Lanes: A, DC10 *Eco*RI; B, DC10 *Eco*RI-*Sst*I; C, DC10 *Sst*I; D, BM71 *Eco*RI; E, BM71 *Eco*RI-*Sst*I; F, BM71 *Sst*I.

EI was missing approximately 150 of a total of 577 amino acid residues from the COOH terminus of the protein.

Further study of mutant DC10 involved examining the cytoplasmic proteins phosphorylated by ^{32}P-PEP. These experiments revealed that EI and HPr were phosphorylated in wild-type BM71, as observed previously in our laboratory (3);

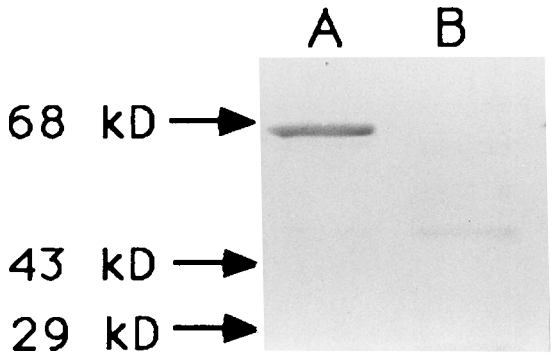

FIG. 3. Western blot of BM71 (lane A) and *ptsI* mutant DC10 (lane B) with anti-EI antibody. Each sample (total protein, 4 μg) was electrophoresed at 200 V for 60 min in a 12% polyacrylamide gel and blotted to polyvinylidene difluoride membranes (Immobilon P; Millipore) for 1 h at 100 V. Immunodetection was performed with a primary antibody against purified *S. salivarius* EI. kD, kilodaltons.

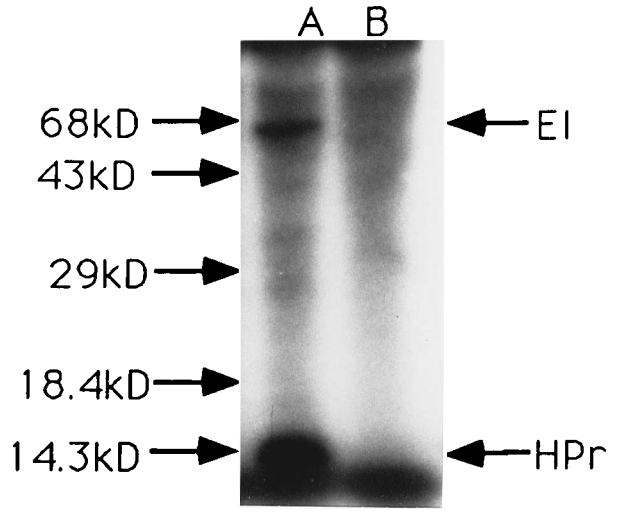

FIG. 4. Polyacrylamide gel electrophoresis of phosphorylated PTS proteins in reactions using ^{32}P-PEP with cytoplasmic extracts of *S. mutans* parent strain BM71 (lane A) and *ptsI* mutant DC10 (lane B). Each sample (35 μg) was incubated with ^{32}P-PEP for 5 min and then electrophoresed on an SDS–12% polyacrylamide gel at 200 V for 1 h. The dried gel was placed on X-ray film for 22 h at −70°C. kD, kilodaltons.

however, no detectable phosphorylation of the truncated EI or HPr was observed in DC10 cell extracts (Fig. 4), further supporting the concept of a dysfunctional PTS in the mutant. The phosphorylated band running below HPr at 10 kDa has not been identified; however, it is probably not a PTS protein since it was observed in the mutant.

Detection of the various forms of HPr. Since the possibility exists that the truncated EI in the phosphorylation experiments was unstable and, therefore, any labelling with ^{32}P-PEP might be limited and not detected by autoradiography, further confirmation of the absence of PEP-dependent phosphorylation of HPr in mutant DC10 was undertaken by measuring the concentration of P~(His)-HPr in DC10 and wild-type *S. mutans* BM71. The various forms of HPr can be detected by employing the crossed immunoelectrophoresis technique of Vadeboncoeur et al. (39) with anti-HPr antibody. Normal cells contain four forms of HPr: nonphosphorylated HPr, P~(His)-HPr, P-(Ser)-HPr, and the doubly phosphorylated derivative P~(His)-P-(Ser)-HPr. P~(His)-HPr, generated by phosphotransfer from PEP via EI, and P-(Ser)-HPr, generated by the action of ATP-dependent (Ser)-HPr kinase, migrate to the same position in the first dimension; however, they can be separated by boiling a portion of the cell extract to degrade the phosphoamidate bond of P~(His)-HPr. Since phosphomonoester bonds are heat stable, P-(Ser)-HPr is unaffected by boiling (26), thus, boiling the sample quantitatively converts P~(His)-HPr to free HPr and P~(His)-P-(Ser)-HPr to P-(Ser)-HPr. Comparison of the areas under the peaks of the unboiled and boiled samples of the same extract permits estimation of the cellular concentration of the four forms of HPr.

All four forms of HPr could be detected in wild-type *S. mutans* BM71 cell extracts; however, only nonphosphorylated HPr and P-(Ser)-HPr could be detected in mutant DC10 cell extracts (Fig. 5). Quantitative analysis of the peaks indicated that the total pool of HPr was not the same in both strains; the wild-type strain possessed 98.6 μg of total HPr mg (dry weight) of cells^{-1}, while the mutant had only 16.8 μg mg^{-1}. For wild-type BM71, the major component was the doubly phosphorylated [P~(His)-P-(Ser)-HPr] fraction (63.0 μg mg^{-1}), while

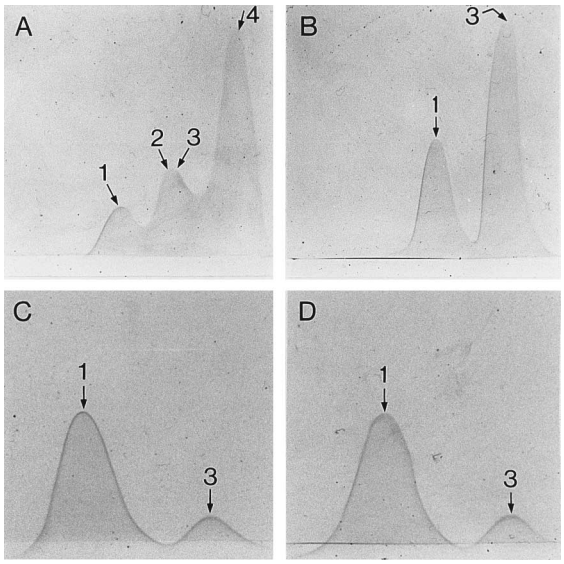

FIG. 5. Crossed immunoelectrophoresis of membrane-free extracts from *S. mutans* BM71 and DC10 cells grown in batch culture. Each sample contained 10 μg of cytoplasmic proteins and was probed with polyclonal anti-HPr rabbit antibodies directed against *S. mutans* DR0001. Panels: A, untreated extract from *S. mutans* BM71; B, same as panel A but boiled for 3 min prior to electrophoresis; C, untreated extract from *S. mutans* DC10; D, same as panel C but boiled for 3 min prior to electrophoresis. The numbers indicate the following immunoprecipitate peaks: 1, nonphosphorylated HPr; 2, P~(His)-HPr; 3, P-(Ser)-HPr; 4, P~(His)-P-(Ser)-HPr.

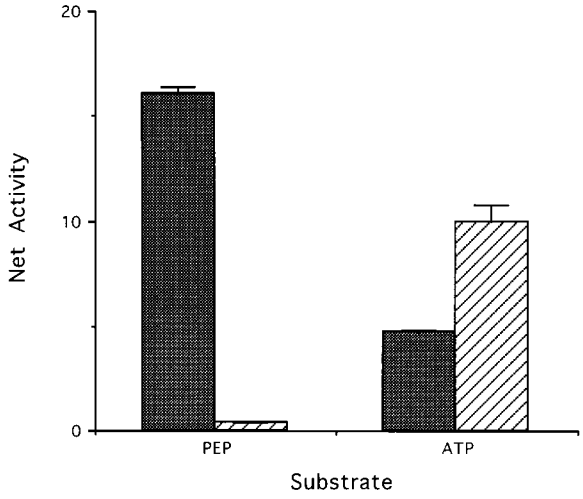

FIG. 6. Glucose phosphorylation by decryptified cells of *S. mutans* BM71 (▨) and *ptsI* mutant DC10 (▨) incubated with PEP or ATP. Net activity is expressed as nanomoles of glucose phosphorylated milligram (dry weight) of cells^{-1} minute^{-1}.

P~(His)-HPr and free HPr were present at 19.5 and 12.1 μg mg^{-1}, respectively, and P~(Ser)-HPr was present at only 4.0 μg mg^{-1}. In mutant DC-10, free HPr was present at 15.0 μg mg^{-1}, while P-(Ser)-HPr was present at only 1.8 μg mg^{-1}. The fact that the mutant had no P~(His)-HPr or P~(His)-P-(Ser)-HPr indicated the absence of PEP-dependent phosphorylation typical of PTS activity, confirming the ^{32}P-PEP data. As a consequence, most (90%) of the HPr in the mutant was in the form of free HPr.

Characterization of the non-PTS glucose transport system. The kinetics of [^{14}C]glucose uptake by intact, glucose-grown BM71 and transformant DC10 cells were determined by measuring initial rates of transport at glucose concentrations ranging from 0.01 to 10 mM. Experiments with BM71 revealed apparent K_s values of 17 and 138 μM and V_{max} values of 39 and 64 nmol mg (dry weight) of cells^{-1} min^{-1}, respectively. Mutant DC10 had only one detectable system, with an apparent K_s of 125 μM and a V_{max} of 0.87 nmol mg of dry cells^{-1} min^{-1}.

Subsequent experiments were directed at determining the intracellular donor employed to phosphorylate glucose in mutant strain DC10 following transport. For this, glucose-grown cells were permeabilized with toluene and incubated with glucose and either ATP or PEP. As shown in Fig. 6, PEP-dependent phosphorylation of glucose was negligible in mutant DC10 but predominant in the parent strain. Substantial glucose-6-phosphate was formed in the presence of ATP in the mutant and to a lesser extent in the parent. The experiment was repeated with cells preincubated with 2-deoxyglucose to deplete the intracellular PEP pool, and the level of phosphorylation by PEP and ATP was similar to that seen with nondepleted cells.

To determine the specificity of non-PTS glucose transport, we incubated glucose-grown cells of wild-type *S. mutans* BM71 and mutant DC10 with [^{14}C]glucose in the presence of a 100-fold excess of various unlabelled sugars. This competition study (Fig. 7A) demonstrated that BM71 was subject to significant inhibition by mannose, a PTS sugar transported at rates similar to those of glucose (41), and moderately by arabinose and fructose. The mutant, however, had a high specificity for glucose, and little inhibition was observed with any of the substrates tested (Fig. 7B). The best competitor for glucose transport via the system was the glucose analog 2-deoxyglucose, which decreased uptake by nearly 40%. Surprisingly, the glucose analog α-methylglucoside inhibited glucose uptake by only 10%. Lactose, galactose, sucrose, raffinose, and the sugar alcohol sorbitol were also tested but failed to demonstrate significant inhibition with either the wild-type or mutant strain.

DISCUSSION

Early studies with decryptified cells of *S. mutans* Ingbritt grown in continuous culture showed that the glucose PTS was repressed under a variety of conditions, including growth at low pH (15), high growth rates, (9) growth with excess glucose (9), and following a transition to growth on sucrose (8). Interestingly, the rates of glucose uptake and glycolysis were reduced, but not to the same degree, suggesting that during PTS repression *S. mutans* employed an alternative, non-PTS glucose transport system (13). Later studies demonstrated that this repression was associated with the synthesis of membrane-bound EII for glucose (EII^{Glc}) and mannose (EII^{Man}) (16, 40, 41). Of particular interest was the concentration-dependent repression of the two activities by glucose itself, with a 40-fold reduction observed with an increase from 3.6 to 271 mM glucose in the growth medium (16). Growth at pH values below 8.0 also reduced the synthesis of EII^{Glc} and EII^{Man}, and cells at pH 5.0 were completely devoid of activity (40). On the other hand, changes in the medium glucose concentration (2.7 to 304 mM), pH (8.0 to 5.0), and growth rate (0.1 to 1.0 h^{-1}) resulted in only fourfold changes in the cellular concentrations of the general PTS proteins HPr and EI (16, 40, 41).

Prior to the present study, evidence for the hypothetical non-PTS glucose transport system was indirect. Early results (18) suggested that glucose uptake via this system was coupled to proton motive force; however, uptake studies with 6-deoxyglucose (6) questioned this model. This latter conclusion was

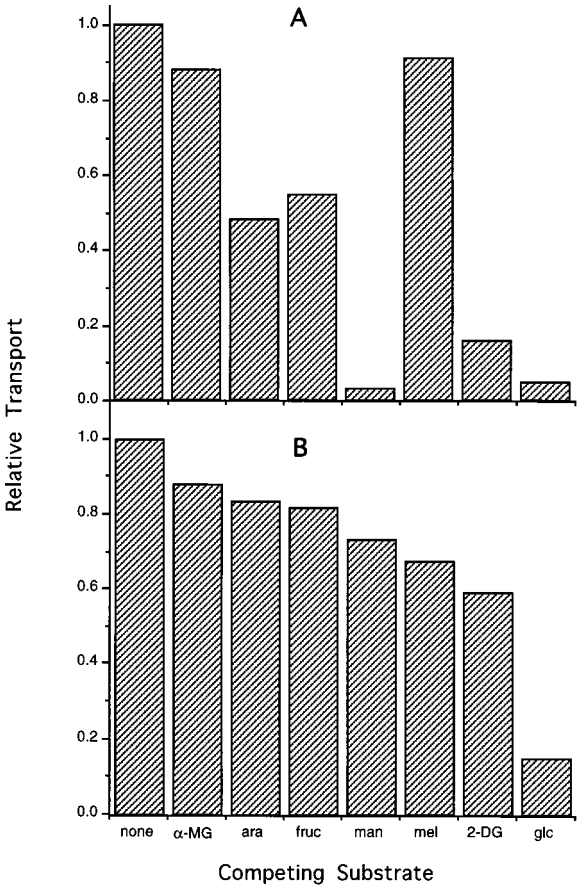

FIG. 7. Sugar competition for [^{14}C]glucose transport with intact cells of wild-type *S. mutans* BM71 (A) and *ptsI* mutant DC10 (B). Competing sugars were used at a concentration 100-fold in excess of that of the labelled glucose. Abbreviations: α-MG, α-methylglucoside; ara, arabinose; fruc, fructose; man, mannose; mel, melibiose; 2-DG, 2-deoxyglucose; glc, glucose.

supported by measurements of the components of the transmembrane electrochemical proton gradients in cells of *S. mutans* and *S. sobrinus* grown in continuous culture under a variety of conditions (14). Proton motive force values were normally low (<70 mV) under all conditions, including those that repress the PTS, suggesting that insufficient proton motive force was generated to support significant sugar transport.

More substantial evidence for the non-PTS system was obtained in experiments with *S. mutans* DR0001 and a PTS-defective mutant, strain DR0001/6, of the organism grown in chemostats at growth rates between 0.04 and 0.6 h^{-1} (18). The wild-type strain possessed two glucose transport processes, one with K_s values for glucose ranging from 6.7 to 8.0 μM and a second with values ranging from 57 to 125 μM, with the high-affinity system shown to be the glucose PTS. The mutant, on the other hand, had only the lower-affinity system (62 to 132 μM). More recently (4), membrane vesicles of *S. mutans* Ingbritt prepared from cells grown in continuous culture under optimum and repressed PTS conditions and devoid of cytoplasmic components and PTS activity exhibited glucose counterflow, indicating the presence of a constitutive transmembrane carrier able to recognize glucose.

Clearly, the characterization of a non-PTS glucose transport system in *S. mutans* requires inactivation of the PTS. Although a mutant of *S. mutans* defective in glucose PTS transport activity has been used in the past, the nature of the mutation was unknown and uptake may have occurred via other PTS permeases (18). The construction of strain DC10 circumvented these problems by eliminating glucose transport and phosphorylation via the PTS since all PTS transport requires a functional EI. The mutant is missing approximately 150 amino acid residues from the COOH terminus of the protein, and while the putative phosphorylation site was present on the truncated protein (3), it was unable to phosphorylate HPr at the histidyl residue as demonstrated by the ^{32}P-PEP phosphorylation and immunoelectrophoresis experiments. These results are in agreement with those obtained with a truncated form of EI from *S. typhimurium* which was not phosphorylated by PEP, although it contained the active His-15 site (24). Thus, it appears that the COOH-terminal domain of the enzyme is a key factor in both catalysis and regulation.

It has been previously reported that PTS-defective strains of *S. typhimurium* (31) and *E. coli* (32) can accumulate mutations that uncouple transport from phosphorylation in EIIs, allowing them to transport glucose via facilitated diffusion. It is unlikely that the ability of *S. mutans* DC10 to grow on glucose is a result of mutations altering the specificity of an EII or uncoupling of an EII from phosphorylation, since mutations of this nature have been shown to be selected only at limiting glucose concentrations under the strong selective pressures presented by growth in continuous culture (31). The growth characteristics of strain DC10 with glucose were also stable regardless of whether the organism was cultured with glucose or raffinose, suggesting that the selective pressure of repeated growth on glucose was not required to generate a glucose-fermenting phenotype.

Data obtained recently with membrane vesicles of *S. mutans* Ingbritt have indicated that the intracellular product of a non-PTS glucose transport process would be free glucose (4). The same study demonstrated that ATP-dependent phosphorylation of glucose was predominant in PTS-repressed cells compared with PTS-optimal cells, indicating that the intracellular glucose is phosphorylated by ATP and a glucokinase. The results obtained with *ptsI* mutant DC10 in this study support this, as demonstrated by the phosphorylation of glucose by ATP, but not by PEP, in decryptified cells (Fig. 6). Furthermore, the K_s value for glucose of 125 μM obtained with mutant DC10 is similar to that observed with the PTS-defective mutant of *S. mutans* DR0001 reported in a previous study, i.e., 57 to 133 μM (18).

The kinetic studies with the mutant strain revealed that the maximum velocity of glucose uptake was 0.87 nmol mg (dry weight) of $cells^{-1}$ min^{-1} compared with a V_{max} for wild-type strain BM71 of 64 nmol mg (dry weight) of $cells^{-1}$ min^{-1} for total glucose transport. The latter value, of course, represents the contribution of both PTS and non-PTS glucose transport processes. This large difference in the maximum rates of glucose transport is difficult to reconcile on the basis of the K_s value for glucose (125 μM), since growing DC10 cells would normally be exposed to glucose concentrations well in excess of this concentration. The data seem to suggest that the PTS has a significant role in regulating the expression or activity of this system. The low growth rate of the mutant on glucose (Fig. 1) tends to support this hypothesis. Thus, it appears that the non-PTS transport process is a secondary system supplementing the PTS and, from the earlier continuous culture data (12, 13), is essential for the organism under conditions of low pH, high glucose concentrations, and high growth rates.

An interesting question is whether the non-PTS mechanism is related to the MSM system recently discovered by Russell and coworkers in *S. mutans* (33). The suggestion has been made that glucose may be transported via the MSM system

because glucose was able to inhibit uptake of radiolabelled melibiose when present in a 100-fold excess concentration (36). These experiments did not consider the possibility that glucose was acting as a catabolic repressor of uptake via the MSM system, a phenomenon that has recently been demonstrated in our laboratory (5). The fact that the MSM system must be induced by one of its substrates (33) also supports the hypothesis that the glucose uptake observed in DC10 occurred via an independent mechanism since the cells were grown in the absence of an MSM system inducer and were also unable to transport radiolabelled raffinose (data not shown). Also, raffinose and melibiose were not efficient inhibitors of glucose transport by DC10 (Fig. 7B), suggesting that glucose entry occurred via an alternate route.

The high degree of specificity of the non-PTS system for glucose is amply demonstrated by the competition experiment depicted in Fig. 7B. The observation that 2-deoxyglucose and, particularly, α-methylglucoside are not effective inhibitors of glucose transport at 100-fold excess concentrations suggests that the permease is probably restricted to glucose uptake alone. The 40% inhibition by 2-deoxyglucose, a mannose analog, probably reflects the close stereospecificity of the mannose and glucose molecules, a factor evident in the competition experiment with wild-type BM71 (Fig. 7A). Mannose and glucose exhibit similar uptake rates via the PTS in cells of *S. mutans* and other oral streptococci (16, 40, 41).

These results confirm the existence of a non-PTS glucose transport system in *S. mutans* possessing an affinity for glucose lower than that of the glucose PTS. The mutant strain *S. mutans* DC10 will be utilized for further study of the regulation of the two systems and should give insight into the various ways that *S. mutans* can transport other non-PTS substrates. Results obtained recently with this strain and the parent BM71 (5) demonstrated that the PTS controls uptake of sugars via the MSM system. This mutant will undoubtably be useful in the study of other non-PTS sugar transport processes, as well as the role of some PTS components, such as HPr, in cellular metabolism.

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