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Channel Specificity and Secondary Structure of the Glucose-Inducible Porins of *Pseudomonas* spp.

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The OprB porin-mediated glucose transport system was investigated in *Pseudomonas chlororaphis, Burkholderia cepacia*, and *Pseudomonas fluorescens*. Kinetic studies of [U-^{14}C]glucose uptake revealed an inducible system of low K_m values (0.3–5 μM) and high specificity for glucose. OprB homologs were purified and reconstituted into proteoliposomes. The porin function and channel preference for glucose were demonstrated by liposome swelling assays. Examination of the periplasmic glucose-binding protein (GBP) components by Western immunoblotting using *P. aeruginosa* GBP-specific antiserum revealed some homology between *P. aeruginosa* GBP and periplasmic proteins from *P. fluorescens* and *P. chlororaphis* but not *B. cepacia*. Circular dichroism spectropolarimetry of purified OprB-like porins from the three species revealed β sheet contents of 31–50% in agreement with 40% β sheet content for the *P. aeruginosa* OprB porin. These findings suggest that the high-affinity glucose transport system is primarily specific for glucose and well conserved in the genus *Pseudomonas* although its outer membrane component may differ in channel architecture and specificity for other carbohydrates.

KEY WORDS: *Pseudomonas* spp; glucose transport; OprB porin; circular dichroism.

INTRODUCTION

The outer membrane of Gram-negative bacteria, such as *Pseudomonas* spp., serves as a permeability barrier to hydrophilic solutes and plays a crucial role in solute transport across the bacterial cell surface (Nikaido, 1994). Several bacterial transport systems specific for inorganic ions, amino acids, peptides, and sugars are well recognized. Small hydrophilic molecules and ions cross the outer membrane barrier by diffusing through the classical nonspecific trimeric porins (Nikaido, 1993). The transport of sugar is known to occur either through the multicomponent bacterial phosphoenolpyruvate-dependent phosphotransferase system (PTS) in which the vectorial transport of sugar across the cytoplasmic membrane is coupled to its phosphorylation (Weigel *et al.*, 1982; Meadow *et al.*, 1990) or via the periplasmic binding protein mediated pathway (Hengge and Boos, 1983).

In *P. aeruginosa*, two separate inducible systems exist for the transport of glucose (Midgley and Dawes, 1973). One is a low-affinity transport system (K_m = 2.8 mM) which involves extracellular oxidation of glucose prior to transport into the cytoplasm. The second is a high-affinity system (K_m = 8 μM) which actively transports glucose directly into the cytoplasm via a periplasmic binding protein-dependent pathway. The outer membrane carbohydrate-inducible OprB porin (Hancock and Carey, 1980) and a periplasmic glucose-binding protein (GBP) (Stinson *et al.*, 1977) have been linked to glucose transport via the latter pathway. The OprB porin presumably interacts physically with the GBP in a manner analogous to the interaction between the maltose-specific LamB porin and

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the periplasmic maltose-binding protein (Bavoil and Nikaido, 1981), both of which specifically facilitate the uptake of maltose and maltodextrins in *E. coli*.

Binding protein-dependent transport mechanisms for the transport of sugars, amino acids, and inorganic ions have been widely reported in Gram negative bacteria, and genes for all known binding protein-mediated transport components are organized as a single operon with the exception of the *E. coli* maltose transport system. High-affinity binding protein-dependent transport systems have been described in *P. aeruginosa* for branched chain amino acids (Hoshino and Kose, 1989, 1990), inorganic phosphate (Poole and Hancock, 1986; Siehnel *et al.*, 1990), and mannitol (Eisenberg and Phibbs, 1982). A major recognition site in these and other binding protein-dependent multicomponent transport systems is the periplasmic binding protein.

The OprB porin, which forms a trimeric water-filled transmembrane channel and plays a central role in carbohydrate transport (Wylie and Worobec, 1995), has been shown to be a carbohydrate-selective porin facilitating the diffusion of glucose, xylose, fructose, mannitol, and glycerol (Trias *et al.*, 1988; Saravolac *et al.*, 1991; Wylie and Worobec, 1993; Williams *et al.*, 1994). Genetic and immunological evidence have also shown that homologues of the OprB porin exist in pseudomonads belonging to the rRNA homology group I including *P. putida, P. fluorescens, P. chlororaphis*, and *P. stutzeri* (Wylie and Worobec, 1994). This is consistent with similar studies done on the OprF porin of *P. aeruginosa* which is well conserved among the same group of pseudomonads (Ullstrom *et al.*, 1991).

The *P. aeruginosa* and *P. putida* OprB porins have been purified and biophysically characterized (Trias *et al.*, 1988; Saravolac *et al.*, 1991; Wylie *et al.*, 1993). The involvement of the periplasmic GBP in glucose transport suggests that both the OprB porin and the GBP may form part of the glucose ABC transporter whose remaining components are yet to be identified in *Pseudomonas*. Bacterial ABC transporters are multicomponent systems composed of three to four membrane-bound proteins in addition to a soluble periplasmic binding protein. The binding protein is proposed to function as a chemotactic response protein and a ligand receptor which connects the entire transport system. The inner membrane components of the transport system are believed to provide the necessary phosphate bond energy for the active transport of the substrate. The *oprB* gene (Wylie and Worobec, 1994) encoding the 47-kDa porin of *P. aeruginosa* is the only high-affinity glucose transport structural gene so far cloned and characterized from the genus *Pseudomonas*. Very little is known about outer membrane permeability and sugar transport in other pseudomonads.

In the present study, we have sought to isolate OprB homologues and to determine whether the specificity of the glucose transport system is well conserved throughout various members of the genus *Pseudomonas*. Here we report the identification of a pore-forming carbohydrate-inducible outer membrane protein (OMP) from *Burkholderia* (formerly *Pseudomonas*) *cepacia* and the structural characterization of this and two other glucose-inducible OMPs earlier identified by Wylie and Worobec (1994) from *P. fluorescens* and *P. chlororaphis*. We also demonstrate the existence of inducible high-affinity glucose transport systems which utilize these OMPs in the three bacterial strains. The prevalence of such carbohydrate transport systems in *Pseudomonas* is discussed in light of the present findings and with particular reference to the well-characterized system in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa H673 ($OprD^-$) (Huang *et al.*, 1992) was used to overexpress the OprB porin following transformation with plasmid pPZSH27 (Wylie and Worobec, 1995) containing the cloned *oprB* gene from *P. aeruginosa* PAO1 strain H103 (Hancock and Carey, 1979). *Burkholderia cepacia* NCTC 10661, *P. chlororaphis* ATCC 9446, and *P. fluorescens* UMCC (University of Manitoba Culture Collection) were used for glucose uptake assays and porin isolation. All bacterial strains were grown overnight at 37°C, with the exception of *P. fluorescens* (grown at 28°C), in basal medium 2 (BM2) minimal medium (Hancock and Carey, 1979) supplemented with 0.4% (w/v) carbohydrate or 20 mM potassium succinate as a carbon source. 15 μg/ml $HgCl_2$ and 500 μg/ml carbenicillin were added to H673/pPZSH27 cultures to respectively prevent mutant reversion and plasmid segregation.

Whole-Cell Glucose Uptake Assays

Incorporation of [U-^{14}C]glucose was studied using a modified membrane filtration assay of Eagon and Phibbs, 1971 as described by Wylie and Worobec

(1993). Briefly, 50 ml overnight cultures of bacterial strains, grown in BM2 supplemented with D-glucose (20 mM), were centrifuged at 10,000 × *g* for 10 min. Harvested cells were washed twice with 10 ml of carbon substrate-free BM2 and resuspended to an absorbance of 0.20 at 600 nm. Cell suspensions were prewarmed to 37°C for 10 min prior to initiation of uptake assays. Calculated amounts of labeled glucose (2.9 mCi/mmol, DuPont Canada Inc., Markam, Ontario) were incubated with resuspended cells and aliquots (0.25 ml) were removed at 20, 40, and 60 s and filtered through a 25 mm, 0.45-μm membrane filter (Gelman Sciences, Ann Arbor, Michigan) in a Millipore manifold (Millipore Corp., Bedford, Massachusetts). Filters were promptly washed twice with 5 ml of substrate-free BM2. Uptake data from formalin-killed cells were used to account for nonspecific filter binding. Each assay was repeated at least three times and average values were used to calculate the uptake rate at different concentrations of labeled glucose. The velocity is expressed in nmol glucose/min/mg dry cell weight.

Glucose Transport Inhibition

A 50-fold excess of test carbohydrate was used to measure the inhibition of [U-^{14}C]glucose uptake in each bacterial strain grown in BM2 supplemented with 20 mM D-glucose. Unlabeled test substrate was added to resuspended cells 20 s prior to addition of [U-^{14}C]glucose. Aliquots were removed as described above and the extent of inhibition by each substrate was determined by comparing the uptake rate in the presence of test susbtrate with uptake rate in the absence of inhibiting substrate.

Preparation of Outer Membrane Proteins (OMP) and Gel Electrophoresis

Cell envelope proteins were prepared by the method of Lugtenberg *et. al* (1975) from a 50-ml overnight culture of each bacterial strain in BM2 supplemented with 20 mM glucose. Cells were harvested by centrifugation at 5000 × *g* for 10 min in a Sorvall centrifuge and resuspended in 50 mM Tris, 2 mM EDTA, pH 8.5. Resuspended cells were French pressed at 15,000 psi and briefly centrifuged to get rid of intact cells. The supernatant was ultracentrifuged at 145,000 × *g* for 1 h at 4°C. Pelleted OMPs were washed and resuspended in 2 mM Tris, pH 7.8. SDS-PAGE analysis of outer membrane proteins utilized the 11% gel system of Lugtenberg *et al.* (1975).

Periplasmic Protein Preparation and Western Immunoblotting

Bacterial periplasmic proteins were prepared by the cold-shock procedure of Hoshino and Kageyama (1980). Bacterial strains were grown overnight in BM2 medium supplemented with 20 mM D-glucose. Cells were harvested and resuspended in 50 mM Tris-HCl, pH 8.0 containing 200 mM $MgCl_2$ followed by two rounds of incubation at 30°C for 10 min and 0°C for 15 min. Cell suspension was then centrifuged at 10,000 × *g* for 10 min and the supernatant was concentrated by ultrafiltration. Shock fluid was subjected to SDS-PAGE and the proteins were transferred onto nitrocellulose filters (Micron Separations Inc.) for immunodetection (Towbin *et al.*, 1979) with polyclonal antibody raised against purified periplasmic glucose-binding protein (GBP) of *P. aeruginosa* (Sly *et al.*, 1993). Diluted goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) was used as a second antibody. Bound antibodies were detected by using hydrogen peroxide and 4-chloro-1-naphthol (Sigma).

Purification of Glucose-Inducible Outer Membrane Proteins

Glucose-inducible OMPs (*P. fluorescens*, 45-kDa, *P. chlororaphis*, 45-kDa, and *B. cepacia*, 40-kDa) were purified according to the modified method of Hancock and Carey (1980) as described by Wylie *et al.* (1993). Cell envelope proteins prepared from glucose-grown bacterial cells were resuspended in 2% (v/v) Triton X-100, 10 mM EDTA, 20 mM Tris-HCl (pH 8.0) and loaded onto a DEAE-Sephacel column (Pharmacia, Sweden) previously equilibrated with 0.1% (v/v) Triton X-100, 10 mM EDTA, and 20 mM Tris-HCl (pH 8.0) (TTE). Each glucose-inducible protein was eluted with a 0.1–0.25 M NaCl gradient in TTE and 2 ml fractions were collected using a Spectra/chrom CF-1 fraction collector. Fractions were analyzed by SDS-PAGE, with total outer membrane proteins from glucose-grown and succinate-grown cultures, to identify fractions containing the glucose-inducible proteins which were succinate repressible.

Liposome Swelling Assays

Purified proteins were reconstituted in proteoliposomes and swelling assays were done as described by Nikaido and Rosenberg (1983). An aqueous solution of each protein (50 μg protein) was added to an oven-dried film of liposomes made from 10 μmol of egg phosphatidylcholine (Sigma) and 0.2 μmol of dicetylphosphate (Sigma). The suspension was sonicated in an ice-bath with an ultrasound sonicator for 30 s, dried in a $CaSO_4$-equipped vacuum pump, and resuspended at room temperature in a buffer solution of 17% (w/v) Dextran T-40, 4 mM sodium-NAD, and 1 mM imidazole-NAD, pH 6.0. Large aggregates were removed from the suspension by passing it through an 8-μm membrane filter (Millipore Corporation, Bedford, Massachusetts). The swelling rates of the proteoliposome suspension were determined by measuring the decrease in optical density at 400 nm upon the dilution of liposome into test solution consisting of 1 mM imidazole-NAD, 1 mM sodium-NAD, and 40 mM test substrate. Absorbance values were recorded every 10 s up to 120 s against a blank solution containing 1 mM imidazole-NAD, pH 6.0 and 1 mM sodium-NAD. Porin-free liposome suspension was used as a negative control in all cases.

Secondary Structure Estimation

Circular dichroism (CD) spectra of purified proteins, precipitated with ice-cold acetone and resuspended in 10 mM Tris-HCl, 0.1% SDS, were obtained from a Jasco J-500A spectropolarimeter using a 0.5-cm cuvette at a protein concentration of approximately 100 μg/ml. The purity of the glucose-inducible proteins was verified by SDS-PAGE which revealed only the presence of the desired bands. Protein concentration was determined with the Scopes method (Scopes, 1974) based on the absorbance measurements at 205 nm. CD spectra were obtained from 200 to 250 nm at 1 nm intervals. Spectral analysis was performed with the convex constraint algorithm (CCA) of Perczel *et al.* (1992) which deconvolutes each spectrum obtained into its pure components and assigned structural components based on comparison with the CD spectra of proteins with known secondary structure.

RESULTS

Investigation of Inducible Glucose Transport Systems in *P. chlororaphis, B. cepacia,* and *P. fluorescens*

Whole-cell [U-^{14}C]glucose uptake assays were used to demonstrate the existence of an inducible high-affinity glucose transport system in the three bacterial strains, *P. fluorescens*, *P. chlororaphis*, and *B. cepacia*. [U-^{14}C]glucose uptake by washed cells was followed for 60 s and the initial uptake rate was calculated for each substrate concentration examined. The velocity (nmol/min/mg dry weight) of glucose uptake was plotted against substrate concentration. Kinetic parameters were determined from Eadie–Hofstee plots of the initial uptake rate (V) versus velocity/substrate concentration (V/[S]) and from fitting the experimental data to theoretical Michaelis–Menten model using the Mathematica software package of Wolfram Research, Inc. USA (Wolfram, 1991). Analysis of the uptake data revealed K_m and V_{max} values of 0.3 μM and 8.8 nmol/min/mg dry weight for *P. chlororaphis*; 5.0 μM and 63.3 nmol/min/mg dry weight for *B. cepacia*; and 1.4 μM and 29.7 nmol/min/mg dry weight for *P. fluorescens*. It was quite obvious that the inducible glucose uptake system in each of the above strains has a very high affinity for D-glucose at low extracellular glucose concentration. The kinetic parameters are similar to data obtained for analogous systems in *P. aeruginosa* with a K_m value of 8 μM (Whiting *et al.*, 1976), in *P. putida* with a K_m value of 8.3 μM (Schleissner *et al.*, 1997), and in *P. fluorescens* with a K_m value of 1 μM (Lynch and Franklin (1978). The high substrate affinity (in μM range) demonstrates that the transport system is binding-protein dependent in each case (Furlong, 1987). The apparent differences in affinities might be due to differences in the concentration of the respective glucose-binding proteins in the periplasm which ultimately represent the substrate recognition sites of the transport systems (Merino *et al.*, 1995).

Inhibition of Glucose Uptake by Various Carbohydrates

The substrate specificity of the glucose uptake systems in the three *Pseudomonas* spp. was investigated using specific unlabeled carbohydrates added 20 s prior to the addition of [U-^{14}C]glucose. As shown in

Table I, uptake of [U-^{14}C]glucose by washed bacterial cells, prepared from BM2-glucose-grown culture, was strongly inhibited by unlabeled D-glucose in all three cases, demonstrating that the systems specifically recognize D-glucose, as earlier observed for *P. aeruginosa* (Wylie and Worobec, 1993). For *P. chlororaphis*, a 50-fold excess of unlabeled substrates over [U-^{14}C]glucose showed that uptake was variably inhibited by different carbon sources (glucose > arabinose > ribose > fructose > maltose) whereas xylose, arginine, and sucrose had virtually no inhibitory effect. Inhibition of D-glucose uptake by L- arabinose is consistent with a previous report on the inhibition of 2-deoxy-D-glucose uptake by L-arabinose in *H. pylori* (Mendz et al., 1995) although arabinose is not known to inhibit glucose uptake in *P. aeruginosa*. In the case of *B. cepacia*, the substrate inhibition pattern (glucose > ribose > fructose > maltose) was similar to that of *P. chlororaphis* except that L-arabinose and D-mannitol showed no inhibitory effect on glucose uptake. Relatively little inhibitory effect was shown by L-arginine and D-xylose. The result obtained with L-arginine was not surprising as liposome swelling studies have shown that the carbohydrate-selective OprB porin of *P. aeruginosa* (Trias *et al.*, 1988) and the *E. coli* maltoporin (Luckey and Nikaido, 1980) are capable of allowing the passage of smaller noncarbohydrate compounds that have no obvious structural resemblance to glucose. In *P. fluorescens*, the uptake of [U-^{14}C]glucose was also inhibited to varying extents by unlabeled sugars (glucose >> arabinose > ribose > maltose). Little or no inhibitory effect was observed with fructose, xylose, sucrose, and mannitol. The extent of inhibition observed for D-glucose, D-maltose, and D-ribose in the three strains was similar to the pattern of inhibition for *P. aeruginosa*. The level of inhibition observed for D-fructose in *P. chlororaphis* and *B. cepacia* suggests that this sugar is transported to a certain extent by the glucose-inducible system, but it is possible that a specific fructose-inducible system exists as is the case for *P. putida* (Vicente *et al.*, 1975) and *P. aeruginosa* (Eagon and Phibbs, 1971). To clarify this observation, we co-induced the two transport systems for glucose and fructose in *B. cepacia* and independently monitored the uptake of [U-^{14}C]glucose and [U-^{14}C]fructose. Uptake of [U-^{14}C]fructose was significantly increased (from 0.008 nmol/min/mg dry cell weight to 17.7 nmol/min/mg dry cell weight) but glucose uptake remained constant in cells co-induced for the two transport systems. This demonstrates the existence of two different transport systems, one for glucose and the other for fructose transport in *B. cepacia*.

Table I. Percent Inhibition of [U-^{14}C]Glucose Uptake by Various Carbon Substrates[a]

	P. chlororaphis	*B. cepacia*	*P. fluorescens*	*P. aeruginosa*[b]
D-glucose	100	100	100	100
L-arabinose	50.6 ± 1.5	0	45.0	ND
D-fructose	32.2 ± 1.5	35.0 ± 1.0	8.5 ± 1.2	9.0 ± 3.0
D-ribose	31.1 ± 2.0	45.0 ± 1.7	26.2 ± 0.3	19.2 ± 3.0
D-maltose	22.2 ± 3.0	27.5 ± 3.5	24.0 ± 2.5	48.1 ± 4.5
L-arginine	1.0	13.0 ± 3.1	ND	13.5 ± 2.3
D-xylose	0	7.0 ± 1.0	0	27.9 ± 1.5
Sucrose	0	0	0	ND

[a] The extent of inhibition of the whole-cell uptake of 2.4 μM [U^{14}C]glucose by various unlabeled carbon substrates (50-fold excess) is expressed as a percentage of the glucose uptake data in the absence of any inhibiting substrate. Values are taken from at least three separate determinations with means and standard deviations shown.

[b] Inhibition data for *P. aeruginosa* are taken from Wylie and Worobec (1993); ND, not determined.

Analysis of the Periplasmic Components of the Glucose Transport Systems

Periplasmic shock fluids were prepared from BM2 glucose-grown cells of *P. chlororaphis*, *B. cepacia*, and *P. fluorescens*. These were subjected to western immunoblotting using polyclonal antibody raised against the purified 44.5-kDa glucose-binding protein (GBP) from *P. aeruginosa* (Sly *et al.* 1993) as a probe to evaluate the conservation of GBP. Only *P. fluorescens* and *P. chlororaphis* possess periplasmic proteins (44-kDa and 46-kDa, respectively) which cross-reacted with the *P. aeruginosa* GBP-specific polyclonal antiserum (data not shown). No cross reaction was observed for *B. cepacia* periplasmic proteins. These data suggest a possible conservation of GBP within members of the *Pseudomonas* rRNA homology group I.

Purification and Reconstitution of the Glucose-Inducible Porins from *P. chlororaphis*, *B. cepacia*, and *P. fluorescens*

Previous studies have identified a 45-kDa carbohydrate-inducible protein from *P. chlororaphis* and a similar protein from *P. fluorescens* (Wylie and Woro-

bec, 1994). In the present study, a ~ 40-kDa outer membrane protein was also identified in *B. cepacia* grown in the presence of myoinositol, glucose, and fructose but not in succinate (Fig. 1). The glucose-inducible outer membrane proteins from these three strains were purified, as explained above, and reconstituted into proteoliposomes to examine their porin function. The three proteins allowed the diffusion of glucose in this model membrane system (Fig. 2), which suggests their capacity to form outer membrane diffusion pores. The rate of diffusion of glucose was determined by measuring the rate of swelling of the liposome upon the addition of liposomes to the appropriate test solution. These rates were compared with that of the OprB porin of *P. aeruginosa*. Relative rates were determined by normalizing individual glucose diffusion rates for each protein to glucose permeability through the OprB porin of *P. aeruginosa* which was assumed to be 100%. Based on this calculation, the relative permeation rates for glucose were: *B. cepacia* OMP (123%) > *P. aeruginosa* OprB channel (100%) > *P. fluorescens* OMP (93%) > *P. chlororaphis* OMP (61%). Thus the *B. cepacia* porin channel appeared more glucose-specific than its homologs in the genus *Pseudomonas*. In terms of channel size and specificity, the *P. fluorescens* porin channel was closer to the *P. aeruginosa* OprB porin channel. It was interesting to note that western immunoblotting with polyclonal antiserum specific for the *P. aeruginosa* OprB porin did not cross-react with either the *B. cepacia* (data not shown) nor with the *P. fluorescens* glucose-inducible porins (Wylie and Worobec, 1994) but cross-reaction was earlier observed for the *P. chlororaphis* porin (Wylie and Worobec, 1994).

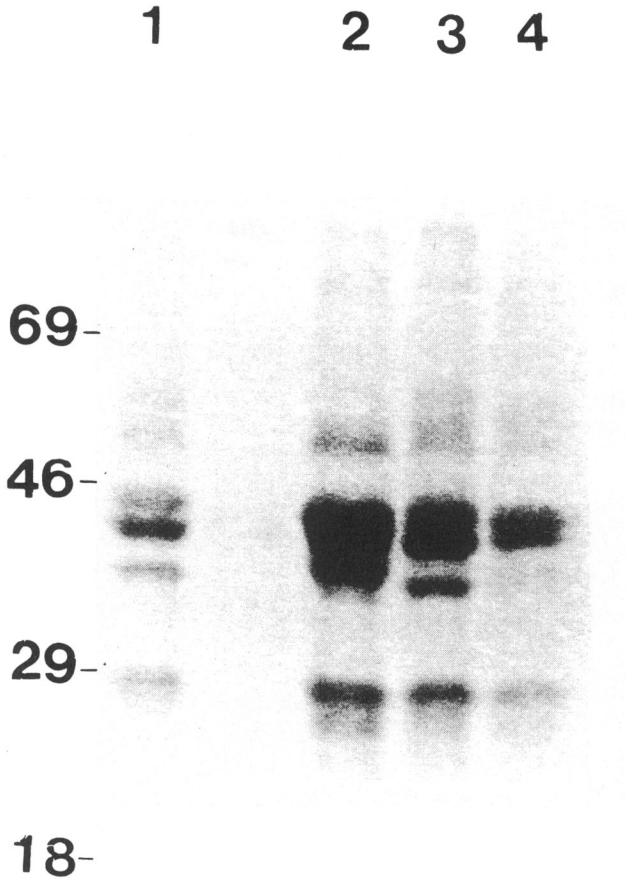

Fig. 1. SDS-PAGE of outer membrane proteins of *Burkholderia cepacia* grown on different carbohydrates. Lane 1, myoinositol-grown cells; lane 2, fructose-grown cells; lane 3, glucose-grown cells; lane 4, succinate-grown cells. Arrow indicates the succinate-repressible protein induced in all carbohydrate-grown cells. Samples (10 μg) were solubilized at 95°C prior to loading on SDS-PAGE. Molecular weight standards are indicated on the left in kDa.

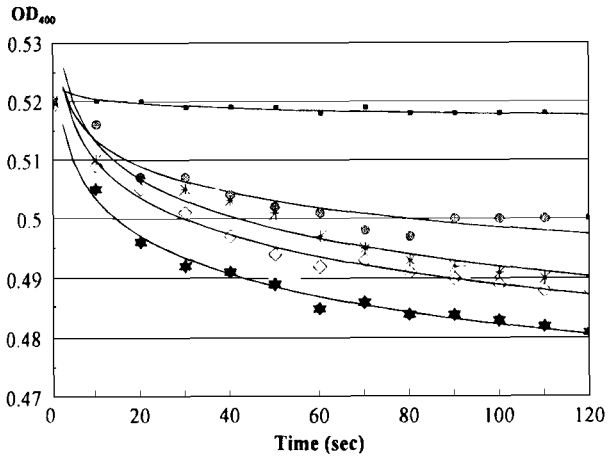

Fig. 2. Liposome swelling assays showing comparative diffusion of glucose through the OprB porin of *P. aeruginosa* and glucose-induced outer membrane proteins from *P. chlororaphis*, *B. cepacia*, and *P. fluorescens*. The rates of glucose diffusion were determined by measuring the swelling rates (decrease in optical density at 400 nm) of reconstituted proteoliposomes made from egg phosphatidylcholine, dicetylphosphate, and 50 μg of each protein. The decrease in optical density at 400 nm upon the addition of proteoliposome suspension to 40 mM glucose solution is plotted against time (in seconds). Liposome suspension made without protein was used as a negative control. Each point is an average of triplicate determinations. ■, negative control; ◇, *P. aeruginosa* OprB; ✶, *B. cepacia* OMP; ○, *P. chlororaphis* OMP; ✳, *P. fluorescens* OMP.

Diffusion of Different Carbohydrates through the Porin Channels

Elucidation of the specificity of porin channels is crucial to understanding their *in vivo* physiological function. Substrate-selectivity studies of the isolated channels would show whether they are substrate-selective or just general diffusion channels. Measurement of the rates of diffusion of carbohydrates of different sizes through the individual porin channel in reconstituted proteoliposomes by liposome swelling assays revealed that only glucose and fructose specifically diffused through the *P. chlororaphis* porin channel (Table II). The channel did not allow significant passage of maltose, sucrose, xylose, and arabinose. The *B. cepacia* porin facilitated the diffusion of arabinose and glucose and, to a limited extent, that of maltose and fructose (Table II). In the case of the *P. fluorescens* porin, the permeation rates of glucose, maltose and xylose were much higher than those of arabinose, fructose, and sucrose. Since carbohydrates of similar sizes diffused to a different extent, we suggest that these channels may be substrate-specific. In view of the fact that the diffusion rate of glucose through the three porins was much higher than for any other carbohydrate, we propose that these channels are all homologues of the OprB porin with different specificities for carbohydrates other than glucose. A comparison of the porin data with the whole-cell glucose uptake and inhibition data showed that glucose is not only the preferred substrate for the transport system but it also binds the isolated porin component with high specificity. Although some of the porin data obtained for other sugars may differ from whole-cell uptake data, this is not unexpected in view of the fact that the binding protein-mediated transport system is a multicomponent system in which isolated components may have different specificities distinct from the specificity of the entire transport system.

Secondary Structure Estimation

High-resolution 3-D structures of several porin proteins have been determined by X-ray diffraction (Cowan, 1993; Schirmer *et al.*, 1995). Porins form trimers in which each monomer consists of a giant 16-stranded antiparallel β-barrel with a pore running through the barrel. In detergent solutions, porin circular dichroism spectra indicate β-sheet contents between 30% and 54% (Park *et al.*, 1992). Estimation of the secondary structure contents of column-purified glucose-selective porins by convex constraint algorithm (CCA) analysis of circular dichroism spectra (Figs. 3A, B, C) of the proteins revealed high β-sheet contents (31–50%) for each isolated porin (Table III). The pure component curves generated by CCA spectral analysis are shown in Fig. 3D. Although this may be an underestimate of the β-sheet content as spectra collected between 200 and 240 nm can be dominated by the strong helical CD bands, the secondary structure values obtained are comparable to those of the OprB porin of *P. aeruginosa* (Wylie *et al.*, 1993). CD analysis of the OprB porin of *P. putida* has also suggested a high β-sheet content (Saravolac *et al.*, 1991). The predominance of β-sheets for each porin is consistent with protein having β-barrel structure.

Table II. Relative Diffusion of Various Sugars through the Glucose-Inducible Proteins of *P. chlororaphis*, *P. fluorescens*, and *B. cepacia*[a]

Sugar	Molecular weight	*P. chlororaphis* protein	*P. fluorescens* protein	*B. cepacia* protein	*P. aeruginosa* OprB[b]
D-xylose	150	16	81	17	123
L-arabinose	150	12	11	120	~85
D-glucose	180	100	100	100	100
D-fructose	180	87	25	34	ND
D-maltose	360	16	60	52	ND
Sucrose	360	08	19	10	<10

[a] Observed rates for all carbohydrates were normalized to the diffusion rate for D-glucose (100%) and data are representative of at least three independent measurements.

[b] From Trias *et al.* (1988); ND, not determined.

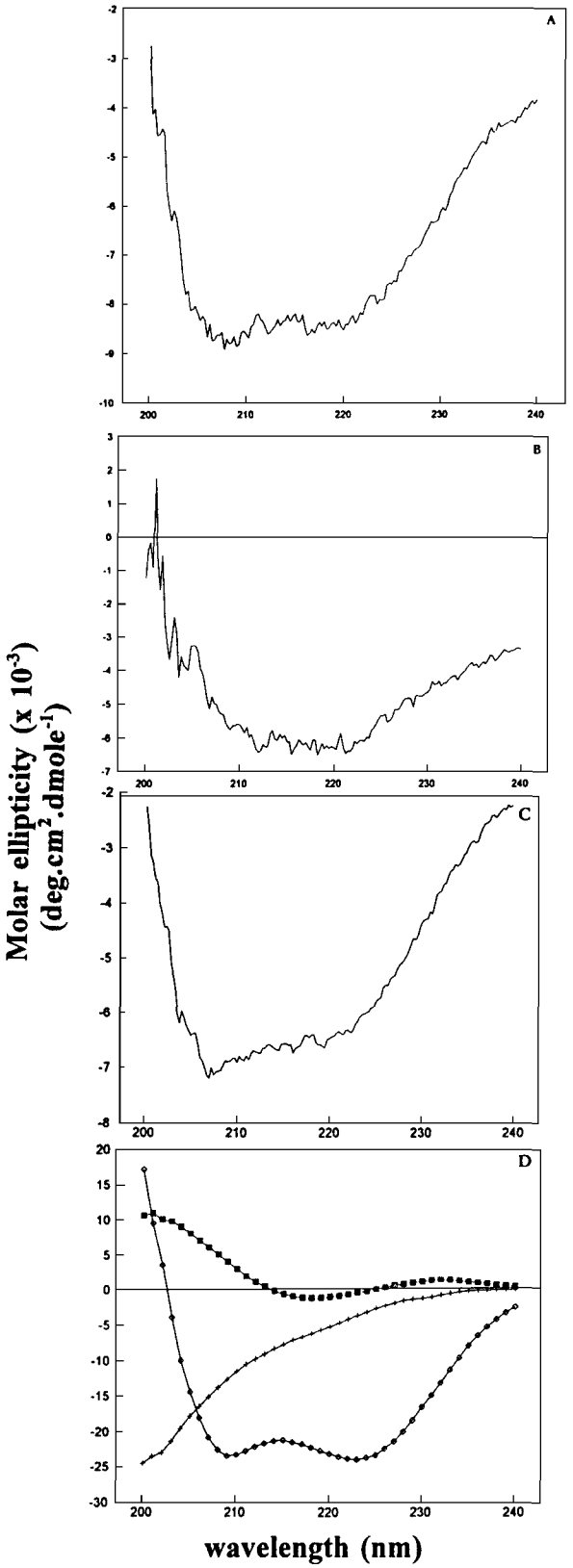

Table III. Secondary Structure Content of the Glucose-Inducible Outer Membrane Proteins from *P. aeruginosa*, *P. chlororaphis*, *B. cepacia*, and *P. fluorescens*[a]

Porin	M_r (kDa)	α-helix	β-sheet	Random coil
P. aeruginosa OprB[b]	47	25%	40%	34%
P. chlororaphis OMP	45	25%	31%	44%
B. cepacia OMP	40	19%	50%	31%
P. fluorescens OMP	45	19%	42%	40%

[a] Data are obtained from circular dichroism spectral analysis of purified proteins using the convex constraint algorithm (CCA) of Perczel *et al.* (1992). Spectral data are typical of five independent determinations.

[b] From Wylie *et al.* (1993).

DISCUSSION

These studies demonstrated the existence of inducible high-affinity glucose transport systems which facilitate glucose passage across the cell envelope of *P. chlororaphis*, *P. fluorescens*, and in *B. cepacia* with K_m values of 0.3, 1.4, and 5 μM respectively. Comparable K_m values have earlier been reported for *P. aeruginosa* (Midgely and Dawes, 1973; Guymon and Eagon, 1974) *P. putida* (Tong, 1995), and *P. fluorescens* (Lynch and Franklin, 1978). The low K_m observed for each strain is suggestive of the existence of binding protein-mediated transport systems analogous to the *P. aeruginosa* and the *P. putida* glucose transport systems. It was noted that the V_{max} for induced glucose uptake was higher for *Burkholderia cepacia* than for other strains examined. This is probably due to the fact that *B. cepacia* is the most nutritionally versatile as it is able to transport, accumulate, and metabolize a wide variety of carbohydrates better than members of the genus *Pseudomonas* (Lessie and Phibbs, 1984) Yabuuchi *et al.*, 1992). The transport systems in the three strains appeared to be inducible

Fig. 3. Secondary structure analyses of glucose-inducible porins. Circular dichroism (CD) spectra of porins from *P. chlororaphis*, *B. cepacia*, and *P. fluorescens* are shown respectively in (A), (B), and (C). Each spectrum was obtained from a Jasco J-500A spectropolarimeter using a 0.5-cm cuvette at a protein concentration of 100 μg/ml in 10mM Tris.HCl, 0.1% SDS. (D) Pure component curves generated by the convex constraint algorithm (CCA) analyses of the individual porin spectra: ◇, α helix; ■, β sheet; †, random.

by glucose which (when available in 50-fold molar excess) is able to effectively block the uptake of radiolabeled glucose in agreement with previous findings that the *P. aeruginosa* glucose transport system is highly specific for glucose (Wylie and Worobec, 1993). It is interesting to note that L-arabinose significantly inhibited whole-cell glucose uptake in *P. fluorescens* (45% inhibition) and *P. chlororaphis* (50.6% inhibition) even though D-xylose, of the same molecular weight, did not affect the uptake. The implication of this finding is that the glucose transport systems in the two *Pseudomonas* spp. are probably utilized to a certain extent to transport L-arabinose while D-xylose has its own specific transport system. Glucose uptake inhibition profile for fructose, maltose, and ribose is similar in *P. chlororaphis* and *B. cepacia*. The uptake inhibition pattern for *P. fluorescens* determined in this study is similar to the *P. aeruginosa* data previously reported (Wylie and Worobec, 1993), suggesting that of all the three systems examined in this study, these two systems are the closest in terms of the specificity of the uptake pathway for glucose.

Examination of the outer membrane carbohydrate-inducible proteins of *B. cepacia* and the previously reported glucose-inducible proteins from *P. fluorescens* and *P. chlororaphis* revealed that these proteins indeed form outer membrane diffusion channels with very high specificity and selectivity for glucose but varying specifity for carbohydrates other than glucose. Considering the relative rates of diffusion of the various carbohydrates through reconstituted proteoliposomes of these porins, the *P. chlororaphis* porin appeared selective for both fructose and glucose while the *P. fluorescens* porin showed high specifity for glucose, maltose, and xylose. On the other hand, the *B. cepacia* porin revealed a preference for glucose, arabinose, and maltose. It is noteworthy that the permeation rates of the different carbohydrates through the porin is not directly proportional to their molecular weights. The *P. fluorescens* porin behaved in a similar manner to the reported channel properties of the OprB porin of *P. aeruginosa* which is also specific for both glucose and xylose (Trias *et al.*, 1988). In terms of the channel sizes, calculated on the basis of the glucose permeation rates, for the isolated porins and the well-characterized OprB porin of *P. aeruginosa*, it appears that the order is *B. cepacia* > *P. aeruginosa* > *P. fluorescens* > *P. chlororaphis*. The implication of these data is that all the glucose-inducible porins are substrate-selective porins having different carbohydrate-binding sites. The finding that the *E. coli* maltose-binding protein does not bind glucose even though maltose and maltodextrins are bound (Spurlino *et al.*, 1991) suggests that the glucose-binding sites in glucose-inducible porins may not necessarily be organized to favor the binding of other carbohydrates.

Investigation of the permeability properties of outer membrane proteins often provides crucial information about their physiological roles. Liposome swelling assays demonstrated the porin functions of the three glucose-inducible proteins reconstituted in proteoliposomes. The *B. cepacia* outer membrane protein was capable of facilitating the diffusion of glucose and a few other carbohydrates. It is interesting to note that while the porin facilitated the diffusion of arabinose, this sugar did not inhibit whole-cell glucose uptake. Considering the significant extent of diffusion through the isolated porin, it is tempting to speculate that the outer membrane glucose-diffusion pore exists in more than one form depending on the presence or absence of the other components of the transport system, most probably the periplasmic GBP. Like the maltose transport system of *E. coli* where the LamB porin is known to physically interact with the periplasmic maltose-binding protein (MBP) (Bavoil and Nikaido, 1981), it is possible that the interaction of the periplasmic receptor with the porin changes the conformation of the carbohydrate-binding site in such a way that arabinose can bind and diffuse through the isolated porin but cannot bind to the GBP-modified form of the porin in whole cells. The fact that *B. cepacia* can utilize arabinose as sole carbon source suggests that it has its own transport system separate from the glucose transport system. Fructose and maltose, on the other hand, seem to be transported to a certain extent through the glucose transport system of *B. cepacia*. Data obtained from isolated porin and whole-cell glucose uptake inhibition are consistent with this deduction. The rest of the sugars tested did not appear to utilize the *B. cepacia* glucose transport system.

In the case of the *P. fluorescens* glucose transport system, the level of glucose uptake inhibition and diffusion rate of maltose through the isolated porin suggest that this system provides another route of entry for maltose in *P. fluorescens*. Possible conformational change in the porin due to the periplasmic GBP would also account for lack of glucose uptake inhibition by xylose and its facilitated diffusion through the isolated porin. Comparison of the glucose uptake inhibition and liposome swelling data reveals that despite the specificity of the isolated porin for glucose and fruc-

tose, possible modification of the carbohydrate-binding site by GBP reduces the specificity of the entire glucose transport system for fructose although this sugar can still be utilized to some extent via this route. The profile observed for the rest of the sugars tested suggests that the entire glucose transport system has a unique specificity which may differ from the specificity of the isolated outer membrane component. Although purely speculative, these findings suggest that the interaction of the porin component of the high-affinity glucose transport system with other component(s) of the system probably modifies the carbohydrate-binding site within the porin channel. This view is corroborated by previous observation that the sugar diffusion rates through isolated *E. coli* LamB channel differ significantly from corresponding rates in intact cells probably due to the interaction between the LamB channel and the periplasmic maltose-binding protein (Bavoil and Nikaido, 1981).

The two outer membrane proteins from *P. fluorescens* and *P. chlororaphis* have earlier been shown to be glucose-inducible even though the *P. fluorescens* protein did not hybridize with OprB porin-specific polyclonal antiserum (Wylie and Worobec, 1994). Secondary structural analysis of these proteins revealed extensive β-sheet contents similar to the secondary structure conformation of the OprB porin of *P. aeruginosa* and *P. putida* (Saravolac *et al.*, 1991, Wylie *et al.*, 1993) and are within the range determined for other bacterial porins. This secondary structural feature would suggest that the proteins form very stable transmembrane channels.

In light of the above findings, we propose that the binding protein-mediated glucose transport system in the genus *Pseudomonas* is very well conserved in terms of their glucose preference but differ in their specificity for carbohydrates other than glucose. This feature is probably due to the different conformations of the carbohydrate-binding sites for the different glucose-inducible porins.

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