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Reevaluation, Using Intact Cells, of the Exclusion Limit and Role of Porin OprF in *Pseudomonas aeruginosa* Outer Membrane Permeability

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Earlier studies that used model membrane reconstitution methods have come to different conclusions regarding the exclusion limit of the outer membrane of *Pseudomonas aeruginosa* and whether OprF is the major channel-forming protein in the outer membrane. In this study, a 6.2-kbp *SalI* fragment, encoding only two cytoplasmic enzymes, α-galactosidase and sucrose hydrolase, and the inner membrane raffinose permease, was cloned behind the *m*-toluate-inducible *tol* promoter of vector pNM185 to create plasmid pFB71. *P. aeruginosa* strains harboring pFB71, when grown with inducer, produced both enzymes encoded by the insert and had acquired the ability to grow on the disaccharide melibiose and the trisaccharide raffinose. The rate of growth was dependent on the concentration and size of the saccharide and was decreased three- to fivefold by the absence of OprF, as examined by measuring the growth on melibiose and raffinose of an isogenic OprF-deficient Ω insertion derivative, H636(pFB71). At high concentrations, di-, tri-, and tetrasaccharides could pass across the outer membrane to plasmolyze *P. aeruginosa*, as measured by light scattering and confirmed by electron microscopy. The initial rate kinetics of light-scattering changes were dependent on the size of the saccharide being used. Furthermore, the rates of change in light scattering due to raffinose and stachyose uptake across the outer membrane for strain H636 were fivefold or more lower than for its OprF-sufficient parent H103. These data are consistent with model membrane studies showing that OprF is the most predominant porin for compounds larger than disaccharides in *P. aeruginosa* and suggest that the exclusion limit for this porin and the outer membrane is greater than the size of a tetrasaccharide. In addition, these data confirmed the existence of other porins with a predominant function in monosaccharide uptake and a more minor function in the uptake of larger saccharides.

The outer membranes of gram-negative bacteria such as *Pseudomonas aeruginosa* are permeability barriers perforated by water-filled channels which determine the size-exclusion limit for hydrophilic compounds (9, 22). These channels are formed by a class of transmembrane proteins called porins (9, 22). The porin protein F (subsequently renamed OprF) of *P. aeruginosa* was isolated and reconstituted into liposomes and shown in equilibrium assays (for which the rate of permeation was not considered) to form channels that were permeable to dextrans with diameters of up to 2 nm (11). In contrast, *Escherichia coli* and *Salmonella typhimurium* under the same conditions were found to possess porins which excluded tetrasaccharides (diameter, 1.17 nm) (11, 16). The data for *P. aeruginosa* porin OprF seemed at first to contradict data indicating that this bacterium had a high degree of intrinsic resistance to antibiotics compared with *E. coli* and *S. typhimurium* (11) as well as subsequent data showing that *P. aeruginosa* had low outer membrane permeability (2, 17, 37). Since OprF was an abundant protein in *P. aeruginosa*, it was proposed that despite its large channel exclusion limit, OprF functioned poorly in uptake of substrates like antibiotics (5, 11). Results consistent with this proposal were provided by both black lipid bilayer (5) and liposome-swelling (19, 38) model membrane studies. Two separate hypotheses were proposed to explain the poor functioning of OprF channels. These were molecular heterogeneity, with individual OprF molecules possessing either large channels (frequency of <1%) or small channels (frequency of >99%) (32), and the specific geometry of OprF channels (19). Since 1986, Nakae and collaborators have produced a series of papers which concluded that the *P. aeruginosa* outer membrane prevents uptake of even disaccharides (8, 33–36), that OprF is not a porin (8, 36), and that the actual porins of *P. aeruginosa* are three proteins named C, D, and E (36). The methodologies that were used to arrive at these conclusions involved both liposome-swelling methodologies (8, 35, 36) and plasmolysis experiments (25, 26). Although each of these studies has been specifically criticized on the basis of flawed methodology (18, 19, 27), it is disturbing that the same technique, i.e., liposome swelling, in the hands of two different groups, could give rise to such different results (36, 38). In addition, OprF-deficient strains generated by chemical (8, 17) or molecular genetic (30) means had either no change or small changes in antibiotic susceptibility and thus failed to support the conclusion that OprF is the major porin protein of *P. aeruginosa*. On the other hand, this lack of change in antibiotic susceptibility was proposed to be due to the substantial alterations in cell permeability, shape, and growth characteristics resulting from the role of OprF in outer membrane and cell structure (8, 31). Therefore, we decided to reexamine this problem by using in vivo experimental protocols to eliminate potential artifacts caused by model membrane studies. The results obtained suggest that the *P. aeruginosa* outer membrane permits the permeation of sugar molecules as large as raffinose (a trisaccharide) or stachyose (a tetrasaccharide) and further suggest that OprF

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is the major but not necessarily the only channel involved in uptake of these saccharides.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. aeruginosa* PAO1 auxotrophic strain H103 (30) was used as the OprF-containing wild-type strain. *P. aeruginosa* H636 was an *oprF*::Ω derivative of strain H103 created by gene replacement with an Ω fragment-mutated *oprF* gene (30). *E. coli* DH5α F′ [F′ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* λ⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA argF*) *U169*] (30) was used for primary cloning, whereas *E. coli* SM10 (28), which contains a mutated RP1 plasmid integrated into the chromosome, was utilized for biparental mating of plasmids from *E. coli* to *P. aeruginosa*. *E. coli* DS25-91 (*lacY melB metB rpsL rafR*) (3) containing the raffinose utilization, 130-kbp plasmid pRSD2-1 with a constitutive mutation was obtained from R. Schmitt (Lehrstuhl für Genetik, Universität Regensburg, Regensburg, Germany). A 6.2-kb *Sal*I fragment from pRSD2-1 (*rafR* [Raf^c] Tra$^+$ InI) has been fully sequenced (GenBank accession number, M27273) and shown to encode only three structural genes, *rafA* (α-galactosidase), *rafB* (inner membrane raffinose permease), and *rafD* (sucrose hydrolase), and a truncated gene, *rafR* (a repressor gene) (3). This fragment was excised from pRSD2-1 and inserted into the *Sal*I site of the *m*-toluate- or benzoate-regulated expression vector pNM185 (Km^r Sm^r *p*mTOL *mob*$^+$ *tra*$^+$) (15) to create pFB71. To create a unique *Sal*I site in plasmid pNM185, an *Eco*RI-*Sal*I adapter had to be inserted into the unique *Eco*RI site of this plasmid. Plasmid pFB71 was used to transform *E. coli* SM10, with selection for plasmid-specific antibiotic resistance markers and growth on raffinose, and then transferred to strains H103 and H636 by conjugation, using the *mob* function encoded on the vectors (3, 7) and *tra* genes inserted into the chromosome of SM10 (28).

Strains were routinely grown on Luria broth medium (0.8% Bacto Tryptone, 0.5% yeast extract), containing 150 mM NaCl, solidified when appropriate with 2% (wt/vol) Bacto agar (30). For experiments involving growth on specific carbon sources, *E. coli* strains were grown on AB minimal medium (6) whereas *P. aeruginosa* strains were grown on BM2 minimal medium (2). For growth experiments, bacteria grown to mid-exponential phase on minimal medium with gluconate (*P. aeruginosa*) or glycerol (*E. coli*) as a carbon source were subcultured 1 in 50 into prewarmed fresh medium containing the specified levels of saccharides and grown with shaking at 37°C. Samples (1 ml each) were taken at regular intervals for A_{600} measurements. For measurement of growth K_m (i.e., the concentration of carbon source yielding one-half of the maximal rate of growth), the reciprocals of the initial growth rates were plotted against the reciprocals of the saccharide concentrations to give lines with a correlation coefficient r exceeding 0.92 (it must be pointed out that these data reflect outer membrane permeation only if this event is rate limiting for growth, as argued in references 20 and 37 and below). Growth K_m values were calculated from the intercept on the ordinate axis ($-1/K_m$). Raffinose and melibiose (>99.5% pure) were obtained from Sigma Chemical Co. (St. Louis, Mo.). The lack of significant contamination by smaller saccharides was revealed by the inability of *P. aeruginosa* and *E. coli* strains to grow on high levels of these saccharides unless they contained the plasmid pFB71.

Light-scattering experiments. Cells grown in BM2 medium containing 10 mM gluconate were collected by centrifugation at 3,000 × *g* and gently resuspended in 10 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.5) containing 5 mM $MgCl_2$ and 300 mosM concentrations of a variety of sugars. Light-scattering measurements were performed by placing 1 ml of the cells into the cuvette holder of a Perkin-Elmer 650-10S Spectrofluorimeter with the emission and excitation wavelengths set at 600 nm and the slit widths set at 2 nm. Light scattering in arbitrary units was recorded continuously for up to 60 min.

Electron microscopy. The cells were incubated in 300 mosM glucose, melibiose, raffinose, or stacchyose with and without 10 mM KCN for 30 min, after which glutaraldehyde was added to a final concentration of 2.5%. Control experiments revealed that KCN did not influence the rate of plasmolysis, as indicated by light-scattering experiments. After 3 h, the cells were washed and fixed in 1% osmium tetroxide for 2 h, dehydrated in an ethanol series (25, 50, 80, 95, and 100%), and then embedded in Araldite. Thin sections were cut with a Reichert Ultramicrotome OM U4 Ultracut, collected on carbon-coated copper grids, and poststained with uranyl acetate and lead citrate. All samples were examined with a Zeiss EM 10C transmission electron microscope operating at 60 kV.

Enzyme assays. Levels of sucrose hydrolase and α-galactosidase activities were determined in exponentially grown cells in the appropriate minimal medium (Table 1) with or without inducer (0.1 and 5 mM *m*-toluate for *E. coli* and *P. aeruginosa*, respectively). Crude extracts were prepared by sonicating the cells three times for 30 s at 0°C with a small probe of a Fisher sonic dismembrator (model 300) at a relative output level of 35%. Sucrose hydrolase and α-galactosidase activities were measured as described elsewhere (24, 25).

RESULTS AND DISCUSSION

Growth on raffinose. Woodruff and Hancock (30, 31) previously suggested that the major structural defects in the *P. aeruginosa* outer membrane, resulting from loss of OprF in strain H636::Ω, precluded definitive conclusions regarding the role of OprF in antibiotic susceptibility. Several antibiotics, including β-lactams, have been suggested to be able to pass through the outer membrane by using nonporin pathways (10) which could have compensated in part for loss of the porin pathway (30). We reasoned, however, that hydrophilic oligosaccharides would not be able to pass through the outer membrane by using nonporin pathways. Thus, if OprF was really the major porin of *P. aeruginosa*, its loss should cause an alteration in growth K_m for saccharides. Unfortunately, *P. aeruginosa* is unable to grow on any saccharides larger than a monosaccharide, a result that could reflect a monosaccharide exclusion limit for the outer membrane, as proposed by Yoneyama et al. (33, 34), or could reflect a lack of suitable enzymes. Therefore, we attempted to provide the appropriate metabolic capabilities by cloning a raffinose utilization operon from *E. coli* plasmid pRSD2-1 into *P. aeruginosa*. The 6.2-kb *Sal*I fragment cloned has been fully sequenced and contains only a part of the *rafR*-encoded repressor in addition to the genes for two cytoplasmic enzymes, α-galactosidase and sucrose hydrolase, and one integral inner membrane permease (7). Originally the fragment was cloned into the expression vector pVDtac (6) behind the *tac* promoter in plasmid pFB15. Although results analogous to those described below were obtained (as briefly reviewed in reference 4), the poor expression of the *tac*

TABLE 1. Sucrose hydrolase and α-galactosidase activities of *E. coli* DH5α(pFB71) and *P. aeruginosa* H103(pFB71) and H636(pFB71)

Carbon source[a]	Sucrose hydrolase activity (μmol of glucose liberated/min/mg of protein)[b]			α-Galactosidase activity (μmol of *p*-nitrophenol liberated/min/mg of protein)[c]		
	DH5α(pFB71)	H103(pFB71)	H636(pFB71)	DH5α(pFB71)	H103(pFB71)	H636(pFB71)
Control[d]	0.5 ± 0.1	1.9 ± 0.3	1.6 ± 0.4	3.5 ± 0.4	4.9 ± 0.1	5.1 ± 0.9
Melibiose	0.8 ± 0.2	2.5 ± 0.4	2.6 ± 0.5	4.1 ± 0.6	9.3 ± 0.8	8.9 ± 0.2
Raffinose	0.9 ± 0.1	3.1 ± 0.4	3.3 ± 0.3	4.5 ± 0.4	8.8 ± 0.6	9.5 ± 0.8

[a] *E. coli* DH5α(pFB71) cultures contained 0.1 mM *m*-toluate as an inducer. *P. aeruginosa* H103(pFB71) and H636(pFB71) cultures contained 5 mM *m*-toluate as an inducer, since 0.1 mM *m*-toluate failed to induce detectable levels of either enzyme. For *E. coli* DH5α(pFB71), 5 mM *m*-toluate was toxic. The concentrations of carbon sources used were 50 mM gluconate, glycerol, and melibiose or 100 mM raffinose.

[b] Means ± standard deviations of three independent assays of specific activity expressed as micromoles of glucose liberated per minute per milligram of protein at 37°C. Since the extracts used all contained cell-derived glucose, background concentrations (approximately 30 to 40% of the given numbers) in plasmid-free or uninduced cultures were subtracted.

[c] Means ± standard deviations of three assays of specific activity expressed as micromoles of *p*-nitrophenol liberated per minute per milligram of protein at 37°C.

[d] α-Galactosidase activities were measured after growth on gluconate, since this was the control carbon source used in growth experiments. However, gluconate interfered with sucrose hydrolase assays, so these were done after growth in glycerol (*E. coli*) or succinate (*P. aeruginosa*).

promoter in *P. aeruginosa* in minimal medium (14) resulted in very slow growth on sugars and long (10 to 24 h) lag times. Therefore, we recloned the 6.2-kb *Sal*I fragment described above behind the *m*-toluate-inducible *tol* promoter in plasmid pNM185. The resulting plasmid pFB71 permitted *E. coli* DH5α to express α-galactosidase and sucrose hydrolase under inducing conditions (Table 1) and to grow on raffinose (Table 2).

After transfer of plasmid pFB71 into *P. aeruginosa* H103, substantial levels of both sucrose hydrolase and α-galactosidase were induced when this strain was grown in succinate or gluconate with 5 mM *m*-toluate added (Table 1). Lower levels of toluate (i.e., 0.1 mM) failed to induce these plasmid-encoded enzymes. Strain H103 containing the plasmid had acquired the ability to grow on the disaccharides melibiose and raffinose (Fig. 1), whereas the plasmid-lacking strain H103 (Fig. 1) or strain H103 containing the vector pNM185 (data not shown) did not. In contrast, plasmid pFB71 did not influence growth on gluconate (Fig. 1). Consistent with these observations, strain H103(pFB71) growing on raffinose or melibiose in the presence of 5 mM *m*-toluate, as an inducer, produced high levels of both sucrose hydrolase and α-galactosidase (Table 1). Acquisition of the ability of H103(pFB71) to grow on melibiose and raffinose implied that both di- and trisaccharides could pass across the outer membrane, since the insert in pFB71 contained only an inner membrane permease and two cytoplasmic enzymes. Consistent with this interpretation, we were unable to identify any enzyme activities in the cell supernatant. Furthermore, at fixed concentrations of saccharides (50 mM), strain H103(pFB71) grew faster on the disaccharide melibiose than on the trisaccharide raffinose (Fig. 1), a finding interpreted as resulting in part from the sizes of these saccharides relative to the sizes of porin channels in the outer membrane.

To test the role of OprF in sugar transport, plasmid pFB71 was introduced into strain H636, an OprF-deficient, Ω insertion mutant of strain H103. H636(pFB71), grown in the presence of 5 mM *m*-toluate as an inducer, expressed levels of sucrose hydrolase and α-galactosidase that were indistinguishable from those of strain H103(pFB71) (Table 1). Strain H636(pFB71) grew at the same rate as strain H103(pFB71) on minimal gluconate medium over a 50-fold range of gluconate concentrations (see Fig. 3). This suggested that porins other than OprF were predominant in gluconate passage across the outer membrane. In contrast, H636 (pFB71) grew more slowly than H103(pFB71) on both raffinose (Fig. 2 and 3) and melibiose (Fig. 3). At growth rate-limiting concentrations, the rate of growth of the OprF-deficient mutant was only 20% (for raffinose) to 33% (for melibiose) that of its OprF-containing parent strain, and

TABLE 2. Growth K_m on different carbon sources for *E. coli* and *P. aeruginosa* strains

Carbon source	Growth K_m (mM)[a]		
	E. coli DH5α(pFB71)	*P. aeruginosa* H103(pFB71)	*P. aeruginosa* H636(pFB71)
Gluconate	0.025	0.150	0.161
Melibiose	0.320	13	35
Raffinose	67	98	>150[b]

[a] Growth K_m, defined in reference 20, is the concentration of carbon source resulting in a 50% maximal rate of growth, taken from results like those shown in Fig. 3.

[b] Because of the toxic effect on *P. aeruginosa* growth of concentrations of raffinose of >200 mM, it was not possible to determine accurately the growth K_m in this case, for which only three datum points were available (Fig. 3).

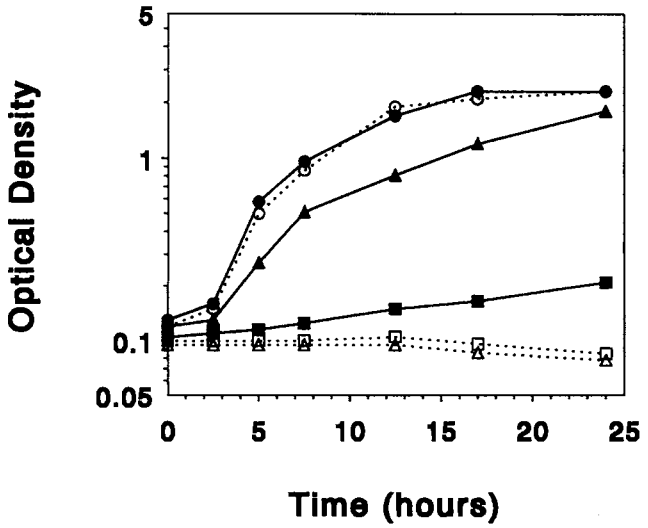

FIG. 1. Growth of *P. aeruginosa* H103 with (solid lines) or without (broken lines) the raffinose utilization plasmid pFB71. Growth substrates (at 50 mM) were gluconate (circles), melibiose (triangles), and raffinose (squares).

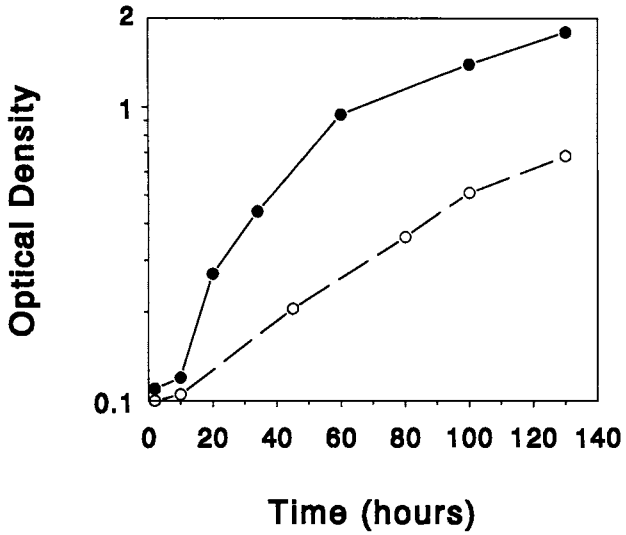

FIG. 2. Growth of strain H103(pFB71) (solid line) and its *oprF*::Ω mutant H636(pFB71) (broken line) on 100 mM raffinose.

these differences were statistically significant ($P < 0.05$ by Student's t test). These data were thus consistent with outer membrane permeation being rate limiting for growth on melibiose and raffinose and further suggested that OprF was the predominant (but presumably not the only) porin involved in passage of these saccharides across the outer membrane of *P. aeruginosa*.

As the initial concentration of either melibiose or raffinose in the medium was increased, the growth rate increased (Fig. 3) for both strains H103(pFB71) and H636(pFB71). It must be noted that the observed growth rates on 5 mM melibiose and on 50 mM raffinose were quite low. This is consistent with the overall low degree of outer membrane permeability of *P. aeruginosa*, i.e., 12- to 100-fold lower than that of *E. coli* (cf. the 40-fold lower growth K_m on melibiose; Table 2). However, the growth rates for the two strains converged with increasing saccharide concentrations and eventually

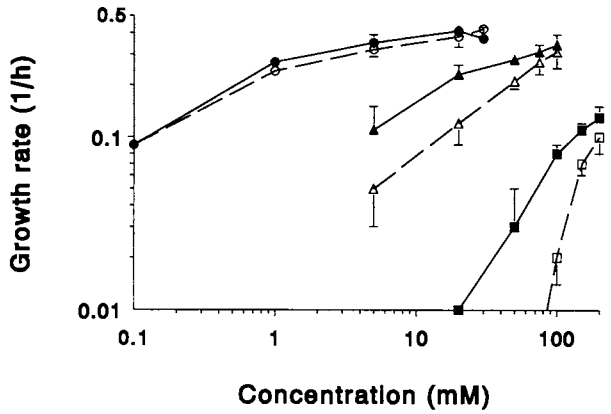

FIG. 3. Influence of growth substrate concentration on the rate of growth of H103(pFB71) (solid lines) and its *oprF*::Ω mutant H636(pFB71) (broken lines) on gluconate (circles), melibiose (triangles), and raffinose (squares). In each case, 5 mM *m*-toluate was added to induce the *tol* promoter of plasmid pFB71. The means of three independent experiments with standard deviations (error bars) are presented.

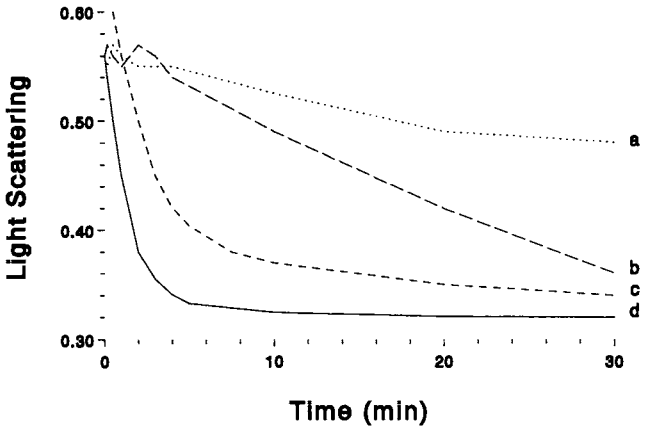

FIG. 4. Changes in light scattering over time after resuspension of *P. aeruginosa* H103 in 300 mosM stachyose (curve a), raffinose (curve b), melibiose (curve c), and glucose (curve d). Cells resuspended into buffer gave a light-scattering value of 0.33, which did not change over time.

became not significantly different at 100 mM melibiose (and within 15% of the maximal growth rate on gluconate; Fig. 3). This is predictable if outer membrane permeation through OprF ceased to become rate limiting at high saccharide concentrations, at which other minor porins could substitute for OprF. In addition, it confirmed that strain H636(pFB71) did not have any intrinsic energization or metabolic defects influencing growth rates. This was in agreement with the similar rates of growth of H103 and H636 on gluconate minimal medium (Fig. 3), Mueller-Hinton medium, or proteose peptone supplemented with 200 mM KCl, sucrose, or potassium succinate (30, 31) and the similar MICs for gentamicin (30), which is strongly affected by alterations in cellular energization.

To independently confirm these results, we reexamined the data and conclusions of Yoneyama et al. (33, 34), who utilized cell plasmolysis by high concentrations of saccharides to conclude that *P. aeruginosa* excluded substrates larger than monosaccharides. In particular, we noted that these authors examined cells by electron microscopy after only 3 or 6 min, a time period that may be too short for following the slow diffusion of solutes across the *P. aeruginosa* outer membrane. We utilized light-scattering measurements to monitor the time course of the osmotic responses of these cells. When cells suspended in low-osmolarity medium (10 mM MOPS [pH 6.5]–5 mM $MgCl_2$) were exposed suddenly to higher-osmolarity medium (the same buffer containing 300 mosM sugar), there was an instantaneous increase in light scattering (from 0.32 to 0.57 arbitrary units on the scale shown in Fig. 4). This was caused by the increase in the average refractive index of cells brought about by the osmotic shrinkage of the cytoplasm (1). However, this increase in scattering could be caused by plasmolysis, accompanied by shrinkage of the cytoplasm alone or by the shrinkage of the whole cells, since the periplasm is in osmotic equilibrium with the cytoplasm (29). We propose that the latter was occurring with *P. aeruginosa* since there was a subsequent slow decrease in scattering over the 30-min period (Fig. 4). This could not be caused by the active uptake of cations such as potassium, because the rate of change of scattering was not affected by cyanide or azide. One plausible explanation would be that the sugars penetrate through the outer membrane, which becomes gradually

TABLE 3. Initial swelling rates of *P. aeruginosa* H103 and its OprF-deficient mutant H636 after resuspension in high concentrations of saccharides

Osmolyte[a]	Initial swelling rate[b]	
	H103	H636
Glucose	0.148 ± 0.014	0.138 ± 0.013
Sucrose	0.092 ± 0.010	0.068 ± 0.011
Melibiose	0.090 ± 0.012	0.066 ± 0.009
Raffinose	0.011 ± 0.002	0.002 ± 0.001
Stachyose	0.004 ± 0.001	<0.001

[a] Present at concentrations of 300 mosM.
[b] Taken from data like those shown in Fig. 4 and expressed as means ± standard deviations of four independent measurements of the initial change in light scattering (in arbitrary units) per minute.

detached from the shrunken cytoplasm and cytoplasmic membrane, creating situations more closely resembling the usual plasmolyzed cells. Indeed, Koch (13) has indicated that if a zone of strongly scattering substance became localized at the cell surface, this would decrease the total scattering of cells. Outer membranes scatter light very strongly, as can be seen by the brilliant white appearance of isolated outer membrane preparations. The extent of scattering is thus determined by the refractive-index difference between the scattering structure and the medium, and thus the smaller extent of scattering change observed for larger sugars (especially stachyose) may be due to the refractive index of stachyose solution, which is higher than that of, for example, glucose solution. This interpretation (increased light scattering due to shrinkage of whole cells followed by a gradual decrease in light scattering due to detachment of the outer membrane from the inner membrane leading to plasmolyzed cells) was supported by electron microscopy studies (see below). On the basis of this interpretation, the initial rate of decrease of scattering in Fig. 4 indicates the rate at which various sugars penetrate through the outer membrane. In the monosaccharide glucose, osmotic swelling occurred very rapidly, i.e., within the time span observed by Yoneyama and Nakae (34). However, osmotic swelling by the disaccharides sucrose and melibiose and the trisaccharide raffinose occurred at easily measurable but lower rates (Fig. 4; Table 3). Osmotic swelling with the tetrasaccharide stachyose occurred very slowly (Fig. 4; Table 3).

Comparison of measurements of the initial rate of osmotic swelling for strain H103 and its *oprF*::Ω derivative H636 demonstrated similar rates with the monosaccharide glucose but progressively greater differences as the size of the saccharide was increased (Table 3). Indeed, osmotic swelling of the OprF-deficient strain H636 by raffinose occurred at only 18% of the rate for the OprF-sufficient parent strain H103. This result was again consistent with a predominant role for OprF in outer membrane permeation of larger saccharides but not for the monosaccharide glucose.

The cells were examined by electron microscopy to confirm the validity of the interpretation of these light-scattering data. Yoneyama and Nakae (34) previously observed that, after 3 or 6 min, cells resuspended in stachyose, raffinose, and sucrose had a crushed appearance, whereas cells resuspended in ribose or α-methylmannoside showed plasmolysis. In contrast, we examined cells after 30 min to permit significant permeation of sugars across the outer membrane and consequent reswelling. In stachyose and raffinose, cells still had a slightly crushed appearance but clear evidence of plasmolysis for most cells was observed (Fig. 5C and D), suggesting that these saccharides could permeate across the outer membrane. In glucose and melibiose, cells demonstrated a more normal, rod-shaped outline but were less obviously plasmolyzed (Fig. 5A and B). Presumably, this reflected slow permeation of glucose and melibiose across the cytoplasmic membrane during the 30 min of the experiment. Consistent with this explanation, when strain H103 cells containing the plasmid pFB71 were incubated in stachyose for 60 min and examined, the cells had the same outline as those seen in Fig. 5D but none of them were plasmolyzed, presumably because of the ability of the raffinose permease encoded by pFB71 to transport stachyose across the cytoplasmic membrane (data not shown).

The data presented in this paper provide two separate lines of evidence, with intact cells, that the outer membrane of *P. aeruginosa* can permit the passage of saccharides much larger than monosaccharides, in contrast to the conclusions of Nakae and colleagues (33–35). Indeed, the exclusion limit in this study apparently equalled or exceeded the size of a tetrasaccharide. Furthermore, the data are consistent with previous conclusions that OprF is the predominant porin protein for the passage of large compounds (but not smaller compounds) across the outer membrane of *P. aeruginosa* (5, 11, 38) but disagree with specific model membrane studies suggesting that OprF has no porin activity (8, 35, 36). Indeed, these studies were previously criticized on technical grounds (19).

The ratio of the permeation rates of melibiose and raffinose through the outer membrane could be estimated from three different measurements presented here (assuming that permeation rather than metabolism is rate limiting for growth, as argued above), namely, the relative growth rates at 50 mM substrate (Fig. 3), the growth K_m values (Table 2), and the relative rates of osmotic swelling (Table 3). These ratios were quite consistent, being 9.0, 7.5, and 8.2, respectively (cf. a ratio of 3 to 4 obtained by in vitro liposome-swelling rates [19, 38]). With an average ratio of 8.2 for the permeation rates from this paper and estimates of the hydrated radii of melibiose and raffinose of 0.56 and 0.70 nm, respectively (26), application of the Renkin equation (20, 23) to these values resulted in an estimate of 1.56 nm for the diameter of the critical outer membrane channel (i.e., OprF). While this estimation is complicated with intact *P. aeruginosa* cells by the apparent existence of other porin proteins, the evidence presented here suggests that OprF is the most effective porin for disaccharides and trisaccharides; thus, this provides an in vivo estimate of the functional channel diameter of OprF porin. This value is larger than the estimated diameter for the *E. coli* OmpF channel (1.16 nm [20]) but somewhat less than previous estimates of 2 nm for the diameter of the OprF channel with model membrane studies (9). Nevertheless, given the uncertainties inherent in such estimates, as discussed by Jap and Walian (12), we feel that our results are consistent with previous data.

Interestingly, despite the rate of permeation of, for example, β-lactams across the outer membrane of *E. coli*, which is 10- to 100-fold higher than that of *P. aeruginosa*, there was little difference in the growth K_m values of these two species on raffinose (Table 2), which approaches the exclusion limit of *E. coli* porin OmpF (9, 16). This is consistent with previous suggestions that the maximal exclusion limit of *P. aeruginosa* OprF is substantially greater than that of *E. coli* OmpF (5, 9, 11, 19, 38), since permeation through a larger channel would be less affected by substrate size. Similar growth K_ms for H103(pFB71) and H636(pFB71) on gluconate implied that OprF was not the predominant porin for

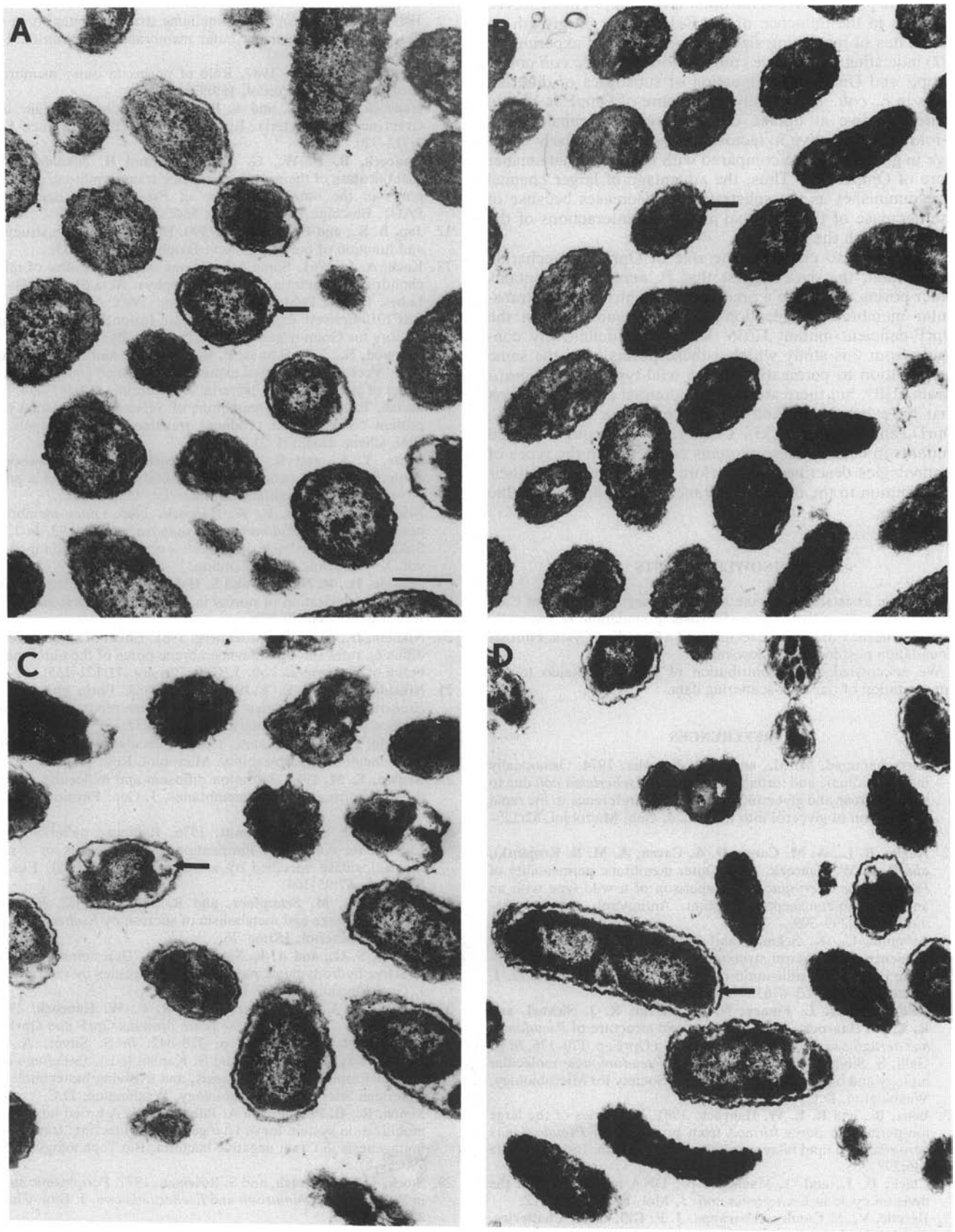

FIG. 5. Electron micrographs of thin sections of *P. aeruginosa* H103 cells showing plasmolysis (arrows) after incubation for 30 min in 10 mM KCN and 300 mosM glucose (A), melibiose (B), raffinose (C), or stachyose (D). The bar in panel A represents 0.25 μm.

gluconate passage across the outer membrane. The observed increase in the influence of OprF deficiency on growth on substrates of increasing size is consistent with experiments (21) indicating the relative contributions of the *E. coli* porins OmpF and OmpC to permeation of substrates of different sizes in *E. coli*. The slightly larger pore of OmpF is 10-fold more effective at uptake of the antibiotic cephaloridine, 2-fold more effective in lactose uptake, and similarly effective in glucose uptake compared with the somewhat smaller pore of OmpC (21). Thus, the advantage of larger channel size diminishes as the substrate size decreases because of the decrease of the frictional and steric interactions of the substrate with the channel.

In addition to clarifying the role of OprF in saccharide permeation, the data suggest that *P. aeruginosa* contains other porins that make a predominant contribution to trans-outer membrane permeation of smaller substrates in the OprF-deficient mutant H636. We cannot definitively conclude from this study whether these porins have the same contribution to permeation in the wild-type *P. aeruginosa* strain H103, but there are several potential candidate porins that have been identified to date, including OprB, OprC, OprD, and OprE (18, 33). Clearly, it will require defined mutants in each of these proteins coupled with the types of technologies described here before we can determine their contribution to the outer membrane permeability of specific substrates.

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