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Identification of Porins in Outer Membrane of *Proteus*, *Morganella*, and *Providencia* spp. and Their Role in Outer Membrane Permeation of β-Lactams

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***Proteus mirabilis*, *Proteus vulgaris*, *Morganella morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens*, which were once classified into the same genus, *Proteus*, were studied. Cefoxitin-resistant mutants from these species were isolated, and it was confirmed that the resistance was attributed to the lack of an outer membrane protein, resulting in a significant decrease in the penetration of hydrophilic cephalosporins through the outer membrane. Comparison of the mutant strains with their parental strains in the diffusion rates of six monoanionic cephalosporins, a zwitterionic cephalosporin (cephaloridine), and a divalent anionic cephalosporin (cephalosporin C) suggested that each species had only one kind of porin protein, with molecular weights of 40,000 (*Proteus mirabilis*) or 37,000 (the other four species) and that the porins formed channels with cation selectivity, except for *Proteus vulgaris*. Porin proteins were purified from all the bacterial species except *Providencia alcalifaciens*, and the radius of the pores formed by the purified porins was estimated by the use of the liposome swelling assay. The pore radii were estimated to be approximately 0.59 nm (*Proteus mirabilis*), 0.63 nm (*Proteus vulgaris*), 0.58 nm (*Providencia rettgeri*), and 0.60 nm (*M. morganii*), similar to the size of the pore radius of *Escherichia coli* porins.**

Proteus mirabilis, *Proteus vulgaris*, *Morganella morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens* were once classified into the same genus, *Proteus* (7), and, together with *Serratia marcescens* and *Pseudomonas aeruginosa*, are known as opportunistic pathogens. Except for *Proteus mirabilis*, these species show low susceptibility to many antibiotics. One of the reasons for such an intrinsic resistance may be the barrier effect of the outer membrane on antibiotic permeation. In the case of *Pseudomonas aeruginosa*, there is evidence suggesting that intrinsic resistance involves the outer membrane (1, 4, 32). However, the characterization of the outer membrane as the permeation route of antibiotics in the former *Proteus* species is incomplete. In our preliminary work (24), *Proteus mirabilis* N-51 was found to produce only a single major porinlike protein, with a molecular weight of 40,000. This 40K protein contributed to the bacterial susceptibility to cephalosporins and tetracycline. The present investigation was undertaken as an extension of the previous study to identify the porin proteins produced by *Proteus vulgaris*, *M. morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens* and to evaluate the porin pores of these four species and of *Proteus mirabilis* as permeation routes for β-lactam antibiotics.

MATERIALS AND METHODS

Bacterial strains and β-lactamase production. *Proteus mirabilis* N-51, *Proteus vulgaris* K22-2, *Providencia rettgeri* RE-18, and *Providencia alcalifaciens* IN-06 are clinical isolates which barely produce β-lactamase activity under usual growth conditions. *M. morganii* 1510/9, which is a mutant strain with lower β-lactamase activity, was isolated from strain 1510 (28). Mutant strains showing significant decreases in production of outer membrane protein were isolated on the basis of cefoxitin resistance (24). The cefoxitin concentrations used for selection were 25 μg/ml for *M. morganii* and 50 μg/ml for the other four species. The mutant strains were termed N-51C1, K22-2C1, RE-18C1, IN-06C1, and 1510/9C1, respectively. For measurement of outer membrane permeability of β-lactams using β-lactamase located in periplasmic space (26), plasmid RGN823 specifying TEM-type β-lactamase was transferred by conjugation to the parental and mutant strains of *Proteus mirabilis*, *M. morganii*, and *Providencia alcalifaciens*. In the case of *Proteus vulgaris* and *Providencia rettgeri*, the species-specific β-lactamase (2, 15, 16, 25) produced in the periplasmic space was used for the measurement. These β-lactamases are inducible enzymes; therefore, the enzymes were induced before cell harvest by using subinhibitory concentrations of 6-aminopenicillanic acid as inducer (100 μg/ml). It was confirmed that the induction treatment had no influence on the form of the bacterial cells and bacterial susceptibility to erythromycin (data not shown).

Antibiotics. The antibiotics used in this study were kindly provided by pharmaceutical companies as follows: benzylpenicillin and ampicillin, Meiji Seika Co., Tokyo, Japan; apalcillin, Sumitomo Chemical Co., Osaka, Japan; cefazolin and ceftezole, Fujisawa Pharmaceutical Co., Tokyo, Japan; cefoperazone and piperacillin, Toyama Chemical Co., Tokyo, Japan; cephaloridine and cefamandole, Shionogi Chemical Co., Osaka, Japan; cephalothin, Torii Pharmaceutical Co., Tokyo, Japan; cefoxitin, Merck Sharp & Dohme Research Laboratories, Rahway, N.J.; cephacetrile, tetracycline, and minocycline, Takeda Chemical Industries, Ltd., Osaka, Japan; chloramphenicol, Yamanouchi Pharmaceutical Co., Tokyo, Japan; erythromycin, Japan-Upjohn Co., Tokyo, Japan. Cephalosporin C (Sigma Chemical Co., St. Louis, Mo.) was commercially available. The hydrophilic character of the β-lactams was expressed by the R_f value, which was measured by reverse-phase thin-

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layer chromatography (24). The greater the R_f value, the higher the hydrophilicity of the molecules.

Preparation of outer membrane and purification of outer membrane proteins. Outer membrane proteins were purified from cell envelopes by a modification of the procedure of Tokunaga et al. (30). A brief description of the procedure follows. The membrane preparation obtained from mid-log-phase cells grown in 7 liters of L broth composed of 1% Proteose Peptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 0.1% glucose, and 0.5% NaCl was suspended in 70 ml of 10 mM Tris hydrochloride buffer (pH 7.2) containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and incubated at 35°C for 30 min. The insoluble fraction was separated as a pellet after centrifugation at 100,000 × *g* for 30 min. The pellet was suspended in 70 ml of 5 mM Tris hydrochloride buffer (pH 7.2) containing 1% (wt/vol) SDS and 5 mM EDTA and then incubated at 37°C for 18 h with gentle shaking. The suspension was centrifuged at 100,000 × *g* for 30 min, and the precipitate was suspended in 20 ml of 10 mM Tris hydrochloride buffer (pH 7.7) containing 1% (wt/vol) SDS, 0.4 M NaCl, 5 mM EDTA, and 0.05% (vol/vol) β-mercaptoethanol. Peptidoglycan-associated proteins were solubilized by incubation of the suspension at 37°C for 2 h, and the solubilized proteins were freed from insoluble materials by centrifugation at 100,000 × *g* for 30 min. The supernatant was concentrated to 2 ml with Ficoll-400 (Pharmacia Fine Chemicals, Piscataway, N.J.), dialyzed for 18 h against 10 mM Tris hydrochloride buffer (pH 7.7), and applied to a Sepharose-CL6B column (3.5 by 40 cm) equibrated with 10 mM Tris hydrochloride buffer (pH 7.7) containing 1% (wt/vol) SDS, 0.4 M NaCl, 5 mM EDTA, and 0.05% (vol/vol) β-mercaptoethanol. Fractions (2.5 ml) were collected and monitored for protein content at 280 nm. Those fractions containing 37K or 40K proteins were concentrated and dialyzed for 3 weeks against distilled water containing 3 mM NaN_3 at room temperature.

Protein assay. The protein content in the membrane preparation and extracts was determined by the method of Lowry et al. (8).

Determination of outer membrane permeability of β-lactams. Measurement of the outer membrane permeability to β-lactams was done as described previously (27). Permeability was expressed by the parameter *C* (cubic centimeters per minute per microgram [dry weight] of bacterial cells) (31).

Measurement of bacterial susceptibility to antibiotics. Bacterial susceptibility to antibiotics was measured by the agar dilution method and expressed as the MIC. An overnight culture of the bacterial strains in heart infusion broth was diluted 100-fold with fresh broth, and 5 μl of the bacterial suspension (about 5×10^6 cells) was inoculated onto agar plates, into which different concentrations of antibiotics had been incorporated by using a replicating device (Microplanter; Sakuma Factory, Tokyo, Japan). MICs were measured after incubation at 37°C for 18 h.

Liposome swelling assay. The permeation rate of various sugars through porin channels incorporated into reconstituted proteoliposomes was determined by the method of Luckey and Nikaido (9). Usually, 4 mg of egg L-α-phosphatidylcholine (Sigma) and 0.4 mg of dicetyl phosphate (Sigma) were dried from a chloroform solution to a thin film under N_2 at reduced pressure for 2 h and suspended in 0.5 ml of an aqueous solution containing one of the purified outer membrane proteins. The suspension was sonicated in a Branson bath-type sonicator and dried to a thin film at 40°C under N_2 at reduced pressure for 2 h. The dried phospholipid-protein mixture was suspended in 0.5 ml of 6% (wt/vol) dextran T-10 (Pharmacia Fine Chemicals) in 10 mM phosphate buffer (pH 7.0). The proteoliposomes (30 μl) were then diluted into 2 ml of an isotonic solution containing a sugar, and the permeation rate of the sugar was determined from the initial rate of change in optical density of liposomes at 450 nm. The results were normalized to the rate of swelling of proteoliposomes in D-glucose. Pore radius was estimated by computer-assisted nonlinear fitting to the Renkin equation (23). The program, named PORERAD, was written in BASIC and is obtainable from us.

RESULTS

Isolation of mutants lacking porin proteins from *Proteus*, *Morganella*, and *Providencia* spp. In previous work (24), we demonstrated that a porin-deficient mutant of *Proteus mirabilis* could be easily isolated on agar medium containing cefoxitin without the addition of any chemical mutagen. Using this method, we isolated mutants defective in outer membrane proteins corresponding to *Escherichia coli* porins from five strains of *Proteus*, *Morganella*, and *Providencia* species. Among the mutants used in this study, the isolation and characterization of *Proteus mirabilis* N-51C1 was reported in part previously (24).

SDS-polyacrylamide gel electrophoretic patterns of the outer membrane proteins from these mutants and their parental strains are shown in Fig. 1. Each mutant lacked only one protein band. The molecular weights of the missing proteins were 40,000 in *Proteus mirabilis* and 37,000 in the other four strains. Because these proteins could be solubilized in 1% SDS solution at 100°C but not at 40°C (data not shown), they seemed to be noncovalently associated with the peptidoglycan, similar to the porins of *E. coli* (5, 10–12, 29) and *Salmonella typhimurium* (11, 17, 21).

Each strain possessed another major outer membrane protein which was easily solubilized in 1% SDS solution even at 40°C. These proteins were heat-modifiable proteins, because the molecular weights of the proteins extracted at 40°C were about 32,000 but the proteins extracted at 100°C migrated at the positions for 38,000 (*M. morganii*), 41,000 (*Proteus mirabilis*), and 40,000 (*Proteus vulgaris*, *Providencia rettgeri*, and *Providencia alcalifaciens*) (data not shown).

The susceptibilities of the parental and mutant strains to β-lactams and other major antibiotics are shown in Table 1. The lack of the 40K protein (*Proteus mirabilis*) and the 37K proteins (*Proteus vulgaris*, *M. morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens*) resulted in a marked decrease in susceptibility to tetracycline and the cephalosporins, except the novel cephalosporin, cefoperazone. This result suggested that the outer membrane proteins are functionally similar to the OmpF porin of *E. coli* and play an important role at least in the outer membrane permeation of cephalosporins and tetracycline. On the other hand, little difference in the susceptibility to penicillins of the parental and mutant strains was observed. This result is similar to that observed for the wild-type strain and porin-deficient mutants of *E. coli* (31), suggesting some difference in the permeation routes for penicillins and cephalosporins.

Outer membrane permeability of cephalosporins. To confirm that the 40K and 37K proteins actually contributed to the outer membrane permeation of the antibiotics, the permeability of cephalosporins through the outer membrane was assayed and compared for the parental and mutant strains.

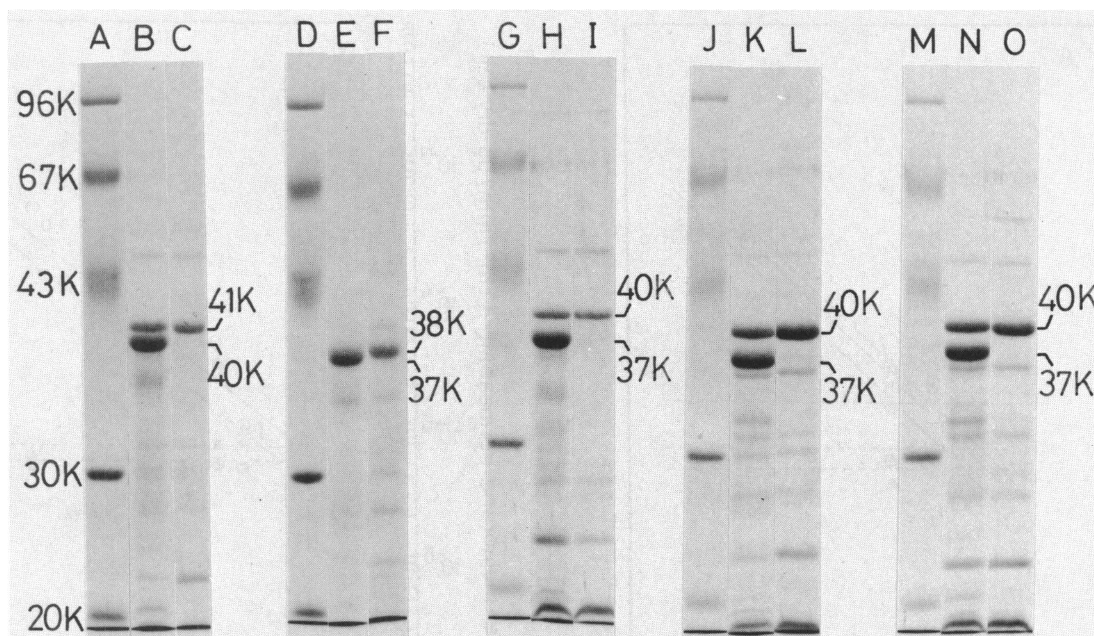

FIG. 1. SDS-polyacrylamide gel electrophoresis of outer membrane proteins. Proteins were prepared from sarcosyl-treated cell envelopes as described in the text and analyzed on an SDS-10% polyacrylamide slab gel. Molecular weight was estimated based on the standards included in the gel. The standards were phospholipase b (96K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and trypsin inhibitor (20K). Lanes: A, D, G, J, and M, standard proteins; B, *Proteus mirabilis* N-51; C, *Proteus mirabilis* N-51C1; E, *M. morganii* 1510/9; F, *M. morganii* 1510/9C1; H, *Proteus vulgaris* K22-2; I, *Proteus vulgaris* K22-2C1; K, *Providencia rettgeri* RE-18; L, *Providencia rettgeri* RE-18C1; N, *Providencia alcalifaciens* IN-06; O, *Providencia alcalifaciens* IN-06C1.

TABLE 1. Susceptibility of wild-type and C1 strains to antimicrobial agents[a]

Strain	MIC (μg/ml)																
	Cephalosporins									Penicillins				TC	MINO	CM	EM
	CEC	CTZ	CEZ	CMD	CET	CPZ	CER	CEP-C	CFX	APC	PCG	PIP	APL				
Proteus mirabilis																	
N-51	12.5	6.3	6.3	1.6	6.3	0.8	6.3	100	3.1	1.6	3.1	0.4	1.6	50	50	3.1	400
N-51C1	25	100	200	25	50	0.8	50	400	50	3.1	6.3	0.4	1.6	200	50	3.1	400
Proteus vulgaris																	
K22-2	800	400	400	200	200	1.6	400	400	3.1	800	800	0.8	6.3	25	6.3	3.1	800
K22-2C1	1,600	1,600	1,600	800	1,600	3.1	1,600	1,600	50	1,600	1,600	0.8	6.3	100	6.3	3.1	800
Morganella morganii																	
1510/9	25	6.3	6.3	0.4	12.5	0.8	6.3	400	3.1	1.6	6.3	0.2	1.6	200	50	12.5	400
1510/9C1	50	25	100	6.3	200	1.6	50	1,600	100	3.1	12.5	0.2	1.6	800	50	12.5	400
Providencia rettgeri																	
RE-18	200	400	200	6.3	400	3.1	200	400	1.6	50	400	3.1	12.5	1.6	6.3	25	400
RE-18C1	1,600	1,600	1,600	200	1,600	12.5	1,600	1,600	50	100	800	3.1	12.5	6.3	6.3	25	400
Providencia alcalifaciens																	
IN-06	25	25	12.5	0.4	50	0.2	50	100	3.1	6.3	25	0.4	3.1	25	100	100	800
IN-06C1	800	400	400	6.3	400	0.8	400	400	50	12.5	50	0.4	3.1	100	100	100	800

[a] CEC, Cephacetrile; CTZ, ceftezole; CEZ, cefazolin; CMD, cefamandole; CET, cephalothin; CPZ, cefoperazone; CER, cephaloridine; CEP-C, cephalosporin C; CFX, cefoxitin; APC, ampicillin; PCG, benzylpenicillin; PIP, piperacillin; APL, apalcillin; TC, tetracycline; MINO, minocycline; CM, chloramphenicol; EM, erythromycin.

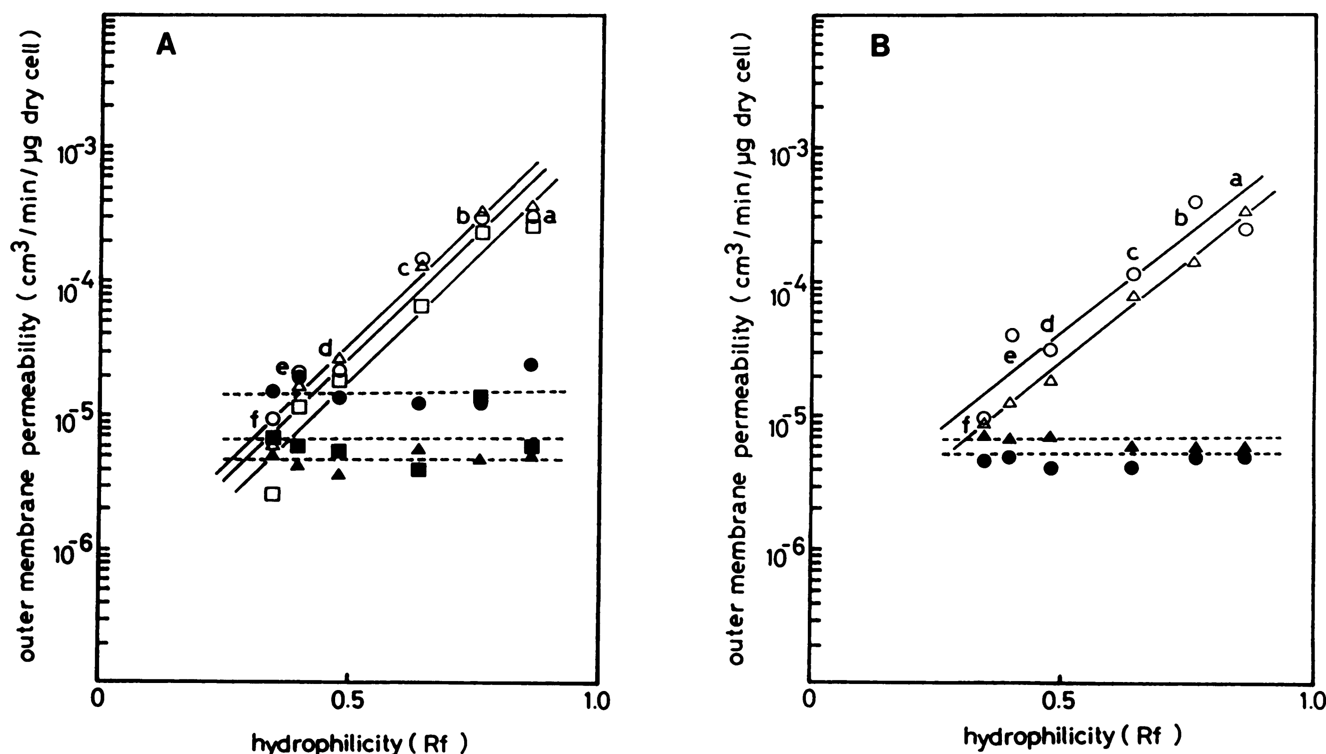

FIG. 2. Outer membrane permeability of β-lactam antibiotics. Solid lines represent the wild-type strains, and dotted lines represent the mutant strains. Closed symbols represent C1 mutants. Symbols: a, cephacetrile; b, ceftezole; c, cefazolin; d, cefamandole; e, cephalothin; f, cefoperazone. (A) Symbols: ○ and ●, *Providencia alcalifaciens* IN-06 and IN-06C1; △ and ▲, *Providencia rettgeri* RE-18 and RE-18C1; ■ and □, *Proteus mirabilis* N-51 and N-51C1. (B) Symbols: ○ and ●, *Proteus vulgaris* K22-2 and K22-2C1; △ and ▲, *M. morganii* 1510/9 and 1510/9C1.

The relationship between outer membrane permeability and hydrophilicity of six monoanionic cephalosporins in the *Proteus*, *Morganella*, and *Providencia* strains is shown in Fig. 2. In the parental strains, permeability was directly proportional to the hydrophilicity of the tested antibiotics, strongly suggesting that cephalosporins passed across the outer membrane via the water-filled channels of protein(s) (31). The dependence of permeability on hydrophilicity was slightly lower in *Proteus vulgaris* and *M. morganii* than in the other species. As shown in Fig. 2, the outer membrane permeability of the monoanionic cephalosporins, such as cephacetrile, ceftezole and cefazolin, was extremely reduced in all the mutants, and the extent of reduction was proportional to hydrophilicity of the drugs. It should be emphasized that the permeability coefficient in the mutant strains was not influenced by the hydrophilicity of the drugs. This fact may suggest that the 40K or 37K protein deleted in the mutant strains was the only major porin protein(s) in the three genera.

In addition to the effect of the hydrophilic property on the outer membrane permeability of cephalosporins, the ion selectivity of the channels was estimated by using cephaloridine as a zwitterionic compound and cephalosporin C as a divalent anionic compound. It was already known that additional positive charge of a solute molecule accelerates the diffusion process through the *E. coli* porin pores but that an increase in negative charge markedly decreases the permeability of the solute (3, 20, 33). The permeability of cephaloridine and cephalosporin C was compared with that of a monoanionic cephalosporin which was similar in

TABLE 2. Outer membrane permeability of monoanionic, divalent anionic, and zwitterionic cephalosporins

β-Lactam[a]	Hydrophilicity[b]	Electrical charge	Permeability coefficient (10^{-5} cm^3/min per μg [dry wt] of cells)[c]					
			N-51	1510/9	K22-2	RE-18	IN-06	KY-2209
CEP-C	0.90	− −	5.7	4.7	10.5	8.4	4.1	46.6
CEC	0.86	−	24.2	32.1	24.8	36.8	30.1	272
CEP-C/CEC			0.24	0.15	0.42	0.22	0.14	0.17
CER	0.41	− +	5.5	5.3	3.9	9.4	12.9	336
CET	0.40	−	1.1	1.2	3.8	1.7	2.0	42.6
CER/CET			4.8	4.5	1.0	5.7	6.6	7.9

[a] CEP-C, Cephalosporin C; CEC, cephacetrile; CER, cephaloridine; CET, cephalothin.
[b] Hydrophilicity is expressed as the R_f value by reverse-phase thin-layer chromatography (24).
[c] Strains: *Proteus mirabilis* N-51, *M. morganii* 1510/9, *Proteus vulgaris* K22-2, *Providencia rettgeri* RE-18, *Providencia alcalifaciens* IN-06, and *E. coli* KY-2209.

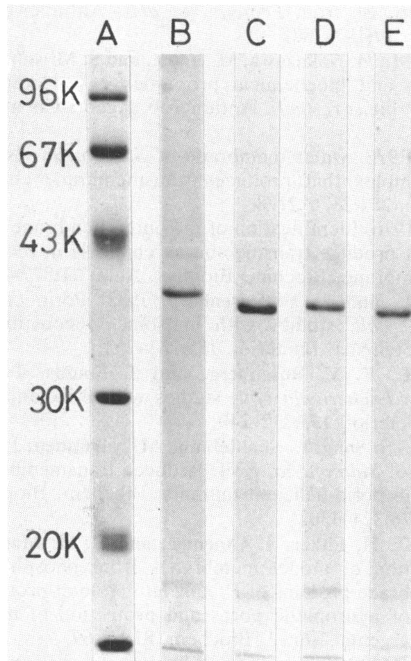

FIG. 3. SDS-polyacrylamide gel electrophoresis of purified outer membrane proteins. Solubilization of the purified protein in the sample buffer was done at 100°C for 5 min, and 5 μg of the protein was applied to each slot. Standards and conditions of gel electrophoresis were similar to those described in the legend to Fig. 1. Lanes: A, standard proteins; B, *Proteus mirabilis* N-51; C, *M. morganii* 1510/9; D, *Proteus vulgaris* K22-2; E, *Providencia rettgeri* RE-18.

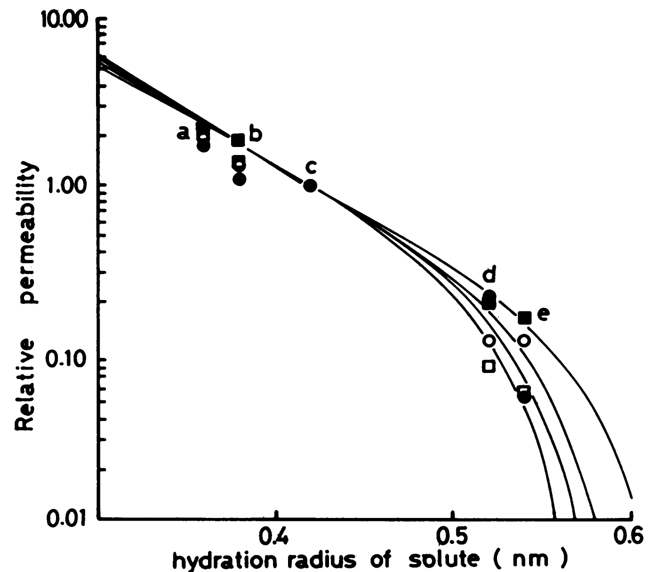

FIG. 4. Rate of permeation of various sugars into proteoliposomes. Permeation rate was calculated from the swelling assay and normalized to the rate of permeation of D-glucose. Symbols: ●, 40K protein of *Proteus mirabilis* N-51; ○, 37K protein of *M. morganii* 1510/9; ■, 37K protein of *Proteus vulgaris* K22-2; □, 37K protein of *Providencia rettgeri* RE-18; a, D-ribose (M_r, 150); b, L-arabinose (M_r, 150); c, D-glucose (M_r, 180); d, sucrose (M_r, 342); e, lactose (M_r, 342).

hydrophilicity, i.e., cephalothin and cephacetrile, respectively. The cephaloridine-cephalothin and cephalosporin C-cephacetrile ratios are shown in Table 2, together with the ratios obtained in *E. coli* KY-2209, a mutant strain which produces only one porin protein, OmpF. The results suggested that the porin pores of organisms of the three genera, except *Proteus vulgaris*, are similar to the OmpF pore in the ion selectivity characteristic.

Determination of porin pore radius. With the exception of those of *Providencia alcalifaciens*, outer membrane proteins presumed to be porins were purified from the bacterial cells by the procedure of Tokunaga et al. (30) with slight modification. The purity of the 40K and 37K protein preparations was judged to be more than 90% by SDS-polyacrylamide gel electrophoresis (Fig. 3).

When the purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis after solubilization at 40°C in the sample buffer, their apparent molecular weights were estimated to be about 100,000 (data not shown). These results suggested that the 40K and 37K proteins formed an oligomeric structure in the outer membrane.

The porin function examined by the liposome swelling assay and proteoliposomes was found to allow penetration of sugars at a rate inversely related to their hydrated radii (Fig. 4). Based on these results, the outer membrane proteins tested were identified as porins, and the radii of the pores formed by the 40K porin and the 37K porin were estimated to be approximately 0.59 nm (*Proteus mirabilis*), 0.63 nm (*Proteus vulgaris*), 0.58 nm (*Providencia rettgeri*), and 0.60 nm (*M. morganii*). Thus, these porin channels have radii similar to that of porin channels of *E. coli*, which were reported to be 0.58 nm (OmpF) and 0.54 nm (OmpC) by Nikaido and Rosenberg (19).

DISCUSSION

The results presented in this study show that the 40K protein in *Proteus mirabilis* and 37K proteins in *Proteus vulgaris*, *M. morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens* are porins. Nixdorff et al. (22) reported that two major outer membrane proteins of *Proteus mirabilis* with apparent molecular weights of 36,000 and 39,000 formed hydrophilic pores in reconstituted membranes, and they claimed that the two kinds of pores have functionally similar properties, although the 39K protein preparation was assumed to contain a large amount of the 36K protein. *Proteus mirabilis* N-51, studied here, produces two major outer membrane proteins, i.e., 40K and 41K, and these two proteins may correspond to the 36K and 39K proteins, respectively, reported by Nixdorff et al. (22). However, *Proteus mirabilis* N-51C1 lacking the 40K porin showed low permeation of hydrophilic cephalosporins, and the diffusion rate of the drugs through the outer membrane was independent of the hydrophilic property of the solute (Fig. 2A). This observation may indicate that the 40K porin is the major porin in strain N-51. A similar assumption is also applicable to the other four species, *Proteus vulgaris*, *M. morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens*. In gram-negative enteric bacteria, such as *E. coli* (5, 6, 10–14, 18), *Salmonella* spp. (11, 17, 21), *Klebsiella* spp. (unpublished data), and *Enterobacter* spp. (24), the production of multiple porin proteins is common. Therefore, the production of a single porin protein may be the characteristic of the five species in this study. The common features among the five species are especially interesting in view of the fact that the species were once classified into a single genus (7).

Our results demonstrated that hydrophilic cephalosporins, such as cephacetrile, ceftezole, cefazolin, and cefamandole, use the porin pore as the main permeation pathway across the outer membrane of the five species. On the other hand, cefoperazone, which has a relatively high hydrophobic character, was weakly affected in its permeation rate and antibacterial activity by the lack of porin. This observation may indicate that cefoperazone can cross through the outer membrane via a nonporin pathway, i.e., the hydrophobic region of the outer membrane, in addition to the porin pathway. A similar conclusion has been offered by us for the outer membrane permeation of some penicillins in *E. coli* (31).

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