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Outer Membrane Permeation of β-Lactam Antibiotics in *Escherichia coli, Proteus mirabilis*, and *Enterobacter cloacae*

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Mutant strains lacking outer membrane protein(s) were isolated from *Escherichia coli, Proteus mirabilis*, and *Enterobacter cloacae*. The outer membrane protein(s) of *P. mirabilis* and *E. cloacae* corresponding to *E. coli* porin were identified on the basis of their function, namely, their ability to allow the permeation of glucose as demonstrated by [^{14}C]glucose uptake by intact cells. *P. mirabilis* has only one outer membrane pore protein (molecular weight, 40,000), but *E. cloacae* has at least two such proteins (molecular weights, 37,000 and 39,000 to 40,000). When the bacteria lost these proteins or porin, the outer membrane permeation of cefazolin was found to be greatly reduced in these three species. Such a change in the outer membrane permeation closely correlated with a significant decrease in the bacterial susceptibility to cephalosporins, including cefoxitin. These results suggested that the main pathway for cephalosporin permeation is the pore made up of these proteins. The 39,000- to 40,000-molecular-weight protein in *E. cloacae* was also assumed to play an important role in the outer membrane permeation of tetracycline and chloramphenicol. On the other hand, the permeation route of penicillins was obscure. The susceptibility to penicillins, except in some cases, was little influenced by the absence of the proteins. Ampicillin was found to pass through the outer membrane via the same route as the cephalosporins, but the possibility that ampicillin and other penicillins possess another unknown route for outer membrane permeation could not be ruled out.

The outer membrane of gram-negative bacteria is known to act as a permeability barrier for various toxic materials, such as antibiotics, detergents, and dyes (13, 14). Recent studies have revealed that several outer membrane proteins of *Escherichia coli* and *Salmonella typhimurium* play a significant role in the permeation of hydrophilic molecules with molecular weights smaller than about 600 (10, 11). Transmembrane channels composed of porin, a major outer membrane protein, are believed to be the route used by β-lactam antibiotics to reach their targets in *E. coli* (14).

We previously devised a method for estimating the outer membrane permeability of β-lactam antibiotics (19). We reported this application to gram-negative bacteria, using seven β-lactam antibiotics, including two penicillins and five cephalosporins (18). These studies demonstrated the significantly higher activity of cephalosporins in passing through the outer membrane than of penicillins. Subsequent investigations revealed another interesting fact: an *E. coli* mutant lacking porin showed high resistance to cephalosporins but not to penicillins. These facts suggested that, in addition to the hydrophobic character of β-lactam antibiotics, the nucleus of these antibiotics plays an important role in their passage through the outer membrane. Furthermore, we are interested in studying the route of β-lactam antibiotic permeation in other pathogenic bacteria and in knowing whether the information obtained from *E. coli* cells can be applied to other pathogenic bacteria.

In this paper, we describe a detailed analysis of the outer membrane permeation of cephalosporins and penicillins. This analysis was made by using mutant strains of *E. coli, Proteus mirabilis*, and *Enterobacter cloacae* which had lost their major outer membrane protein(s).

MATERIALS AND METHODS

Bacterial strains and bacteriophage. *E. coli* CS109 and CS197 were kindly provided by A. P. Pugsley. CS109 is an *E. coli* K-12 substrain, and CS197 has a mutation at the *ompB* locus and is derived from CS109. CS197 lacks outer membrane proteins OmpF and OmpC (Ia and Ib, respectively, in Schnaitman's

nomenclature). Its properties have been described in detail by Pugsley and Schnaitman (16). *E. coli* CU204 was isolated from CS109 as the phage TuII*-resistant mutant lacking the outer membrane protein OmpA, which is the phage receptor. *E. coli* CU205 is a double mutant lacking three major proteins: OmpC, OmpF, and OmpA; this mutant was isolated from CU204 by selection for cefoxitin resistance. Phage TuII* was kindly provided by T. Nakae.

P. mirabilis N-51 is a clinical isolate which produces hardly any β-lactamase activity. The mutant N-51C1, a significantly decreased producer of an outer membrane protein, was selected on the basis of cefoxitin resistance.

E. cloacae 206 is a clinical isolate and was selected from our stock cultures on the basis of its inability to produce β-lactamase. Three mutants lacking one or two major proteins(s) were isolated from strain 206 on the basis of cefoxitin resistance.

Antibiotics. The antibiotics employed in this study were kindly provided by the following pharmaceutical companies: benzylpenicillin, ampicillin, cloxacillin, streptomycin, and tetracycline, Meiji Seika Co., Tokyo, Japan; carbenicillin and cefazolin, Fujisawa Pharmaceutical Co., Osaka, Japan; cephalothin and cephaloridine, Torii Pharmaceutical Co., Tokyo, Japan; piperacillin, Toyama Chemical Co., Tokyo, Japan; cefoxitin, Merk Sharp & Dohme Research Laboratories, Rahway, N.J.; chloramphenicol, Yamanouchi Pharmaceutical Co., Tokyo, Japan; erythromycin, Japan-Upjohn Co., Tokyo, Japan.

Assay of outer membrane permeation of β-lactam antibiotics. The assay of the outer membrane permeation of β-lactam antibiotics was carried out as described previously (19), except that the bacterial cells were grown in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) instead of heart infusion broth.

Reverse-phase TLC. The hydrophobic character of various antibiotics was expressed as the R_f value which was measured by reverse-phase thin-layer chromatography (TLC). The polar mobile phase was acetate-Veronal buffer (pH 7.0)-methanol (4:1, vol/vol). Merck TLC silica gel 60 F254 plates were used as the nonpolar stationary phase. A sample was dissolved in the acetate-Veronal buffer or methanol to give about 3 mg/ml, and 1 to 2 μl of the solution was loaded on the TLC plate. After development at room temperature, the antibiotic was detected on the plate by using iodine vapor.

Preparation of outer membrane proteins and their analysis by polyacrylamide gel electrophoresis. Membranes were prepared by a modification of the procedure of Matsuhashi et al. (8) from mid-log-phase cultures grown in antibiotic medium 3 (Difco). The crude outer membrane was purified by treatment with sodium lauroyl sarcosinate (Sarkosyl NL-97) as follows. The crude outer membrane obtained from 1 liter of the bacterial culture was suspended in 3 ml of 0.01 M phosphate buffer (pH 7.0) containing 2% Sarkosyl NL-97 and incubated at 20°C for 20 min. The suspension was then centrifuged at 100,000 × *g* for 40 min at 10°C. The pellet was suspended, washed two times with 1% Sarkosyl NL-97 solution, and then used as the purified outer membrane preparation.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the outer membrane was preformed by a modification of the method of Laemmli (4). Thin (0.8 mm) slab gels were prepared by the procedure described by Laemmli. They were composed of a lower separation gel of 10% acrylamide and 0.1% SDS in 0.375 M Tris-hydrochloride buffer (pH 8.8) and an upper stacking gel of 5% acrylamide and 0.1% SDS in 0.125 M Tris-hydrochloride buffer (pH 6.8). Purified outer membrane solubilized in the same volume of sample buffer (about 2 to 3 mg of protein per ml) was heated in boiling water for 2 min before application. The sample buffer was composed of 12.5 ml of 0.5 M Tris-hydrochloride buffer (pH 6.8), 20 ml of 10% SDS, 5 ml of mercaptoethanol, 10 ml of glycerol, and 0.002% bromophenol blue.

SDS-urea-8% acrylamide tube gel electrophoresis was carried out as described by Nakamura and Mizushima (12).

Measurement of β-lactamase activity. β-Lactamase activity was assayed by the microiodometric method of Novick (15) with slight modifications.

Assay for penicillin-binding proteins. The procedure described by Matsuhashi et al. (8) was used for the assay of penicillin-binding proteins.

Protein assay. The method of Lowry et al. (5) was used to assay for protein in the outer membrane preparations.

Uptake of labeled glucose. Cells were grown in 200 ml of M9 medium supplemented with 0.1% Casamino Acids (Difco) and harvested at mid-log phase. Cells were than washed three times with the same medium but without glucose and to which 50 μg of chloramphenicol per ml was added. The washed cells were resuspended at a density of approximately 0.4 mg (dry weight) of bacteria per ml in M9 medium in which glucose was replaced by 1 mM glycerol (modified M9 medium). The labeled glucose solution (50 μl) was added to 5 ml of the cell suspension prewarmed at 25°C and incubated for 30 s at 25°C. A 0.5-ml sample was taken and filtered through a 0.45-μm (Millipore Corp., Bedford, Mass.) membrane filter. The filter was rinsed with the modified M9 medium, dried, and counted. The labeled glucose was D-[1-^{14}C]glucose (8.2 mCi/mmol; New England Nuclear Corp., Boston, Mass.), and the mixture for the uptake experiment contained 4 × 10^{-3} to 6 × 10^{-2} μCi/ml, depending on experimental conditions.

Measurement of bacterial susceptibility to antibiotics. The susceptibility of bacteria to antibiotics was measured by two distinct methods. One was a serial agar dilution method, and the susceptibility was expressed as the minimum inhibitory concentration (MIC). An overnight culture of the bacterial strain in nutrient broth was diluted 100-fold with fresh broth, and 5 μl of the bacterial suspension (about 3 × 10^6 cells per ml) was spotted on heart infusion agar, using an inoculum replicating device (Microplanter; Sakuma Factory, Tokyo, Japan). MICs were measured after incubation at 37°C for 18 h.

The other method was the measurement of the minimum concentration of antibiotic which resulted in complete lysis of bacterial cells (a decrease in the optical density to less than 10% of the initial density). Antibiotic was added to the bacterial culture in exponential phase (about 5 × 10^5 cells per ml) in heart infusion broth at 37°C, and cell lysis was followed for 4 h by the measurement of the optical density with the aid of a Bio-Log II photometer (Jobin-Yvon, Paris, France).

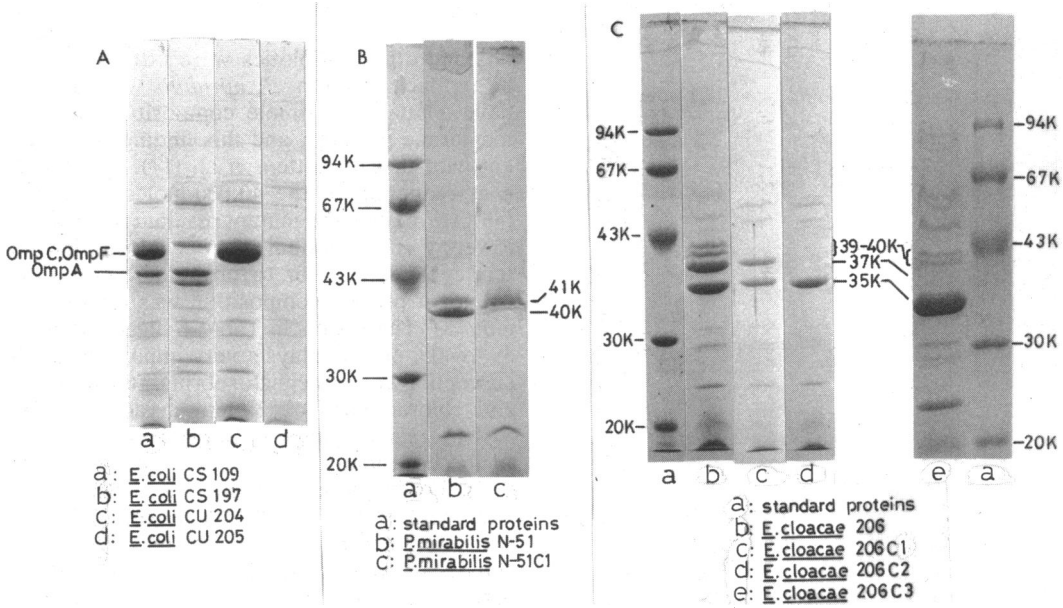

FIG. 1. SDS-polyacrylamide gel electrophoresis of the outer membrane proteins. Proteins were prepared from Sarkosyl-treated cell envelopes as described in the text and analyzed on an SDS-10% polyacrylamide slab gel. Molecular weight was estimated from the standards included in the gel. The standards were phospholipase b (94K), bovine serum albumin (67K), ovalbumin (45K), carbonic anhydrase (30K), and trypsin inhibitor (20K). (A) A 20- μg to 30-μg amount of protein from *E. coli* CS109, CS197, CU204, and CU205 was applied to each slot. Strain CS109 is the parent wild-type strain from which the other strains were derived. (B) A 10-μg amount of protein from *P. mirabilis* strains N-51 and N-51C1 was applied to each slot. Strain N-51 is the parent wild-type strain. (C) A 10-μg to 30-μg amount of protein from *E. cloacae* 206, 206C1, 206C2, and 206C3 was applied to each slot. Strain 206 is the parent wild type.

RESULTS

Isolation and properties of outer membrane mutants from *E. coli*, *P. mirabilis*, and *E. cloacae*. Guided by the fact that the *E. coli* strains lacking porin show low susceptibility to cephalosporins, we found that the mutant lacking porin could be easily selected on agar medium containing cefoxitin without the addition of any chemical mutagen. Strangely, cefoxitin was the most effective for this purpose, and other cephalosporins, such as cefazolin, did not give successful results. We applied this method to *P. mirabilis* and *E. cloacae* and isolated mutant strains defective in the outer membrane protein(s) functionally corresponding to the porin of *E. coli*.

P. mirabilis N-51C1 and *E. cloacae* 206C1 are the mutants isolated from the parent wild-type strains by selection on agar medium containing 3.1 μg of cefoxitin per ml. *E. cloacae* 206C2 was isolated from strain 206C1 by further selection on agar medium containing increasing concentrations of cefoxitin (25 μg/ml). *E. cloacae* 206C3 was spontaneously isolated from strain 206C2 as a revertant with restored susceptibility to cephalosporins with the aid of the replica-plating technique.

E. coli CU204 was selected from strain CS109 as a spontaneous mutant resistant to phage TuII*, which is known to use the OmpA outer membrane protein as the receptor for infection (1, 10). *E. coli* CU205, which was isolated from strain CU204 by selection on cefoxitin medium (3.1 μg/ml), lacks both OmpA and porin.

Sarkosyl-purified outer membrane of various mutants mentioned above and those of their parent wild-type strains were solubilized, and their protein composition was analyzed by SDS-slab gel electrophoresis. The results are shown in Fig. 1. The outer membrane protein profile of *E. coli* is almost identical with that reported by other workers (7).

Table 1 shows pertinent properties of all of the strains studied, i.e., the composition of their outer membrane proteins, their doubling time in heart infusion broth, and their susceptibility to various β-lactams and other important antibiotics. The growth rates of these strains generally decreased as the number of outer membrane proteins increased. The considerable variation in the MICs could be correlated with a particular outer membrane protein(s). In the case of *E. coli*, the lack of porin (OmpC and OmpF pro-

TABLE 1. Properties of outer membrane mutants of *E. coli*, *P. mirabilis*, and *E. cloacae*

Strain	Missing outer membrane protein(s)	Doubling time (min)	MIC (μg/ml) of:											
			Penicillins[a]					Cephalosporins[a]				TC	CM	SM	EM
			PCG	APC	CPC	PIP	CLX	CEZ	CET	CER	CFX				
E. coli															
CS109[b]	None	30.2	25	3.1	3.1	0.8	200	1.6	3.1	3.1	3.1	1.6	3.1	3.1	50
CS197	OmpC, OmpF	33.2	50	6.3	12.5	0.8	200	25	50	25	25	3.1	3.1	3.1	50
CU204	OmpA	42.0	25	3.1	3.1	0.8	200	0.8	3.1	3.1	3.1	1.6	3.1	3.1	50
CU205	OmpC, OmpF, OmpA	>60	25	3.1	12.5	1.6	400	25	50	25	50	3.1	3.1	3.1	100
P. mirabilis															
N-51[b]	None	34.5	3.1	1.6	0.8	0.4	200	3.1	3.1	25	3.1	50	3.1	6.3	400
N-51C1	40K	46.5	12.5	3.1	1.6	0.4	400	12.5	25	200	25	200	3.1	6.3	400
E. cloacae															
206[b]	None	31.5	100	50	200	6.3	800	1.6	3.1	3.1	3.1	3.1	3.1	3.1	100
206C1	39–40K	46.8	200	50	400	12.5	1,600	3.1	25	6.3	25	25	25	3.1	200
206C2	39–40K, 37K	54.9	200	100	800	25	1,600	50	100	100	200	50	50	3.1	200
206C3	37K	32.4	50	50	200	3.1	400	1.6	3.1	6.3	3.1	1.6	1.6	3.1	100

[a] Abbreviations: PCG, benzylpenicillin; APC, ampicillin; CPC, carbenicillin; PIP, piperacillin; CLX, cloxacillin; CEZ, cefazolin; CET, cephalothin; CER, cephaloridine; CFX, cefoxitin; TC, tetracycline; CM, chloramphenicol; SM, streptomycin; EM, erythromycin.

[b] Wild type.

teins) closely correlated with a significant increase in the MIC of cephalosporins, but penicillins and other antibiotics were little influenced by the lack of porin. *P. mirabilis* was found to have a relatively simple composition of outer membrane proteins, and this finding is consistent with that of Rottem et al. (17). The significant reduction of the 40,000-molecular-weight (40K) protein in the mutant resulted in a marked lowering of susceptibility to cephalosporins and tetracycline, but not to penicillins. The most abundant protein composition was found in *E. cloacae*. The results in Table 1 suggest that the 39 to 40K protein plays a very important role in susceptibility to cephalosporins, tetracycline, and chloramphenicol. When, in addition, the 37K protein was missing, the low susceptibility to these antibiotics was significantly amplified even though the lack of the 37K protein alone had no effect on the MIC of the antibiotics.

It was confirmed that detectable β-lactamase activity was not found in the mutant strains nor in their parent strains, and there was no essential difference in the property of penicillin-binding proteins between the mutant strains of *E. coli* and their parent strain. The penicillin-binding proteins of the other strains have not yet been confirmed, but the morphological properties of these strains were found to be normal by microscopic observation.

Effect of outer membrane mutation on glucose uptake. To elucidate the function of the outer membrane proteins, we measured glucose uptake in the strains mentioned above, and the results are shown in Fig. 2. *E. coli* mutants CS197 and CU205 showed significantly lower glucose uptake activity, suggesting that outer membrane proteins OmpC and OmpF contribute to the outer membrane permeation for such a low molecular weight and hydrophilic substance. However, glucose uptake was not affected by the absence of OmpA protein.

P. mirabilis N-51C1, which is a lower producer of the 40K protein, showed significantly lower activity for glucose uptake. This result suggests that the 40K protein is functionally similar to the porin of *E. coli*. The 40K protein could not be separated into multiple bands by SDS-urea-polyacrylamide tube gel electrophoresis; therefore, it seems that *P. mirabilis* has only one kind of protein corresponding to porin.

E. cloacae has two kinds of outer-membrane proteins which are responsible for glucose uptake, namely, 37K and 39 to 40K. Although the 39 to 40K protein was separated into two bands by gel electrophoresis, they were always lost together as a single mutational event. The glucose uptake in the *E. cloacae* strains decreased stepwise as 39 to 40K, 37K, and then 37K and 39 to 40K were lost. The results shown in Fig. 2

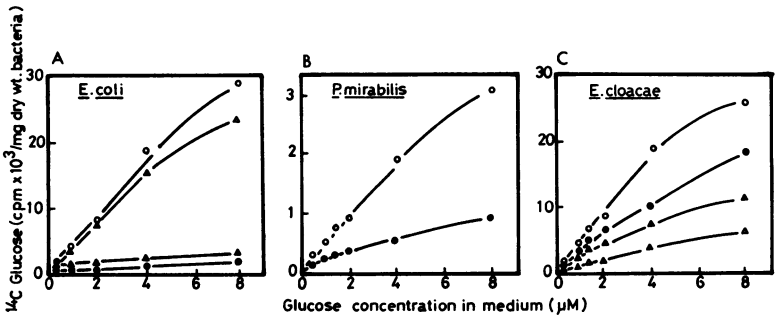

FIG. 2. Glucose uptake by *E. coli*, *P. mirabilis*, and *E. cloacae*. Details are given in the text. Symbols: (A) ○, *E. coli* CS109; ●, *E. coli* CS197; △, *E. coli* CU204; ▲, *E. coli* CU205; (B) ○, *P. mirabilis* N-51; ●, *P. mirabilis* N-51C1; (C) ○, *E. cloacae* 206; ●, *E. cloacae* 206C1; △, *E. cloacae* 206C2; ▲, *E. cloacae* 206C3.

suggest that the 37K protein plays a more important role in glucose uptake than the 39 to 40K protein; however, *E. cloacae* seems to possess multiple proteins functionally corresponding to porin.

Effect of outer membrane mutation on β-lactam antibiotic permeation. To assay outer membrane permeation of β-lactam antibiotics by the method of Sawai et al. (19), we infected all of the strains employed in this study with an R plasmid, RGN823, which mediates type Ib penicillinase (TEM-2 penicillinase) production (20).

Figure 3 shows the cefazolin concentrations that were achieved in the periplasmic space at various drug concentrations in the medium. The outer membrane of the wild-type *E. coli* (CS109) was permeable to cefazolin with high efficiency, and an approximately linear relationship between periplasmic and external drug concentrations was observed. This result is in agreement with that found in our earlier study (18). When the *E. coli* strain lost porin, cefazolin permeation was significantly decreased as observed in the cases of glucose uptake in CS197 and CS205. Similar results were also observed with cephaloridine and cephalothin (data not shown).

In *P. mirabilis*, the results shown in Fig. 2 and 3 strongly suggest that cefazolin passes through the outer membrane by the same route as does glucose, and this permeation function is attributed to the 40K protein. *E. cloacae*, which has two kinds of outer membrane proteins responsible for glucose uptake, presented somewhat complicated results. Contrary to the case of glucose uptake, the 37K protein made little contribution to cefazolin permeation, and the 39 to 40K protein played a significant role in the permeation function. The decrease in the outer membrane permeation of cephalosporins was well expressed by the increase in the drug MICs (Table 1).

As reported in a previous paper (18), penicillins have significantly lower rates for outer membrane permeation than do cephalosporins. Therefore, it is not an easy matter to obtain exact data on the outer membrane permeation of penicillins. In addition, we recently found that trace amounts of penicillinase leak out from the

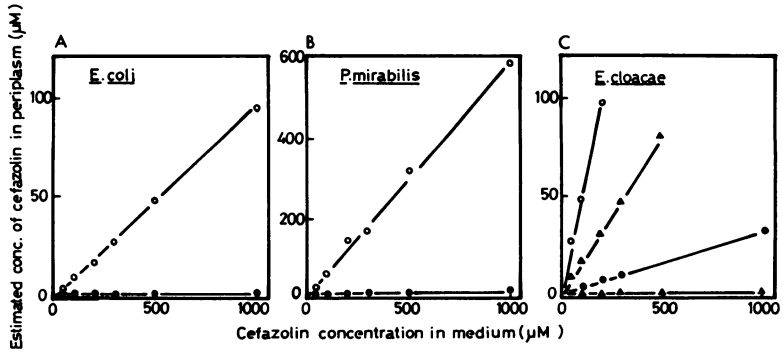

FIG. 3. Outer membrane permeation of cefazolin. Symbols: (A) ○, *E. coli* CS109; ●, *E. coli* CS197; (B) ○, *P. mirabilis* N-51; ●, *P. mirabilis* N-51C1; (C) ○, *E. cloacae* 206; ●, *E. cloacae* 206C1; △, *E. cloacae* 206C2; ▲, *E. cloacae* 206C3.

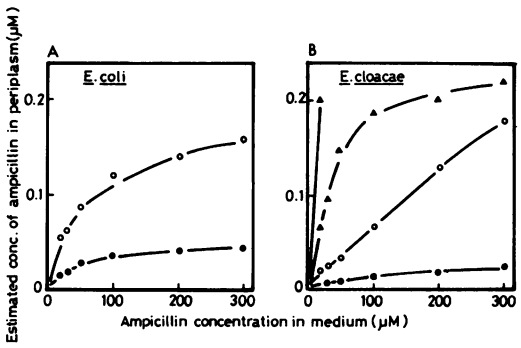

FIG. 4. Outer membrane permeation of ampicillin. Symbols; (A) ○, *E. coli* CS109; ●, *E. coli* CS197; (B) ○, *E. cloacae* 206; ●, *E. cloacae* 206C1; △, *E. cloacae* 206C2; ▲, *E. cloacae* 206C3.

intact cells (less than 0.5% of total activity in the cells) and interfere with the assay of low permeable materials, such as penicillins. Figure 4 shows the ampicillin permeation in *E. coli* CS109 and its porin-lacking mutant (CS197). The penicillinase leakage was compensated for as much as possible by subtracting the leaked penicillinase activity from the total enzyme activity in the cell suspension. The results indicate that ampicillin passes through the outer membrane via porin. However, considerable ampicillin permeation was also found in the mutant lacking porin. The possibility that ampicillin is capable of passing through the outer membrane via a route other than that of porin cannot be ruled out. We could not perform exact measurements for penicillin permeation in other *E. coli* strains or in the *P. mirabilis* strains because of the difficulty mentioned above.

Ampicillin permeability in the *E. cloacae* strains is shown in Fig. 4, and even more complicated results are seen. The 39 to 40K protein appears to play an important role in ampicillin permeation as in the case of cefazolin, but the barrier effect of the outer membrane against the drug was significantly reduced by the lack of the 37K protein. At present, we have no simple explanation for these observations.

To examine the correlation between a particular outer membrane protein(s) and the outer membrane permeation of β-lactams with respect to bacterial susceptibility to the drugs, we carried out a finer assay for bacterial susceptibility to ampicillin and cefazolin, using the Bio-Log II photometer, an automatic spectrophotometer for the assay of bacterial growth. The results obtained are shown in Table 2. The decrease in the outer membrane permeation of cefazolin due to the lack of outer membrane protein(s) was well expressed by the lowering of susceptibility to the drugs in the three bacterial species; however, susceptibility to ampicillin was only slightly affected by the lack of the protein(s).

DISCUSSION

The selective permeability of various nutrients across the outer membrane of gram-negative bacteria has been investigated mainly with *E. coli* and *S. typhimurium* cells or the reconstituted vesicles made from the bacterial membranes (6, 9–11, 22). These investigations have revealed that porin, a major outer membrane protein of *E. coli* and *S. typhimurium*, constitutes a pore for the permeation route of small hydrophilic substances, such as glucose and amino acids. Recently, we found that the mutational loss of porin production in *E. coli* could be easily detected on the basis of lower susceptibility to cefoxitin. Such a mutation was found to be a relatively frequent event and was estimated to be about 10^{-6} per cell division. The application of this method to eight species of gram-negative bacteria allowed us to identify the specific outer membrane protein(s) functionally similar to porin of *E. coli* in these bacteria. The molecular weights of these proteins, other than those of the three species mentioned in this paper, were found to be 36,000 for *Proteus morganii*, 37,000 for *Proteus vulgaris*, 40,000 for *Klebsiella pneumoniae*, 37,000 and 38,000 for *Citrobacter freundii*, and 40,000 and 41,000 for *Serratia marcescens*. Detailed properties of the proteins produced by these five species are under investigation.

In this study, we compared ampicillin and cefazolin with respect to their outer membrane permeation and employed these two β-lactams as representative of penicillins and cephalosporins, respectively. The results strongly suggested that cefazolin passes through the outer membrane by the same pathway as does glucose (Fig. 2 and 3). Similar results were also obtained for cephaloridine and cephalothin. By analogy with the porin of *E. coli*, it can be assumed that the

TABLE 2. Minimum concentrations leading to complete lysis of bacterial cells in liquid medium

Strain	Concn (μg/ml)of:	
	Cefazolin	Ampicillin
E. coli CS109	34	10
E. coli CS197	250	22
E. coli CU204	10	8
E. coli CU205	60	18
P. mirabilis N-51	30	100
P. mirabilis N-51C1	400	250
E. cloacae 206	20	800
E. cloacae 206C1	200	1,000
E. cloacae 206C2	1,200	1,000
E. cloacae 206C3	25	1,000

40K protein of *P. mirabilis* and the 37K and 39 to 40K proteins of *E. cloacae* build a pore across the outer membrane, and the main pathway for cephalosporins and glucose is the pore. This assumption is supported by the fact that the absence of these proteins resulted in a significant lowering of bacterial susceptibility to cephalosporins (Tables 1 and 2).

On the contrary, the ampicillin susceptibility was only slightly reduced by the loss of these proteins. Because of its low permeability, we could not determine the exact relationship between ampicillin susceptibility and the decrease in its outer membrane permeation. Such low permeability of penicillins cannot be attributed to their molecular size or hydrophobic character because the molecular weight of penicillins is generally lower than that of cephalosporins and there was no significant difference in the R_f on TLC (ampicillin, 0.53; carbenicillin, >0.60; cefazolin, 0.76; cefoxitin, 0.63; cephaloridine, 0.40). Furthermore, the results in Fig. 4 and Tables 1 and 2 suggest the possibility that ampicillin uses another pathway in addition to the pore mentioned above for its outer membrane permeation.

To examine other possible pathways for the outer membrane permeation of β-lactam antibiotics, we isolated two additional types of outer membrane mutants of *E. coli*, a mutant lacking the *lamB* protein and a mutant lacking the *tsx* protein (unpublished data). The *lamB* protein is known to produce a transmembrane channel and to function in the transport of maltose (21), and the *tsx* protein is known to produce a specific diffusion pathway for nucleosides and amino acids (2, 3). We could not detect any difference in the susceptibility to ampicillin and cefazolin nor in the outer membrane permeability of the antibiotics between these two mutants and the parent wild-type.

Another possible pathway for the passage of β-lactams across the outer membrane is passive diffusion through the phospholipid bilayer in the outer membrane. Our preliminary results demonstrated a significant passage of ampicillin through an artificial phospholipid layer but not of cephalosporins, such as cefazolin and cephaloridine (unpublished data). The facilitated permeation of ampicillin in the mutants of *E. cloacae* (Fig. 4) may be explained by such a property of ampicillin.

It was an interesting fact that porin and protein(s) corresponding to porin appeared not to have necessarily the same functional property. When the *E. cloacae* strain lost its outer membrane proteins, the bacteria showed significantly decreased susceptibility both to tetracycline and chloramphenicol, in addition to cephalosporins. Our preliminary experiments showed that the amount of tetracycline incorporated into the mutant cells was in fact less than one-fourth of that in the wild-type strain (unpublished data). On the other hand, the porin-lacking mutants of *E. coli* were little affected by mutation with respect to susceptibility to tetracycline and chloramphenicol. In the case of *P. mirabilis*, the significant decrease in the production of the 40K protein resulted in a lowering of the tetracycline susceptibility. The difference in functional property between the two proteins in the same species was also observed in *E. cloacae*. The 37K protein seemed to play a more important role in glucose uptake than did the 39 to 40K protein. However, the situation was reversed in the case of cefazolin permeation.

These findings underscore the delicate difference in the functional properties of these outer membrane proteins. They also lend support to the hypothesis that the intrinsic susceptibility of bacteria to an antibiotic can be attributed in part to the characteristics of the outer membrane proteins.

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