THE BACTERIAL OUTER-MEMBRANE PERMEABILITY OF β-LACTAM ANTIBIOTICS

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Two penicillins and 5 cephalosporins were evaluated for their ability to pass through the outer-membranes of *Proteus morganii, Citrobacter freundii* and *Escherichia coli*. Cefazolin, ceftezole and cephaloridine showed high permeability through the outer-membranes of these Gram-negative bacteria. Benzylpenicillin and cephalothin, on the contrary, showed low permeability. The outer-membrane permeability of ampicillin and cephalexin varied from species to species. *C. freundii* was found to have the highest barrier against both the penicillins and the cephalosporins, and *E. coli* appeared to have a low barrier against the cephalosporins. The hydrophobic character of the β-lactam antibiotics, which was estimated by a reversed-phase thin-layer chromatography was closely related to the outer-membrane permeability. In general, the more hydrophilic antibiotic showed the higher outer-membrane permeability. However, cephaloridine, the most lipophilic compound among the antibiotics tested, showed good permeability.

The outer-membrane of the Gram-negative bacteria is believed to be a barrier to the penetration of β-lactam antibiotics to their targets in the bacterial inner membrane. The ability of the antibiotics to pass through the outer-membrane is an important property which decides antibacterial activity or antibacterial spectrum. In a previous paper[1], we reported a method for estimating the outer-membrane permeability of β-lactam antibiotics. The method is based upon the fact that β-lactamase of Gram-negative bacteria is located in the periplasm which lies between the outer- and the inner-membranes, and that the reaction velocity of the enzyme is proportional to the substrate concentration when the concentration is lowered to a level of the *Km*. A similar way of measuring the outer-membrane permeability of β-lactam antibiotics has been reported by ZIMMERMAN and ROSSELET[2], who used an *Escherichia coli* strain as the test organism.

In this paper, we describe the application of the method to the cells of *Proteus morganii*, *Citrobacter freundii* and *E. coli*. Seven β-lactam antibiotics were evaluated for outer-membrane permeability, and the relationship between the permeability and the hydrophobicity of the antibiotics was also studied.

Materials and Methods

Bacterial strains, media and growth conditions.

Proteus morganii 1510[3] and *Citrobacter freundii* GN346[4,5] are strains which produce their species-specific cephalosporinase constitutively. *P. morganii* 1510/9[5] and *C. freundii* GN346/16[4,5] are mutant strains which show very low cephalosporinase activity and were derived from *P. morganii* 1510 or *C. freundii* GN346, respectively, with the aid of N-methyl-N′-nitro-N-nitrosoguanidine. *C. freundii* GN346/16 RGN823 and *E. coli* ML1410 RGN823 are strains harboring an R plasmid RGN823. The plasmid mediates a constitutive synthesis of type Ib penicillinase in the host bacteria[6].

Heart infusion broth was used for bacterial cultivation in liquid medium, and heart infusion agar was used for the determination of bacterial susceptibility to antibiotics. Bacterial cultivation

was performed at 37°C. The media used were all products of Eiken Chemical Co., Tokyo, Japan.

β-Lactam antibiotics.

Penicillins and cephalosporins were kindly provided by the following pharmaceutical companies: benzylpenicillin and ampicillin, Meiji Seika Co., Tokyo, Japan; cefazolin and ceftezole, Fujisawa Pharmaceutical Co., Osaka, Japan; cephalothin and cephaloridine, Torii Pharmaceutical Co., Tokyo, Japan; cephalexin, Toyama Chemical Co., Tokyo, Japan.

Assay of β-lactamase activity.

β-Lactamase activity was assayed by a modification of the microiodometric method of NOVICK[7]. Substrate dissolved in 0.95 ml of 13 mM phosphate buffer (pH 7.0) containing 0.68% NaCl and 1 mM $MgSO_4$ was preincubated for 5 minutes at 30°C, and enzyme reaction was then initiated by adding 0.05 ml of enzyme solution. The enzyme solution used was either the intact cells or the sonically disrupted cells suspended in the phosphate buffer. After incubation for 15 minutes at 30°C, the enzyme reaction was stopped by adding 0.5 ml of 0.15 M sodium tungstate dissolved in 2 M acetate buffer (pH 4.0). Immediately, 1.5 ml of starch-iodine reagent was added to the mixture. The starch-iodine reagent consists of 1 ml of 20 mM iodine – 160 mM potassium iodide, 20 ml of 2% starch solution (Stärke löslich, Art. 1252, Merck) and 79 ml of 0.05 M phosphate buffer (pH 6.0). After standing for 20 minutes at room temperature, absorbance of the reaction mixture was measured at 595 nm by Beckman spectrophotometer model 24. As a control, the absorbance of mixture having the same composition as that of the reaction mixture but to which the enzyme solution was added after the sodium tungstate was measured. The amount of hydrolyzed substrate in the reaction mixture was calculated by the iodine consumption of a given substrate, which was determined experimentally[8,9]. In all the assay experiments, the substrate consumed during the enzyme reaction was less than 20% of the added substrate.

Preparation of intact cell and sonically disrupted cell suspensions.

Exponential-phase cells grown in heart infusion broth at 37°C were harvested by centrifugation for 15 minutes at $7,000 \times g$ at 25°C. The bacterial cells were washed once with 13 mM phosphate buffer containing 0.68% NaCl and 1 mM $MgSO_4$ (pH 7.0) and resuspended in the buffer to give about 5×10^8 cells per ml. The cell suspension was divided into 2 portions. One was the intact cell sample and used for assay of the permeability within 1 hour. The other was used as the disrupted cell sample after treated with an ultrasonic disintegrator (Ohtake Works, Tokyo. 20 Kc/s) for 2 minutes with ice cooling. The complete disruption of cells was confirmed microscopically.

Measurement of bacterial susceptibility to β-lactam antibiotics.

The determination of levels of bacterial susceptibility to β-lactam antibiotics was performed according to the procedure described previously[10] except that minimal inhibitory concentration (MIC) was expressed in μM.

Reversed-phase thin-layer chromatography.

The hydrophobic character of β-lactam antibiotics was expressed as the Rf value which was measured by means of a reversed-phase thin-layer chromatography[11]. The polar mobile phase was sodium acetate-Veronal buffer (pH 7.0) containing 2% acetone and saturated with silicone oil TSF451, 10 cs (Toshiba Silicone Co., Tokyo). The nonpolar stationary phase was a silica gel plate (Merck silica gel 60) impregnated with the silicone oil. Before the coating, the silica gel plate was activated by heating for 15 minutes at 110°C. β-Lactam antibiotics were dissolved in distilled water to give about 3 mg/ml, and about 1 μl of solution was spotted on the thin-layer plate. The development was carried out for 15 minutes at 25°C. After the developed plate was dried, the antibiotic was detected by spraying 5% $KMnO_4$ solution.

Toluene treatment of bacterial cells.

The washed bacterial cells were prepared as described above, and suspended in 13 mM phosphate buffer (pH 7.0) containing 0.68% NaCl, 1 mM $MgSO_4$ and the appropriate amount of toluene (1.0 μl/ml for the *P. morganii* cells, 0.5 μl/ml for the *C. freundii* cells and 0.3 μl/ml for the *E. coli* cells). The suspension was incubated for 4 minutes at 30°C, and then diluted 30-fold with the phosphate buffer to remove the effect of toluene.

Results

Stability of Intact Cells

For the assay of the outer-membrane permeability of β-lactam antibiotics by the method reported previously[1], the outer-membrane of the tested microorganism must be stable during the assay. Intact cells of *P. morganii* 1510, *C. freundii* GN346, and *E. coli* ML1410 RGN823 suspended in 13 mM phosphate buffer (pH 7.0) containing 0.68% NaCl and 1 mM $MgSO_4$ were examined for β-lactamase activity and leakage of the enzyme into the surrounding medium over 2 hours at 25°C. During standing for 2 hours, β-lactamase activity of the intact cells of *C. freundii* and *E. coli* to benzylpenicillin increased 1.4- and 2.1-fold, respectively. The enzyme activity expressed by the intact cells of *P. morganii* was unchanged even though benzylpenicillin, cephalothin or cephaloridine were used as substrate. However, no detectable β-lactamase activity could be found in the supernatant of the centrifuged suspensions of these bacterial cells even at the end of the standing, and if any it was less than 0.1 per cent of the total activity. The increase in apparent β-lactamase activity of the *C. freundii* and the *E. coli* cells could not be protected by replacing the phosphate buffer with 0.01 M Tris-HCl buffer (pH 7.0) or 0.05 M HEPES-NaOH buffer (pH 7.0), containing 1 mM $MgSO_4$.

Ethylenediaminetetraacetic acid (EDTA) is known as the reagent which causes the decrease in the barrier effect of outer-membrane[12]. In the presence of 1 mM EDTA, β-lactamase activity expressed by the intact cells of *C. freundii* and *E. coli* increased 4.7- and 11-fold, respectively. However, leakage of the enzymes from the cells into surrounding medium could not be detected. In the case of *P. morganii*, β-lactamase activity of the intact cells was not affected by the presence of 1 mM EDTA. Toluene is also known to decrease the barrier effect of biological membranes. The bacterial cells were treated with toluene under mild conditions in which leakage of β-lactamase from the cells could not be detected. The toluene treatment raised the apparent β-lactamase activity of the *P. morganii* cells 2.1-fold. Similarly the enzyme activity of the *C. freundii* and the *E. coli* cells increased 4.9-and 2.1-fold, respectively.

Measurement of the Outer-membrane Permeability of β-Lactam Antibiotics

The experimental results described above suggested that the outer-membrane of the *P. morganii* cell is the most stable among those of the three species. Therefore, the permeability of 7 β-lactam antibiotics through the outer-membrane was first measured by using the *P. morganii* cell as the test organism, and the results are presented in Table 1. Although the *E. coli* and the *C. freundii* cells are less suitable for such an experiment, differences in the barrier effect of the outer-membrane on antibiotic penetration among these bacterial species is a subject of much interest. These data from the *C. freundii* and the *E. coli* cells are shown in Tables 2 and 3, respectively.

As described in a previous paper[1], β-lactam antibiotic penetrates into the periplasm at a constant diffusion rate after a short lag time and the concentration of the antibiotic achieved in the periplasm is estimated from a formula with the aid of the ratio Vd/Vi, the antibiotic concentration in the reaction medium and the *Km* value (Table 1). The ratio S_1/S_2 is an indicator of the ability of the antibiotic to pass through the outer-membrane. Lower values of the ratio indicate higher permeability.

Benzylpenicillin and cephalothin showed lower outer-membrane permeability in the 3 species. Cephaloridine, cefazolin and its derivative, ceftezole, exhibited higher permeability against all the tested strains. Cephalexin has very high permeability into the *E. coli* cell, but less so in the cells of

Table 1. Permeability of β-lactam antibiotics through the outer-membrane of *Proteus morganii* 1510[a].

Antibiotic and (*Km*)[b]	Conc. of S_1 (μM)	Estimated conc.[c] of S_2 (μM)	S_1/S_2	MIC to *P. morganii* 1510/9 (μM)
Benzylpenicillin (426 μM)	200 50 20	0.53 0.11 0.05	377 455 400	32
Ampicillin (90 μM)	200 50 20	7.5 1.6 ND[d]	27 31 ND	8
Cephalothin (12 μM)	200 50 20	ND 0.15 0.08	ND 333 250	32
Cefazolin (28 μM)	200 50 20	ND 2.1 1.2	ND 24 17	16
Cephalexin (14 μM)	200 50 20	ND 0.97 0.38	ND 52 53	63
Cephaloridine (84 μM)	200 50 20	11 2.4 1.0	18 21 20	8
Ceftezole (102 μM)	200 50 20	65 12 7.8	3.1 4.2 2.6	16

[a] Part of these data were reported previously as communications to the editor of this journal[1].

[b] The MICHAELIS constants (*Km*s) of the *P. morganii* cephalosporinase for the antibiotics were taken from the data of FUJII-KURIYAMA *et al.*[3] and our unpublished data.

[c] The antibiotic concentration in the periplasm (S_2 μM) was estimated from the following formula[1]. Where, S_1 is the antibiotic concentration (μM) outside the bacterial cells. Vi and Vd are defined as the enzyme activities of a bacterial cell suspension at the substrate concentration of S_1 μM before and after disruption, respectively.

$$[S_2]=\frac{Vi}{Vd}\left(\frac{Km\cdot[S_1]}{Km+[S_1]-(Vi/Vd)\cdot[S_1]}\right)$$

[d] ND, Not determined.

Table 2. Permeability of β-lactam antibiotics through the outer-membrane of *Citrobacter freundii* GN346.

Antibiotic and (*Km*)[a]	Conc. of S_1 (μM)	Estimated conc. of S_2 (μM)	S_1/S_2	MIC to *C. freundii* GN346/16 (μM)
Benzylpenicillin[b] (24 μM)	200 50 20	ND[c] 0.11 0.07	ND 455 286	63
Ampicillin[b] (32 μM)	200 50 20	ND 0.13 0.09	ND 385 222	16
Cephalothin (16 μM)	200 50 20	ND 0.08 0.06	ND 625 333	32
Cefazolin (435 μM)	200 50 20	1.8 0.58 0.26	111 86 77	8
Cephalexin (77 μM)	200 50 20	1.4 0.39 0.13	143 128 154	63
Cephaloridine (455 μM)	200 50 20	3.9 0.51 0.23	51 98 87	16
Ceftezole (95 μM)	200 50 20	3.2 0.57 0.11	63 88 182	8

[a] The *Km* values were taken from previous[4] and unpublished data.

[b] The outer-membrane permeability of benzylpenicillin and ampicillin were measured by using *C. freundii* GN346/16 RGN823 as the test organism because the penicillins are so poor substrate for the cephalosporinase of *C. freundii*.

[c] ND, Not determined.

P. morganii and *C. freundii*. Ampicillin, the first semisynthetic penicillin active against Gram-negative bacteria, has about 15-times higher permeability than benzylpenicillin in *P. morganii*, and the difference of antibacterial activity between ampicillin and benzylpenicillin can be accounted for by this difference in permeability. However, in the cases of *E. coli* and *C. freundii*, this characteristic of ampicillin is not seen. The barrier effect of the outer-membrane against the antibiotics-penetration varies from species to species. The *C. freundii* cell has extremely high barrier against both penicillins and cephalosporins.

The MICs of the antibiotics for a cephalosporinase-less mutant from *P. morganii* 1510 shows that there is a close relationship between the permeability and antibacterial activity except in the case of

cephalexin. In *E. coli* and *C. freundii*, similar relationship are observed except in the cases of ampicillin and cephalexin.

The Permeability-hydrophobicity Relationship in β-Lactam Antibiotics

The relationship between the chemical structure and the outer-membrane permeability of

Fig. 1. The relationship between hydrophobicity of β-lactam antibiotics and their permeability to the *P. morganii* outer-membrane.
Abbreviations: PCG, benzylpenicillin; APC, ampicillin; CET, cephalothin; CEZ, cefazolin; CEX, cephalexin; CER, cephaloridine; CTZ, ceftezole.

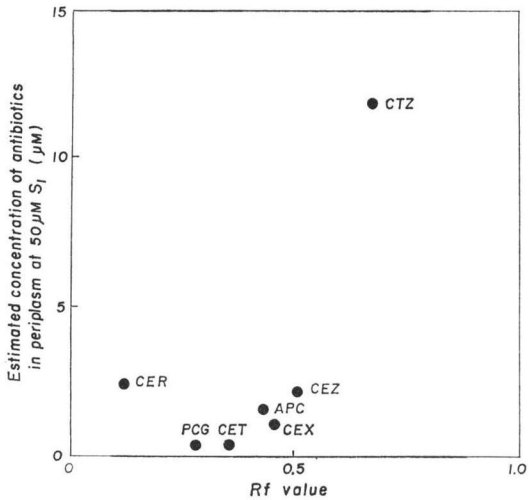


Fig. 2. The relationship between hydrophobicity of β-lactam antibiotics and their permeability to the *C. freundii* outer-membrane.

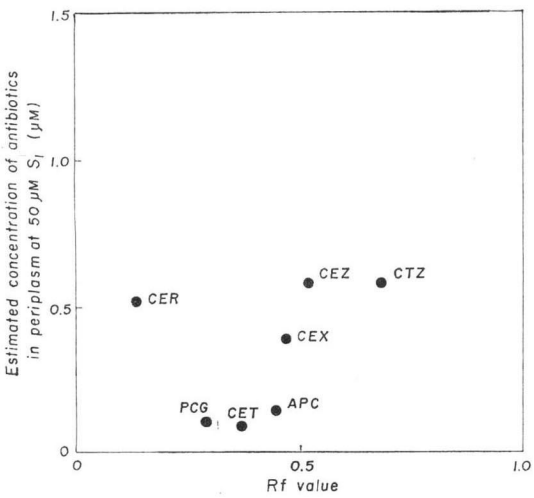
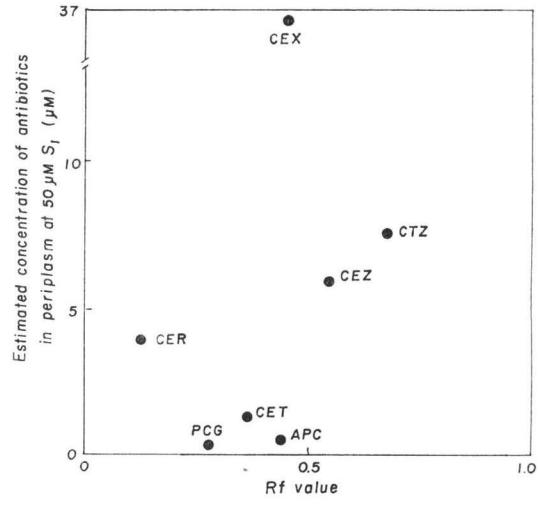


Table 3. Permeability of β-lactam antibiotics through the outer-membrane of *Escherichia coli* ML 1410 RGN823.

Antibiotic and (*Km*)[a]	Conc. of S_1 (μM)	Estimated conc. of S_2 (μM)	S_1/S_2	MIC to *E. coli* ML 1410 (μM)
Benzylpenicillin (24 μM)	200	ND[b]	ND	63
	50	0.15	333	
	20	0.10	200	
Ampicillin (32 μM)	200	ND	ND	16
	50	0.32	156	
	20	0.24	83	
Cephalothin (67 μM)	200	2.5	80	16
	50	1.2	42	
	20	0.46	43	
Cefazolin (250 μM)	200	34	5.9	4
	50	5.9	8.5	
	20	5.0	4.0	
Cephalexin (740 μM)	200	200	1.0	16
	50	37	1.4	
	20	18	1.1	
Cephaloridine (400 μM)	200	41	4.9	8
	50	3.9	13	
	20	1.2	17	
Ceftezole (333 μM)	200	ND	ND	4
	50	7.4	6.8	
	20	1.9	11	

[a] The *Km* values were taken from a previous paper[6] and unpublished data.
[b] ND, Not determined.

Fig. 3. The relationship between hydrophobicity of β-lactam antibiotics and their permeability to the *E. coli* outer-membrane.



β-lactam antibiotics is particularly interesting. The hydrophobic character of the 7 β-lactam antibiotics was measured by means of reversed-phase thin-layer chromatography. Hydrophobicity of the tested antibiotics were expressed as the chromatographic value. The Rf values were widely distributed from 0.68 of ceftezole to 0.13 of cephaloridine. The Rf values of the 7 antibiotics, ceftezole, cefazolin, cephalexin, ampicillin, cephalothin, benzylpenicillin and cephaloridine were 0.68, 0.52, 0.47, 0.45, 0.37, 0.29 and 0.13, respectively. Lower value indicates higher hydrophobicity. The relationship between hydrophobicity and permeability is graphically illustrated in Figs. 1, 2 and 3. Benzylpenicillin and cephalothin are the antibiotics which exhibited lower-membrane permeability among the tested antibiotics, and the 2 antibiotics also have similar Rf values. It was generally found that increasing the Rf value tends to increase the outer-membrane permeability. On the other hand, cephaloridine which has a lower Rf value indicating higher hydrophobic property showed higher permeability than other antibiotics except ceftezole.

Discussion

As mentioned in a previous paper[1], the ratio S_1/S_2 is an useful parameter for the outer-membrane permeability of β-lactam antibiotics. On the basis of the parameter, it is assumed that ceftezole, cefazolin and cephaloridine possess good outer-membrane permeability. On the contrary, benzylpenicillin and cephalothin showed lower permeability. These facts are consistent with relatively low antibacterial activity of the 2 antibiotics.

In the *C. freundii* strain, no significant difference in the ratio S_1/S_2 between benzylpenicillin and ampicillin was observed. Such a lower permeability of ampicillin was also observed in the *E. coli* strain. These are unexpected results because the MIC value of ampicillin in significantly higher than that of benzylpenicillin and both the penicillins are believed to possess about the same level of inhibitory activity against their target enzymes. These results may be attributed to the presence of R plasmid in the cells. Recently, KENWARD *et al.*[13] reported that an R plasmid, RP1, confers a change in composition of the outer-membrane of *Pseudomonas aeruginosa*, and reduces the sensitivity of the host bacteria to chelating agents, polymyxin B and cold shock treatment.

The barrier effect of the outer-membrane on antibiotic penetration varies from species to species. The data in Tables 1~3 indicate that the *C. freundii* strain has the highest barrier against both penicillins and cephalosporins. Stability of the bacterial outer-membrane in the buffer solution or to EDTA is not necessary correlative with the level of the barrier against the antibiotics.

There is a close relationship between hydrophobic character and antibacterial activity against Gram-negative bacteria in β-lactam antibiotics, more hydrophilic β-lactam antibiotics are generally more active against Gram-negative bacteria[14]. It was also assumed that a more hydrophilic β-lactam antibiotic is able to pass more easily through the outer-membrane of Gram-negative bacteria[14]. Direct comparison between the outer-membrane permeability and the hydrophobic character of 7 β-lactam antibiotics in this study supports this assumption. However, cephaloridine, the most hydrophobic antibiotic among the 7 β-lactam antibiotics, showed good permeability. This finding may suggest different penetration routes for β-lactam antibiotics through the outer-membrane of Gram-negative bacteria.

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