

Novel Method for Measurement of Outer Membrane Permeability to New β -Lactams in Intact *Enterobacter cloacae* Cells

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The ability of five new β -lactams to permeate the outer membrane of intact *Enterobacter cloacae* β -lactamase-overproducing cells was measured by using a high-pressure liquid chromatography (HPLC)-based technique that avoided certain possible artifacts of the traditional methods. Low concentrations of antibiotics were mixed with bacterial suspensions, and at different times, the cells were removed from the medium by filtration. Residual β -lactam concentrations in the medium were then assessed by HPLC and UV detection. The assay was performed under conditions in which no β -lactamase activity was detected in the filtrate and the number of viable cells remained constant during the experiment. Outer membrane permeability was assessed with the Zimmermann-Rosselet equation, in which outer membrane permeability was rate limiting for hydrolysis of the β -lactam by periplasmic β -lactamase. Thus, the rate of disappearance of β -lactam was equal to the rate of outer membrane permeation. Preincubation of bacterial suspensions with 300 μ g of cloxacillin per ml inhibited the hydrolysis of β -lactams by intact cells, demonstrating that β -lactam hydrolysis by periplasmic β -lactamase was essential in order to allow measurement of outer membrane permeability by this method. Permeability coefficients (P) were calculated from the Zimmermann-Rosselet equation and were independent of the external concentration of antibiotic over a 100-fold concentration range. Cefepime and cefpirome exhibited rates of outer membrane permeation 5- to 20-fold higher than those of carumonam, ceftriaxone, and cefotaxime. Thus, the presence of a positive charge in the 3-lateral chain increased the permeation ability of β -lactam molecules considerably.

The outer membrane plays an important role in the physiology of gram-negative bacteria (16). Its structure is asymmetric, and it is essentially composed of proteins associated with an external lipopolysaccharide monolayer and an internal phospholipid monolayer (2, 4). The bacterial cell is surrounded by this asymmetric bilayer which controls, largely through channel-forming porin proteins, the influx of nutrients and the efflux of metabolic products. It also constitutes a permeation barrier against certain antibiotics and other noxious molecules. The ability to cross the outer membrane is a prerequisite for the action of antibacterial agents on gram-negative cells (6, 21). A large number of hydrophobic and amphiphilic antibiotics such as macrolides, rifamycins, lincosamides, fusidic acid, and novobiocin are thought to be inactive against many gram-negative pathogens because of their slow penetration through the narrow porin channels (16). In the case of β -lactams, outer membrane permeability, in conjunction with affinity for target penicillin-binding proteins and resistance to β -lactamase, is an important codeterminant of efficacy (11). Therefore, quantitative appraisal of outer membrane permeability is essential for understanding how β -lactams act in gram-negative bacteria and for elaborating efficient drug design strategies.

Several methods to measure the ability of β -lactams to penetrate the outer membrane have been designed. Hamilton-Miller (3) and Richmond and Curtis (17) proposed the determination of "crypticity," or a "permeability index," defined as the ratio of hydrolysis rates of antibiotics by disrupted cells to hydrolysis rates of antibiotics by intact

cells. However, this approach produced misleading conclusions, since crypticity varies according to the antibiotic concentration in the external medium (11, 25). Since the water-filled channels of porins control the penetration of small hydrophilic molecules into the periplasm, another method took advantage of reconstituted proteoliposomes containing purified porins to study the diffusion of β -lactams (14, 24). The structural complexity of barriers to antibiotic penetration through the outer membrane was somewhat oversimplified by this approach. In addition, direct penetration of some β -lactams through the lipid bilayers and proteoliposomes (12) and charge and counterion effects have limited the utility of this method (5). Nevertheless, it provided an assessment of the relative importance of the gross physicochemical properties influencing permeation of molecules through porin channels.

Of the methods used to assay the permeability of intact cells, the method of Zimmermann and Rosselet (25) and Sawai et al. (18) was probably the most theoretically correct, since the integrity of the cell wall was maintained during the experiment. The authors postulated that the penetration of β -lactams into bacterial cells equilibrated at a steady state in which the rate of diffusion of β -lactams through the outer membrane was balanced by the hydrolysis of β -lactamase in the periplasmic space. These events were mathematically described by Fick's first law of diffusion and the Michaelis-Menten equation, respectively. The Zimmermann-Rosselet method has allowed the comparison of outer membrane permeabilities among different gram-negative bacteria, but only for antibiotics which were readily hydrolyzed. Unfortunately, permeability parameters for the recently developed β -lactams that were much more β -lactamase stable could not be accurately determined under the same experimental con-

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TABLE 1. HPLC conditions for analyzing β -lactams

β -Lactam	Mobile-phase ratio (0.1 M NaClO ₄ : methanol)	Retention ^b time (min)	UV detection wavelength (nm)
Ceftriaxone	65:35	4.8	254
Cefotaxime	65:35	4.6	254
Cefpirome	65:35	3.8	254
Cefepime	70:30	3.6	254
Carumonam	65:35	5.0	295

^a 0.1 M NaClO₄ was adjusted to pH 2.5 with concentrated sulfuric acid.

^b At room temperature and at a flow rate of 1 ml/min.

ditions. The modification proposed by Kojo et al. (8) to circumvent this problem provided data that were internally inconsistent, as discussed by Nikaido (11).

In this paper, we propose a new method which allows the study of the outer membrane permeation by new β -lactams of intact cells by using a high-pressure liquid chromatography (HPLC) technique.

MATERIALS AND METHODS

Bacterial strain and growth medium. Of the strains used in a previous study of the mechanisms of resistance in *Enterobacter cloacae*, we selected strain 218 R1 because of its high level of chromosomal β -lactamase production (240,000 molecules per cell) and its unmodified outer membrane protein pattern compared with that of the wild type (10). Antibiotic medium no. 3 was purchased from Difco Laboratories, Detroit, Mich.

Diffusion of β -lactam antibiotics into intact cells. Cells were grown in antibiotic medium no. 3, harvested in late exponential phase (optical density at 650 nm, 0.7 to 0.8), washed twice at 4°C, and gently suspended (30 ml/g of drained pellet) in the same buffer (10 mM morpholinepropanesulfonic acid [MOPS], pH 6.5). The dry weight of bacterial cells was determined by drying 1.00 ml of bacterial suspension in an oven at 105°C to constant weight. To prevent damage to the outer membrane and β -lactamase release, growth media, wash, and suspension buffer contained 5 mM MgCl₂. The cells were maintained at room temperature for 30 min. Five hundred microliters of antibiotic solution at an appropriate concentration (10 to 1,000 μ M for most antibiotics, according to the experiment) was added to 4.5 ml of bacterial suspension. At different time intervals, samples were withdrawn and rapidly filtered (0.45- μ m-pore-size disposable filters; Alltech Inc., Deerfield, Ill.). Before and after the last sample, cell viability was checked by determination of CFU. In the filtrate, β -lactamase activity was detected by using the chromogenic β -lactam nitrocefin (10), and β -lactam compounds were assayed by HPLC. HPLC was performed at a flow rate of 1 ml/min with a System Gold apparatus (Beckman Instruments, Inc., San Ramon, Calif.) which included a model 126 programmable solvent delivery system and an on-line NEC controller. Isocratic chromatography analyses were carried out on a reverse-phase Beckman Ultrasphere IP ion pair (5 μ m; 4.6 by 150 mm) analytical column. Samples were injected with a 20- μ l loop syringe loading-sample Beckman injector (model 210A). The aqueous-organic mobile phase consisted of 0.1 M sodium perchlorate, adjusted to pH 2.5 with concentrated sulfuric acid, and methanol at an appropriate ratio (Table 1). β -Lactams were detected at their absorbance maxima by UV detection with a Beckman variable-wavelength monitor (model 166). Quanti-

tation was performed by integration of the peak areas. Standard curves with known concentrations of antibiotics were used to test the linearity of the response of the HPLC systems. All experiments were performed at β -lactam concentrations within the limits giving rise to linear responses.

Permeability studies. Permeability coefficients (P) for cefpirome, cefotaxime, and the other β -lactams were calculated from the Zimmermann-Rosselet equation:

$$V = P \times A \times (S_o - S_p) = V_{\max} \times \frac{S_p}{S_p + K_m} \quad (1)$$

The rate of hydrolysis by intact cells (V) was measured directly by the HPLC method, V_{\max} and K_m values were determined as in reference 1, and S_o and S_p were the external and internal (periplasmic) concentrations of antibiotic, with values of S_p as determined as in reference 1. A represented the area of cell surface per unit of weight (132 cm² · mg⁻¹). This value was determined for *Salmonella typhimurium* (20), and it has been assumed to be identical for all the other enteric gram-negative bacteria. Although this assumption has never been proven, we used this value in order to be able to compare our permeability coefficients with those previously published (13, 23–25).

RESULTS

In this new method, after bacterial cells and β -lactam were mixed for various periods, the cells were removed from the medium by filtration, and the residual β -lactam in the supernatant was then assayed by HPLC and UV detection. This guaranteed a specific and accurate analysis. The solvent systems used in this study allowed the chromatography of all of the β -lactams tested within a few minutes (Table 1) and provided excellent resolution between antibiotic and uncharacterized peaks (Fig. 1). The uncharacterized peaks were also detected when no hydrolysis was measured (e.g., in the presence of cloxacillin) and were probably waste products excreted by the bacteria. The response of the HPLC system was linear within the range of antibiotic concentrations used. Furthermore, the antibiotic disappearance in the medium could be monitored very precisely at concentrations as low as 1 μ M, depending on the UV detector sensitivity and the extinction coefficient of each molecule. Initial rates could be measured within 1 or 2 min for fast-penetrating compounds such as cefpirome and cefepime. For the other compounds, 10 min or even longer was necessary to accurately determine the initial rates. No significant β -lactamase activity (<0.01 mU/ml) was detected in filtrates with the sensitive nitrocefin test, indicating that almost no enzyme was released into the medium during the experiment. The number of viable cells (2×10^9 to 3×10^9 CFU/ml) remained stable during the 30 min of measurements. Preincubation of bacterial suspensions for 10 min within 300 μ g of the β -lactamase inhibitor cloxacillin per ml inhibited the disappearance of β -lactams from the medium (Fig. 2), showing that β -lactam hydrolysis was required for this assay method. The HPLC separation profiles of two β -lactams with closely related structures at different times of incubation with intact cells are shown in Fig. 1. Cefpirome was hydrolyzed much more rapidly than cefotaxime. Hydrolysis rates by intact cells were similarly measured for five diverse β -lactam antibiotics and allowed the calculation of outer membrane permeability coefficients (Table 2). Carumonam, and to a lesser extent cefotaxime and ceftriaxone, exhibited low outer membrane permeation. On the other hand, P values for cefepime and cefpirome were 5-

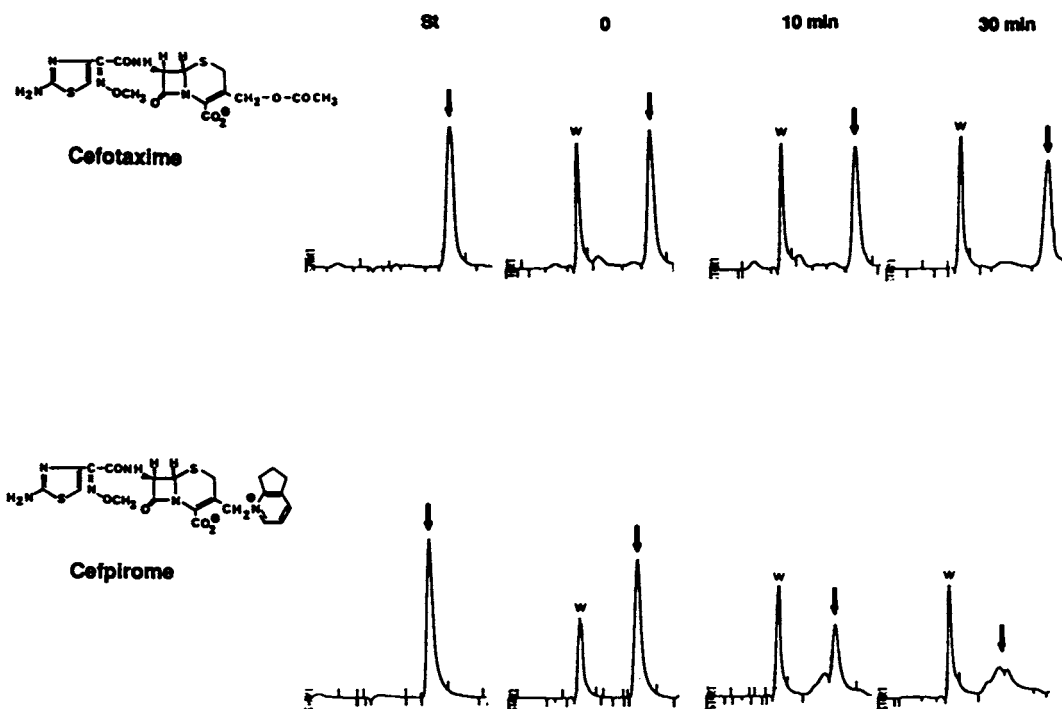


FIG. 1. HPLC separation profiles of cefotaxime and cefpirome at three different times of incubation with intact *E. cloacae* 218 R1 cells. Initial concentrations of both antibiotics were 15 $\mu\text{g/ml}$. Arrows, Peaks corresponding to the antibiotics; St, standard concentration (15 $\mu\text{g/ml}$) of antibiotic (control); w, waste products excreted by the bacteria.

to 20-fold higher. These results (Table 2) clearly indicated that the presence of a net positive charge in the β -lactam structure increased its ability to permeate. The two compounds exhibiting the highest P values, i.e., cefpirome and cefepime, possessed a fixed positive charge borne by the quaternary ammonium of a pyridine ring. Negatively charged β -lactams exhibited reduced permeation, while carumonam, a monobactam compound with two negative charges, exhibited the lowest P value of all of the β -lactams tested. This result was consistent with the assumptions that the major porins of *E. cloacae* are cation selective, like those of *Escherichia coli* (14), and that the high concentration of fixed negatively charged molecules in the periplasm creates

a Donnan potential (interior negative) of around -40 mV (22).

To demonstrate that we were measuring outer membrane permeability and not just hydrolysis by external, outer-membrane-associated β -lactamase, we assessed the effect on the permeability coefficient of changing the cefpirome concentration. If hydrolysis by surface-attached β -lactamase was being measured, then according to Michaelis-Menten kinetics, hydrolysis rates should have saturated at high concentrations (thus decreasing the calculated P value). Conversely, Fick's first law of diffusion predicts that diffusion rates across the outer membrane should be a linear function of external β -lactam concentrations (resulting in a constant P value). The permeability coefficients were determined at seven different antibiotic concentrations ranging from 7 to 700 $\mu\text{g/ml}$ (Table 3), i.e., 0.05 to 5 times the K_m . No

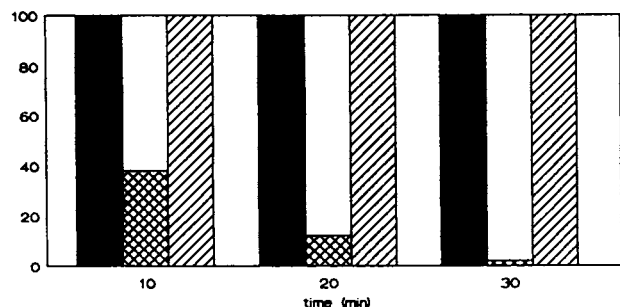


FIG. 2. Effect of preincubation of 300 μg of cloxacillin per ml on the hydrolysis of cefpirome (15 $\mu\text{g/ml}$ = 100%) by intact *E. cloacae* 218 R1 cells. Symbols: ■, cefpirome incubated without cells (blank control); ▨, R1 cells without cloxacillin; ▩, R1 cells with cloxacillin.

TABLE 2. Outer membrane permeability coefficients of β -lactams in *E. cloacae* 218 R1

β -Lactam	Ionic charge ^a	Molecular mass ^b (g/mol)	Permeability coefficient ^c (nm/s)
Cefpirome	-+	500	37
Cefepime	-+	466	29
Cefotaxime	-	454	5.6
Ceftriaxone	--	552	5.1
Carumonam	--	466	1.9

^a These compounds may also carry, at physiological pH, an additional partial positive charge on the primary amine of their aminothiazole moieties.

^b Molecular masses are those of free ionic molecules.

^c Values were determined with external antibiotic concentrations ranging from 10 to 20 μM for most antibiotics.

TABLE 3. Effects of different external ceftiprome concentrations on outer membrane permeability coefficient in *E. cloacae* 218 R1

Concn (μ g/ml)	Permeability coefficient (nm/s) \pm SD ^a
7	37 \pm 4
12	45 \pm 6
25	34 \pm 5
50	29 \pm 6
75	35 \pm 5
100	40 \pm 7
700	31 \pm 6

^a From three determinations.

significant differences were observed ($P < 0.05$; Student *t* test). These results suggested that we were measuring permeability and further indicated that there was no detectable β -lactamase activity on the external outer membrane surface of *E. cloacae* 218 R1.

DISCUSSION

Quantitative aspects of how new β -lactams penetrate into gram-negative bacteria have remained largely unknown because of difficulties in measuring hydrolysis rates with intact cells. In part, these difficulties have resulted from technical limitations. Earlier studies (15, 25) spectrophotometrically assessed the disappearance of antibiotic in microcuvettes containing dense bacterial cell suspensions. Although this method has been used for readily hydrolyzable compounds, it seems impractical for more β -lactamase-stable antibiotics.

Alternatively, outer membrane permeation values extrapolated from assays of uptake of β -lactams into proteoliposomes incorporate assumptions (12) which, on the basis of comparison of literature data with the numbers obtained in this paper, may not be entirely correct (13). In this study, we have proposed a technical approach based on the HPLC analysis of residual cell-free β -lactams. The use of this new protocol eliminated several sources of potential artifacts created by traditional methods, including extensive light scattering of bacterial suspensions in microcuvettes, which considerably reduced the sensitivity of the measurements; interference by fluctuant waste products that were excreted by bacteria over the time of the assay and that absorbed at the same wavelength as β -lactam molecules (Fig. 1); and the necessity, in order to monitor the hydrolysis by intact cells, of using high concentrations (0.1 to 1 mM) of antibiotics that are in the majority of cases pharmacologically irrelevant and do not mimic the actual physiological conditions of *in vivo* hydrolyses (i.e., periplasmic antibiotic concentrations lower than the K_m values).

Another advantage of this new method is the development of a simple HPLC system suitable for essentially all new β -lactams. Most of these molecules, which are as structurally diverse as monobactams and aminothiazole cephalosporins, can be resolved in a few minutes without changing any chromatographic parameter.

Permeability coefficients can be calculated from equation 1, assuming that at steady state, the rate of β -lactam diffusion through the outer membrane is balanced by the rate of β -lactamase hydrolysis in the periplasmic space. Since the validity of this postulate has never been experimentally assessed, we tested it in two different ways. Firstly, if the penetration of antibiotics through the outer membrane

obeyed Fick's first law of diffusion, the rate of influx should be proportional to the antibiotic concentration gradient and the calculated permeability coefficient should remain constant at any external concentration. Secondly, if the β -lactam hydrolysis by intact cells was driven only by the β -lactamase and no other physical or chemical phenomenon interfered with the reaction, one would expect that the presence of a strong class 1 β -lactamase inhibitor, such as cloxacillin, would completely prevent the hydrolytic process during permeability-limited disappearance of β -lactam from the medium (note that because of the small volume of the periplasm, equilibration of β -lactam molecules across the outer membrane gradient in the absence of β -lactamase would be accomplished within a second or so and would have virtually no effect on the external β -lactam concentration). Our data strongly suggest that despite its technical limitations, the theoretical bases underlying the Zimmermann-Rosselet method are rigorously correct (Table 3; Fig. 2). These results were in contrast with those published by Hewinson et al. (7). These authors, using the traditional Zimmermann-Rosselet method to measure the permeation of cephalosporinase C in *Pseudomonas aeruginosa*, found that the permeability coefficient varied according to the external antibiotic concentration. However, these results could probably be due to the activity of β -lactamase on the outer membrane surface, since the hydrolysis rates plotted in a linear reciprocal plot as a function of cephalosporin C concentration (i.e., a Lineweaver-Burk plot) predict a K_m similar to the K_m of free β -lactamase for this β -lactam.

Vu and Nikaido (23) and Nikaido et al. (13) published permeability coefficients of intact *E. cloacae* cells for some expanded-spectrum and "fourth-generation" cephalosporins, respectively. It should, however, be noted that these values were not determined experimentally but were derived by normalizing the proteoliposome permeation rate of these products by using the permeability coefficients of intact cells with cephaloridine (23) or cephalozine (13) while making adjustments for the influence of Donnan potential. Actually, several limitations could bias the results obtained by the liposome swelling assay (for a discussion, see references 5 and 10). Nevertheless, this method apparently allowed a reasonable estimate of relative penetration rates of certain antibiotics. Our data (Table 2) differed from those determined previously by plus or minus twofold. Discrepancies between the two methods could be explained by two potential problems related to extrapolation of liposome swelling data. Firstly, despite attempts to correct for them, charged compounds and associated counterions create problems in the liposome swelling method (5). Secondly, the physiological Donnan potential effect cannot be reproduced in proteoliposomes. This electric gradient across the outer membrane (19, 22), which should slow down the penetration of negatively charged molecules into the periplasm (12), was corrected for by using a factor of 2.66. However, there is no proof that such a factor adequately corrects for the effect of Donnan potentials in every case. Another source of variation could result from the great fluctuation in the amounts of outer membrane proteins that are expressed from one *E. cloacae* strain to another (10). Recently, Komatsu et al. (9) have isolated an *E. cloacae* gene, *romA*, that greatly influences the posttranscriptional regulation of the outer membrane proteins. Modulations in the expression of this gene could explain the variability in the expression of outer membrane proteins from one strain to another.

The determination of the outer membrane permeation of

five compounds permitted a preliminary analysis of structure-activity relationships. Our data partly confirmed previous results obtained with *E. coli* OmpF-containing proteoliposomes (24) in that the presence of a positive charge in the 3-lateral chain increased considerably the permeation of β -lactams. The influence of charge was especially noticeable for the new fourth-generation cephalosporins cefpirome and cefepime, which bore a net positive charge in the quaternary ammonium of a pyridine ring, in contrast to the expanded-spectrum cephalosporins cefotaxime and ceftriaxone.

We have developed a method that allows for the first time the reliable determination of the *in vivo* outer membrane permeation of β -lactamase-resistant β -lactams in intact cells. Using the same approach, we have been able to measure decreased outer membrane permeability in a porin-altered strain of *E. cloacae* and to assess the role of outer membrane permeability in the efficacy of β -lactam antibiotics (1). One possible limitation in applying this method to other gram-negative bacteria would be the presence of β -lactamase activity at the external outer membrane. Hence, care should be taken that this source of artifact does not bias the data, by measuring β -lactamase in the filtrates and by assessing the concentration dependence of *P* values.

ACKNOWLEDGMENTS

This work was made possible by a postdoctoral fellowship and grant (31-28007-89) from the Fonds National Suisse de la Recherche Scientifique to F.B. and J.C.P., respectively, and by financial assistance from the Canadian Cystic Fibrosis Foundation and the Natural Sciences and Engineering Research Council of Canada to R.E.W.H.

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