Diffusion of Meropenem and Imipenem through the Outer Membrane of *Escherichia coli* K-12 and Correlation with Their Antibacterial Activities

GIUSEPPE CORNAGLIA,[1]* LIMING GUAN,[1,2] ROBERTA FONTANA,[1] AND GIUSEPPE SATTA[3]

Istituto di Microbiologia, Università degli Studi di Verona, Strada Le Grazie 8, I-37134 Verona,[1] and Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Largo F. Vito 1, I-00168 Rome,[2] Italy, and Department of Microbiology, China Medical University, Shenyang, People's Republic of China[2]

The outer membrane permeability to meropenem and imipenem in *Escherichia coli* K-12 was investigated, and its porin-deficient mutants were transformed with a constructed vector carrying the carbapenem-hydrolyzing CphA metallo-β-lactamase gene. By using the method of Zimmermann and Rosselet, meropenem was shown to penetrate through the outer membrane of *E. coli* K-12 five times faster than cephaloridine but twice as slowly as imipenem. Lack of one or both porins significantly reduced the penetration of both carbapenems. No evidence of specific porin pathways of the type described in *Pseudomonas aeruginosa* was found. Despite its slower penetration, meropenem was two to eight times more active than imipenem against both parent and porin-defective mutants, whether harbouring CphA β-lactamase or not. Meropenem was also more active than imipenem against *E. coli* DC2, a strain with a breakdown in the outer membrane permeability which made periplasmic concentrations of β-lactams similar to the external concentrations. In this strain, meropenem caused a more than 50% reduction in cell number increase at a concentration very close to the 50% inhibitory concentration for penicillin-binding protein type 2 (PBP 2), whereas imipenem, at the same concentration, did not significantly inhibit cell growth. This result was explained by the higher affinity of meropenem for PBP 3 compared with imipenem and supports the conclusion that synergistic inhibition of both PBPs was the main mechanism in the better antibacterial activity of meropenem.

Meropenem is a new carbapenemic compound with a spectrum of activity comparable to that of imipenem but with enhanced activity against many gram-negative species (7, 15, 22). This activity is related mainly to its high affinity for penicillin-binding proteins (PBPs) (34) and to its high resistance to most bacterial β-lactamases (7, 22). In several bacterial species, alterations of outer membrane permeability have also been shown to make an efficient contribution to carbapenem antimicrobial activity. In *Pseudomonas aeruginosa*, these compounds prove capable of penetrating cells via alternative, specific pathways in addition to the widely known, nonspecific porins, and the absence of these specific channels implies a reduced outer membrane permeability to carbapenems but not to cephaloridine (35). Conversely, the permeability coefficient of imipenem through the *Escherichia coli* outer membrane had been reported to be very similar to that of cephaloridine, even though the data were obtained not with intact cells, as was the case in *P. aeruginosa*, but with reconstituted proteoliposomes and only subsequently normalized to intact cell values (23, 40).

The existence of specific pathways for the carbapenems has never been demonstrated in members of the family *Enterobacteriaceae*, although at times it has been postulated in both wild and mutant strains, on the basis of evident discordances between permeability patterns and susceptibility data (8–10). Conversely, nonspecific porins have been shown to play a role in resistance to carbapenems in *Providencia rettgeri* (27) and in two *Enterobacter* species (12, 18, 27).

Recently, a carbapenem-hydrolyzing β-lactamase (CphA)

has been cloned from *Aeromonas hydrophila* into *E. coli* (21). This makes it possible to study the penetration kinetics of carbapenems in intact *E. coli* cells, since the method devised for this purpose by Zimmermann and Rosselet (26, 42) requires the strain under investigation to harbor a suitable β-lactamase. In this work, we investigated the permeability of intact *E. coli* cells to meropenem and imipenem by transferring the CphA β-lactamase into *E. coli* K-12 and its porin-deficient mutants. We also investigated the poor correlation between the antibiotic influx kinetics and the susceptibility patterns shown by these strains.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the experiments on outer membrane permeability are listed in Table 1. Strains pop1010, B1466, B1449, B1478, B1467, and pop1389 were previously described derivatives of *E. coli* K-12 (14) and were obtained from A. Jaffé. They differed from one another in their outer membrane protein patterns, but no differences in patterns of PBPs or in β-lactamase activity could be detected between the mutant and the parental strains. Strains LGC10, LGC66, LGC49, LGC78, LGC67, and LGC89 were constructed by transforming *E. coli* K-12 and its porin-deficient derivatives with plasmid pAA20R, which contained the gene coding for a carbapenem-hydrolyzing β-lactamase (CphA), cloned from *A. hydrophila* (21). Strain LGC01 was constructed by transforming *E. coli* K-12 with the recombinant plasmid pAS50, which had been obtained in the very first phases of the *cphA* gene cloning and which coded for both CphA and TEM-1 β-lactamases (21). In the experiments with the Coulter Counter (see below), we used the *E. coli* mutant DC2 (kindly donated by David

---

* Corresponding author.

TABLE 1. Designation, relevant genotype, and origin of strains

Strain	Relevant genotype[a]	Phenotype	Source or reference
DC2		OmpF$^+$ OmpC$^+$	28
pop1010	Hfr *his metA rpoB*	OmpF$^+$ OmpC$^+$	38
B1478	Hfr *his metA aroB rpoB ompC*::Tn5	OmpF$^+$ OmpC$^-$	14
B1449	Hfr *his metA aroB rpoB ompF*::Tn5	OmpF$^-$ OmpC$^+$	14
pop1389	Hfr *his metA rpoB ompB101 lac*	OmpF$^-$ OmpC$^-$	38
LGC01	pAS50 transformant of pop1010	OmpF$^+$ OmpC$^+$ CphA$^+$ TEM$^+$	This study
LGC10	pAA20R transformant of pop1010	OmpF$^+$ OmpC$^+$ CphA$^+$	This study
LGC78	pAA20R transformant of B1478	OmpF$^+$ OmpC$^-$ CphA$^+$	This study
LGC49	pAA20R transformant of B1449	OmpF$^+$ OmpC$^+$ CphA$^+$	This study
LGC89	pAA20R transformant of pop1389	OmpF$^-$ OmpC$^-$ CphA$^+$	This study

[a] All strains are *E. coli* K-12 derivatives. Genetic nomenclature is from Bachmann and Low (1).

Clark), which has been shown to have a breakdown in the outer membrane permeability barrier (6). Each strain was kept frozen in brain heart infusion broth plus 10% glycerol at −80°C until used.

Culture media and growth conditions. Luria-Bertani (LB) broth containing 5 mM MgSO$_4$ was used for all tests. LB broth is 1% tryptone (Difco Laboratories, Detroit, Mich.)–0.5% yeast extract (Difco Laboratories)–1% NaCl adjusted to pH 7.5 with NaOH. Culture medium for *E. coli* strains harboring plasmids or transposons was supplemented with the appropriate antibiotic for selection and maintenance of the transferable elements.

Antibiotics. Working solutions were prepared on the day of use from laboratory standard powders of all compounds as specified by the manufacturers. Meropenem was provided by ICI-Pharma, Milan, Italy. Cylastatin-free imipenem was provided by Merck Sharp & Dohme, Rome, Italy. All other antibiotics were from commercial sources.

Susceptibility tests. Antibiotic susceptibility tests were performed by serial twofold dilution in Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) essentially as described by Sahm and Washington (29). The inoculum consisted of either 10^6 or 10^4 CFU per spot applied with a Steers replicator (33). The MIC was defined as the lowest concentration that prevented visible growth after incubation for 18 h at 35°C in air.

Plasmid extraction and bacterial transformation. Alkalidenaturing methods described by Kado and Liu (16) and Birnboim and Doly (3) were used for plasmid extraction. Cells were made competent for transformation as described by Sambrook et al. (30), i.e., with a variation of the calcium chloride procedure of Cohen et al. (6). The selective tetracycline concentration was 50 μg/ml.

Permeability assays with intact cells. The cells were grown until the mid-exponential growth phase and then harvested by centrifugation. They were washed twice with 10 mM sodium phosphate buffer (pH 7) supplemented with 5 mM MgCl$_2$, resuspended in the same buffer, and sonicated with a Labsonic 2000 ultrasonic disrupter (B. Braun Melsungen AG, Melsungen, Germany) by three or four 15-s pulses of sonication with intervening 30-s periods in ice to minimize β-lactamase damage. Centrifugation or filtration to remove cellular debris was omitted, since both these procedures involve substantial loss of enzyme activity (roughly 50%); the untreated sonic fluid caused no scattering or other drawbacks when spectrophotometric assays were performed, and thus we used it as the crude enzyme preparation.

The hydrolysis rates of intact cells and sonic extracts of

cells by 10 μM antibiotic were obtained with a Beckman DU-7 UV spectrophotometer, with readings recorded at 10-s intervals for 5 min, operating at the wavelength at which the difference between the extinction values of the hydrolyzed and nonhydrolyzed molecule was maximal. The exact wavelengths used were as follows: cephalothin, 265 nm; cephaloridine, 260 nm; and imipenem and meropenem, 298 nm. The millimolar differential absorbance value for all the β-lactams tested was 8.0 mM^{-1} cm^{-1} at the respective wavelengths. To measure the extent of leakage of enzyme into the medium, the rate of β-lactam hydrolysis was measured with supernatants obtained by centrifugation of the intact-cell suspension and the intact-cell hydrolysis rates were corrected for the contribution by extracellular enzyme. The permeability coefficient was calculated by using the method of Nikaido et al. (26) with the modifications proposed by Yoshimura and Nikaido (41).

β-Lactamase catalytic properties. The Michaelis constant (K_m) and the maximum rate of hydrolysis (V_{max}) were determined by using the Eadie-Hofstee plot of initial velocity (v) at six different substrate concentrations. We used 20-fold diluted crude cellular extracts as the enzyme sources, and the hydrolysis of β-lactam antibiotics was followed by a spectrophotometric assay under the aforementioned conditions. Alternatively, the K_m values were obtained directly from a time course of the hydrolysis data, as previously described (25, 37), with no significant differences in the values obtained. Table 2 lists the catalytic properties of the β-lactamases used in the present study.

The influences of pH and ionic strength on CphA β-lactamase activity were evaluated by using 10 mM sodium phosphate buffer supplemented with 5 mM MgCl$_2$ and with variable amounts of NaCl.

Inhibition of carbapenem transport. The possible influence of basic amino acids on carbapenem transport was studied by monitoring the hydrolysis of imipenem by the intact cells as described above. Inhibition was determined by adding

TABLE 2. Catalytic properties of the β-lactamases used in this study

β-Lactam	Enzyme	V_{max} (μmol/mg/min)	K_m (μM)
Meropenem	CphA	5.6	962
Imipenem	CphA	0.8	281
Cephaloridine	TEM type	3.9	865
Cephalothin	TEM type	0.3	300

inhibitors to the cell suspension (at a final concentration of 1 mM) either prior to the addition of imipenem or simultaneously.

Purification of the outer membrane. Cells were grown in LB broth up to the late exponential phase of growth, harvested by centrifugation, washed with 10 mM phosphate buffer (pH 7.0), resuspended in a small amount of the same buffer, and broken with the aforementioned ultrasonic disrupter by four 30-s pulses of sonication with intervening 30-s intervals in ice. The unbroken cells were removed by centrifugation at 8,000 × g for 20 min, and crude membranes were pelleted by centrifugation of the supernatant at 100,000 × g for 30 min at 4°C. Pellets were resuspended in 500 μl of 10 mM phosphate buffer (pH 7.0) containing 15 μl of 35% (wt/vol) sodium lauryl sarcosinate (Sarkosyl NL-30; Serva, Heidelberg, Germany), kept for 20 min at 37°C, centrifuged at 100,000 × g for 30 min at 4°C, reextracted at room temperature for 5 min with 10 ml of phosphate buffer plus 1% (vol/vol) Sarkosyl, and centrifuged at 100,000 × g for 30 min at 4°C. Finally, the pellets were resuspended in 1 ml of 10 mM phosphate buffer (pH 7.0) and stored at −80°C.

Alternatively, the rapid procedure described for *P. aeruginosa* by Trias and Nikaido (36) was used.

Assay of PBPs. PBPs in cells envelopes of *E. coli* DC2 were determined as described by Spratt (32), except that the PBP labeling was done by using [³H]benzylpenicillin (20 Ci/mmol; Amersham International plc, Amersham, United Kingdom). The affinity of PBPs for meropenem and imipenem was determined by using the standard competition procedure (32). Cell envelopes were incubated for 10 min at 30°C with a range of twofold-increasing concentrations of unlabeled β-lactams and, thereafter, for 10 min at 30°C with [³H]benzylpenicillin at a final concentration of 30 μg/ml. PBPs were detected by fluorography after exposure of the gel to an X-ray film (X-OMAT AR; Kodak) for 2 weeks at −80°C.

The relative intensities of the PBP bands on the fluorograms were quantified with a densitometer and corrected for variations in width. The IC_{50} was defined as the concentration of β-lactam required to reduce the binding of ³H-labeled penicillin to PBPs by 50%.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (17). For analysis of the outer membrane preparations, solid urea was added to both stacking and resolving gels (at 4 M) and to the sample buffer (at 8 M). Proteins were detected by Coomassie blue staining.

Cell number counting. The effect of antibiotic on cell number increase was determined by counting the cell number with a Coulter Counter (model ZBI) equipped with a 50-μm orifice, after different incubation times in the presence of different concentrations of antibiotic. Serial culture samples (0.5 ml) were diluted in 0.5 ml of ISOTON II (Coulter Scientific) containing 0.5% formaldehyde. Cell suspensions were fixed at room temperature for 20 min and then diluted 200-fold in ISOTON II, and cell numbers were counted.

Other methods. The protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Transformations. Transformation was fully successful with all strains when plasmid pAA20R was used. With plasmid pAS50, we were able to profitably exploit only the
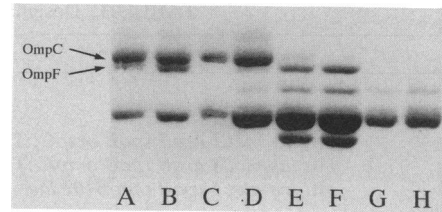

FIG. 1. Outer membrane proteins of *E. coli* K-12 derivatives, separated on a 12% acrylamide gel containing 4 M urea. Outer membranes were prepared from various strains grown in LB broth, as described in Materials and Methods. Lanes: A, pop1010 (OmpF⁺ OmpC⁺ CphA⁻); B, LGC10 (OmpF⁺ OmpC⁺ CphA⁺); C, B1449 (OmpF⁻ OmpC⁺ CphA⁻); D, LGC49 (OmpF⁻ OmpC⁺ CphA⁺); E, B1478 (OmpF⁺ OmpC⁻ CphA⁻); F, LGC78 (OmpF⁺ OmpC⁻ CphA⁺); G, pop1389 (OmpF⁻ OmpC⁻ CphA⁻); H, LGC89 (OmpF⁻ OmpC⁻ CphA⁺). Lane C contains a poorly concentrated sample.

transformant obtained from the parental *E. coli* K-12 but not its porin-deficient mutants, since the transformants obtained from the mutants showed strong autolysis during washing and resuspension and were thus unsuitable for the Zimmermann-Rosselet experimental procedure. No significant difference in outer membrane profile was detectable at electrophoresis between strains that were transformed with plasmid pAA20R and those that were not transformed (Fig. 1).

Determination of permeability coefficients. Unlike other metalloenzymes (2, 4, 39), and to an even greater extent than a similar enzyme (A2) previously purified from *A. hydrophila* (13), the CphA activity spectrum is virtually restricted to carbapenems (21). For this reason, the hydrolysis of cephalosporins in strains producing only CphA β-lactamase could not be measured, and for this purpose we used strain LGC01, harbouring the pAS50 plasmid which codes for a TEM-type enzyme too. The permeability coefficients for the carbapenems proved virtually the same regardless of the plasmid used for transformation (Table 3).

To rule out the possibility that the CphA enzyme exhibited significantly different activity in periplasm from that in the buffer used in the assay, which would have produced huge errors in determinations of permeability coefficients, we determined the effect of pH and salt concentration on the enzyme activity and found no significant differences throughout the ranges explored (Fig. 2).

In the transformed *E. coli* K-12 (strain LGC01), the permeability to imipenem was roughly 2, 10, and 200 times greater than that to meropenem, cephaloridine, and cephalothin, respectively. As regards the porin-deficient derivatives, in both OmpC⁻ and OmpF⁻ strains the permeability to imipenem was roughly half that of the nondeficient strain and the permeability to meropenem was three to five times lower than that of the nondeficient strain; greater reductions (up to roughly 90%) could be observed for both carbapenems in the OmpF⁻ OmpC⁻ strain LGC89 (mutated in the *ompB* regulatory locus).

Investigation of a possible carbapenem-specific saturable porin pathway in *E. coli* cells. The hydrolysis tests we carried out with intact *E. coli* cells showed the permeability coefficients of the two carbapenems to be noticeably higher than that of cephaloridine, whereas the permeability coefficients of imipenem and cephaloridine had proved almost identical when measured in proteoliposomes and normalized to intact-cell values (23). Since our results suggested a possible specific pathway of the kind described by Trias et al. for intact *P. aeruginosa* cells (35), we investigated this possibil-

TABLE 3. Rates of permeation of carbapenems and cephalosporins through the outer membranes of *E. coli* strains
with different porin patterns

Strain	Phenotype	Antibiotic	Permeability coefficient $(10^{-5}$ cm/s) $\pm$ SD[a]	Relative rate of permeation[b]
LGC01	OmpF⁺ OmpC⁺ CphA⁺ TEM⁺	Imipenem	149.3 ± 44.6	93.9
		Meropenem	67.3 ± 20.5	42.3
		Cephaloridine	12.8 ± 5.1	8.1
		Cephalothin	0.7 ± 0.3	0.4
LGC10	OmpF⁺ OmpC⁺ CphA⁺	Imipenem	158.9 ± 52.4	100.0
		Meropenem	54.3 ± 23.8	34.2
LGC78	OmpF⁺ OmpC⁻ CphA⁺	Imipenem	70.9 ± 32.4	44.6
		Meropenem	15.3 ± 6.0	9.6
LGC49	OmpF⁻ OmpC⁺ CphA⁺	Imipenem	61.8 ± 6.9	38.9
		Meropenem	10.5 ± 0.5	6.6
LGC89	OmpF⁻ OmpC⁻ CphA⁺	Imipenem	7.2 ± 2.1	4.5
		Meropenem	6.2 ± 2.1	3.9

[a] The permeability coefficients are averages of at least three different determinations; permeability coefficients for cephaloridine and cephalothin could be determined only in strain LGC01. SD, standard deviation.

[b] The relative permeation rates are normalized to the permeability coefficient of imipenem in strain LGC10 (which presents a normal porin pattern).

ity. However, unmodified use of the procedure of Trias et al. (35) was not possible, since our previous experiments had shown the hydrolysis rates by the sonic extracts of the cells to be very close to those measured in the intact cells, i.e., a condition under which the intact-cell rates alone are not satisfactorily representative of the permeation across the outer membrane.

To explore the possibility of a carbapenem-specific channel, which would allow high permeation rates even at low substrate concentrations, we measured the apparent permeability coefficients at different substrate concentrations in strain LGC01 (which does not present any apparent porin defect) and strain LGC89 (in which only trace levels of porin are present and, therefore, the influence of a possible specific channel should be more appreciable). Figure 3 shows that the measured permeability coefficients were indeed higher as the substrate molarity decreased, in accordance with findings previously reported and discussed by several authors (11, 19), but no relation could be established between the extent of the differences and the porin content of the strains. Moreover, similar variations in the permeability coefficients measured at different substrate concentrations could be observed, in the same strains, between carbapenemic and noncarbapenemic compounds. These findings seem to rule out the possibility of carbapenem-specific

pathways, at least in the range we considered (namely 10 to 1,000 µM).

Since the specific channel formed by the D2 protein in *P. aeruginosa* has a binding site for basic amino acids (36), resulting in a competitive inhibition of carbapenem diffusion into the periplasm, we tested whether these amino acids were also able to inhibit the transport of carbapenems and, consequently, their hydrolysis rates by intact cells of *E. coli*. Such inhibition turned out to be negligible (less than 7%) compared with that observed in *P. aeruginosa*, and, moreover, no distinction could be made between basic and nonpolar amino acids. It is worth noting that our test was performed with an antibiotic concentration five times lower than that used by Trias and Nikaido (36), so that the amino acids were present at concentrations 100 times higher than the carbapenem concentrations.

Relationship between permeability defects and susceptibility to carbapenems. Table 4 shows the MICs of the two carbapenems we tested and those of some cephalosporins for all the strains described in this work. In the nontransformed strains, which lack a carbapenem-hydrolyzing β-lactamase, no defect in outer membrane permeability was sufficient to produce large increases in carbapenem MICs, whatever the inoculum, whereas up to 600-fold increases in cephalosporin MICs could be seen in the OmpF⁻ mutants. The presence of
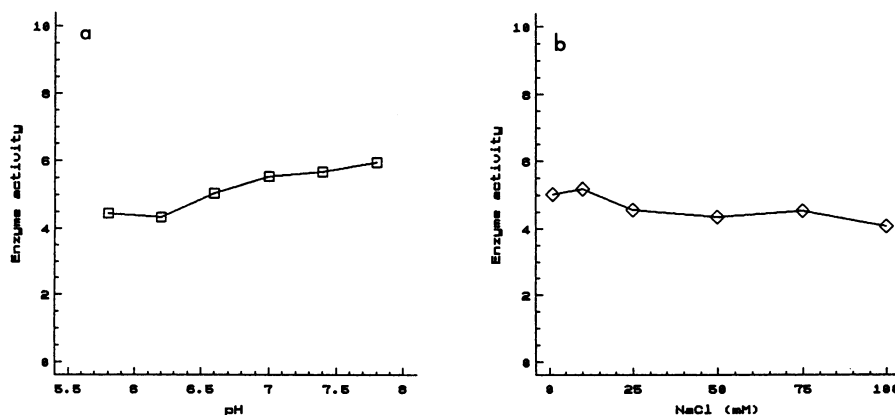

FIG. 2. Influence of pH (a) and ionic strength (b) on the rate (micromoles per milligram per minute) of the hydrolysis of meropenem catalyzed by CphA β-lactamase. Similar results were obtained with imipenem.
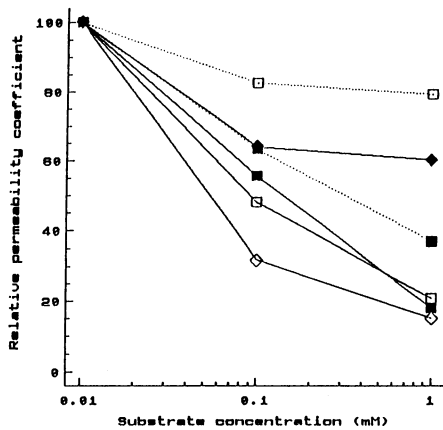
FIG. 3. Relative rate of permeation of meropenem (■), imipenem (□), cephaloridine (◆), and cephalothin (◇) measured at different substrate concentrations in strains LGC10 (solid lines) and LGC89 (dotted lines). For each strain, the relative permeation rates are normalized to the permeability coefficient measured with a 10 μM concentration of the drug considered.

CphA β-lactamase had almost no further effect on the MICs of cephalosporins in any of the porin-deficient isolates whatever the inoculum used, whereas, for the carbapenems, the results were strongly inoculum dependent. When the lower inoculum was used (10^4 CFU per spot), the CphA β-lactamases had only a negligible influence on the MICs of carbapenems for the non-porin-deficient strain LGC10 and for the two strains (namely LGC49 and LGC78) in which the ompF or ompC structural genes had been inactivated by the insertion mutation element Tn5 (14), but a significant (roughly 10-fold) decrease in meropenem susceptibility could be seen in strain LGC89, in which the regulatory locus ompB is mutated (38). When the higher inoculum was used (10^6 CFU per spot), the susceptibility of strain LGC89 to both carbapenems was even lower and a 16-fold increase in the MICs of both imipenem and meropenem was also detected in the OmpF-deficient strain LGC49. However, even with this inoculum, only very minor increases were observed in strain LGC10, which has a normal porin pattern, and in the OmpC-deficient strain LGC78.

Relationship between the PBP affinity of carbapenems and their antibacterial activity. In E. coli imipenem and meropenem have the highest affinity for PBP 2 and very similar IC_{50} for this protein, but meropenem also has a higher affinity than imipenem for PBP 3 (34). To investigate the
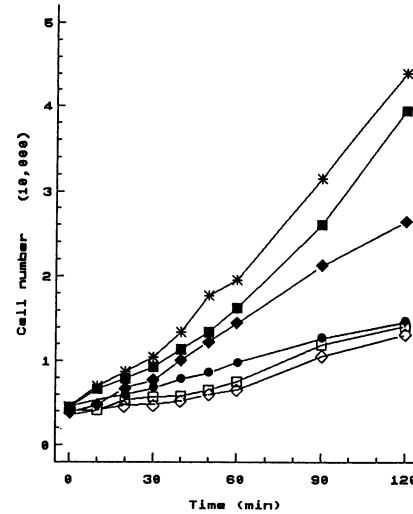

FIG. 4. Effects of meropenem and imipenem on cell growth and division. Cells were incubated in the presence of different concentrations of antibiotic and counted as described in Materials and Methods. Symbols: *, control; ■, imipenem at 0.02 μg/ml; ◆, imipenem at 0.04 μg/ml; □, imipenem at 0.08 μg/ml; ●, meropenem at 0.01 μg/ml; ◇, meropenem at 0.02 μg/ml;

effect of the main target inhibition on E. coli growth, we used the E. coli mutant DC2, which had been shown to have a breakdown in the outer membrane permeability barrier (5, 28), assuming that in this strain PBPs are exposed to β-lactam concentrations very close to those in the medium where bacteria are grown. Susceptibilities of this strain to meropenem and imipenem were 0.015 and 0.125 μg/ml respectively. The IC_{50}s of both carbapenems for PBPs were similar to the ones reported in reference 34 for E. coli K-12 strain C600. The IC_{50} of both carbapenems for PBP 2 was 0.02 μg/ml, whereas for PBP 3 the meropenem IC_{50} was 0.6 μg/ml and the imipenem IC_{50} was >16 μg/ml; the inhibitory effect of carbapenems on E. coli DC2 growth was determined by counting the cell number with a Coulter Counter after different incubation times in the presence of different concentrations of antibiotic. Figure 4 shows that the effects of a concentration (0.02 μg/ml) very close to the IC_{50} for PBP 2 were very different for meropenem and imipenem, since at that concentration meropenem inhibited the cell number increase more significantly than imipenem did. Moreover, meropenem showed virtually identical effects even with a

TABLE 4. Susceptibility to carbapenems and cephalosporins of strains with different porin patterns harboring or not harboring CphA β-lactamase and of their parents

Strain	Phenotype	MIC (μg/ml) of antibiotic at given inocula									
		Meropenem		Imipenem		Cefoxitin		Ceftazidime		Ceftriaxone	
		10^4 CFU	10^6 CFU	10^4 CFU	10^6 CFU	10^4 CFU	10^6 CFU	10^4 CFU	10^6 CFU	10^4 CFU	10^6 CFU
pop1010	OmpF⁺ OmpC⁺ CphA⁻	0.03	0.125	0.06	0.25	4	4	0.06	0.125	0.015	0.03
B1478	OmpF⁺ OmpC⁻ CphA⁻	0.03	0.06	0.06	0.125	8	8	0.125	0.125	0.015	0.03
B1449	OmpF⁻ OmpC⁺ CphA⁻	0.06	0.06	0.25	0.5	>128	>128	32	64	8	16
pop1389	OmpF⁻ OmpC⁻ CphA⁻	0.03	0.03	0.06	0.25	>128	>128	16	16	2	2
LGC10	OmpF⁺ OmpC⁺ CphA⁺	0.03	0.125	0.125	0.5	4	8	0.06	0.125	0.015	0.015
LGC78	OmpF⁺ OmpC⁻ CphA⁺	0.06	0.125	0.125	0.25	4	8	0.06	0.125	0.015	0.03
LGC49	OmpF⁻ OmpC⁺ CphA⁺	0.125	1	0.5	8	>128	>128	32	>128	8	16
LGC89	OmpF⁻ OmpC⁻ CphA⁺	0.5	8	0.5	16	>128	>128	16	32	2	4

0.01-μg/ml dose, whereas such effects could be reproduced only by an imipenem dose roughly four times higher than its IC$_{50}$ for PBP 2 (0.08 μg/ml).

DISCUSSION

Making every allowance for the limitations due to experimental errors, there seems to be no doubt that, in this study, very high permeability coefficients for meropenem and imipenem could be measured in intact *E. coli* cells transformed with pAA20R, a plasmid coding for CphA metallo-β-lactamase, whereas the permeability coefficients for cephalosporins were of the same order of magnitude as in other studies (23, 26). In the *E. coli* K-12 derivative LGC01, which shows no defect in its outer membrane permeability, the permeability coefficients for meropenem and imipenem under such conditions were 5 and 14 times, respectively, higher than that for cephaloridine; this is in sharp contrast with previous reports of very similar values for imipenem and cephaloridine (23). The fact that these values were calculated not in intact cells, as in our study, but in proteoliposomes and only subsequently normalized to intact-cell values may indicate that the whole-cell kinetics of carbapenem penetration are much faster than in reconstructed systems, possibly implying the existence of specific channels of the type described in *P. aeruginosa* (36), in addition to the well-known nonspecific porins OmpF and OmpC. A comparison of the influx kinetics of the two carbapenems at different substrate concentrations, however, seems to contrast with the hypothesis of a specific channel (which, because it has a specific binding site, would be saturable). The higher apparent permeability coefficients we obtained at low concentrations of the substrate are in accordance with suggestions by Liu and Nikaido about the contribution of the cell surface-associated enzyme in the Zimmermann-Rosselet assay (19). Another feature of the *P. aeruginosa* D2 channels, i.e., their specificity for the basic amino acids, which competitively inhibit imipenem diffusion into the periplasm, could not be demonstrated by our studies in intact *E. coli* cells.

Our findings cannot rule out the existence of specific channels selective for substances other than the basic amino acids, nor can they rule out the existence of specific channels operating at antibiotic concentrations below those for which we were able to obtain precise measurements; however, with the antibiotic concentrations used throughout our work (namely 10 μM), this possibility cannot realistically account for the strong difference we found between the intact-cell permeability coefficients for imipenem and cephaloridine. Such a difference could suggest that in intact *E. coli* cells the nonspecific porins are much more permeable to carbapenems than when they are purified and used in reconstituted proteoliposomes, unlike current opinion and experience to date with the noncarbapenemic β-lactams.

The preeminent role of nonspecific porins in the permeability to carbapenems was further confirmed by using the porin-deficient strains, since the lack of either OmpF or OmpC significantly reduced the permeability coefficients of both compounds.

As regards the OmpC⁻ OmpF⁻ mutants, it is worth recalling that they are not totally porin deficient but that they simply synthesize very little OmpF and OmpC protein because of a mutation in the *ompB* regulatory locus (38). In such strains, the permeability coefficients for imipenem and meropenem were reduced by roughly 90%, thus confirming with the two carbapenems under investigation what had been observed for several other β-lactams, i.e., that mutants

with trace levels of porin show only 5 to 10% of the permeability of the wild-type cells (26).

The correlations between the antibiotic influx kinetics and the susceptibility patterns fully confirm the postulate that the effect of decreased permeability must be amplified by the presence of an effective enzymatic inactivation barrier (24). Our results with nontransformed strains, which lack a carbapenem-hydrolyzing β-lactamase, show that decreases in outer membrane permeability did not produce large increases in carbapenem MICs even in the OmpC⁻ OmpF⁻ mutants, which retain only trace levels of porins and show less than 10% of the permeability of the wild-type cells (see above). On the other hand, in the strains transformed with the plasmid pAA20R, specifying the carbapenem-hydrolyzing CphA β-lactamase, porin defects were associated with a more significant decrease in carbapenem susceptibility, whereas the transformed strains did not show any significant change in cephalosporin MICs compared with the nontransformed strains, in accordance with the aforementioned poor affinity of these compounds for CphA β-lactamase.

The MICs of meropenem for the various mutants were in fairly good agreement with the measured permeability coefficients, since the isolated OmpC and OmpF defects did influence the susceptibility, although both were less important than the combined OmpC OmpF defect.

Comparative analysis of the aforementioned permeability and susceptibility data clearly shows that meropenem has a higher antibacterial activity than imipenem despite the lower permeation rate. We explain these discrepancies by showing that meropenem could exert the same inhibitory effect on cell number increase at periplasmic concentrations lower than those required by imipenem (Fig. 4). As suggested in reference 34, the different inhibitory effects of the two carbapenems may be related to their different PBP affinity patterns. In fact, the IC$_{50}$s of imipenem and meropenem for PBP 2 are virtually the same (0.02 μg/ml for both compounds), but it can be reasonably argued that, at that concentration, meropenem also binds PBP 3 to a significant extent (IC$_{50}$, 0.6 μg/ml) whereas imipenem does not (IC$_{50}$, >16 μg/ml). Even though we are not aware of the proportion of lethal target(s) necessary for survival of the cell, it has already been demonstrated that the kinetics of cellular killing by various β-lactams shows a strict correlation with the extent of the binding of different essential PBPs (20, 31).

REFERENCES

1. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44:**1–56.
2. **Bandoh, K., Y. Muto, K. Watanabe, N. Katoh, and K. Ueno.** 1991. Biochemical properties and purification of metallo-β-lactamase from *Bacteroides fragilis*. Antimicrob. Agents Chemother. **35:**371–372.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Bush, K.** 1989. Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob. Agents Chemother. **33:**271–276.

5. Clark, D. 1991. Permeability and susceptibility of *Escherichia coli* to beta-lactam compounds. Antimicrob. Agents Chemother. 19:369–370.

6. Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69:2110.

7. Edwards, J. R., P. J. Turner, C. Wannop, E. S. Withnell, A. J. Grindey, and K. Nairn. 1989. In vitro antibacterial activity of SM-7338, a carbapenem antibiotic with stability to dehydropeptidase I. Antimicrob. Agents Chemother. 33:215–222.

8. Frère, J.-M. 1989. Quantitative relationship between sensitivity to β-lactam antibiotics and β-lactamase production in Gram-negative bacteria. I. Steady-state treatment. Biochem. Pharmacol. 38:1415–1426.

9. Frère, J.-M., B. Joris, M. Crine, and H. H. Martin. 1989. Quantitative relationship between sensitivity to β-lactam antibiotics and β-lactamase production in Gram-negative bacteria. II. Non-steady-state treatment and progress curves. Biochem. Pharmacol. 38:1427–1433.

10. Hechler, U., M. Van den Weghe, H. H. Martin, and J.-M. Frère. 1989. Overproduced β-lactamase and the outer-membrane barrier as resistance factors in *Serratia marcescens* highly resistant to β-lactamase-stable β-lactam antibiotics. J. Gen. Microbiol. 135:1275–1290.

11. Hewinson, R. G., D. C. Lane, M. P. E. Slack, and W. W. Nichols. 1986. The permeability parameter of the outer membrane of *Pseudomonas aeruginosa* varies with the concentration of a test substrate, cephalosporin C. J. Gen. Microbiol. 132:27–33.

12. Hopkins, J. M., and K. J. Towner. 1990. Enhanced resistance to cefotaxime and imipenem associated with outer membrane protein alterations in *Enterobacter aerogenes*. J. Antimicrob. Chemother. 25:49–55.

13. Iaconis, J. P., and C. C. Sanders. 1990. Purification and characterization of inducible β-lactamases in *Aeromonas* spp. Antimicrob. Agents Chemother. 34:44–51.

14. Jaffé, A., Y. A. Chabbert, and O. Semonin. 1982. Role of porin proteins OmpF and OmpC in the permeation of β-lactams. Antimicrob. Agents Chemother. 22:942–948.

15. Jones, R. N., K. E. Aldridge, S. D. Allen, A. L. Barry, P. C. Fuchs, E. H. Gerlach, and M. A. Pfaller. 1989. Multicenter in vitro evaluation of SM-7338, a new carbapenem. Antimicrob. Agents Chemother. 33:562–565.

16. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for the detection and isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.

17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

18. Lee, E. H., M. H. Nicolas, M. D. Kitzkis, G. Pialou, E. Collatz, and L. Gutmann. 1991. Association of two resistance mechanisms in a clinical isolate of *Enterobacter cloacae* with high-level resistance to imipenem. Antimicrob. Agents Chemother. 35:1093–1098.

19. Liu, W., and H. Nikaido. 1991. Contribution of the cell-surface-associated enzyme in the Zimmermann-Rosselet assay of outer membrane permeability to β-lactam antibiotics. Antimicrob. Agents Chemother. 35:177–179.

20. Lleò, M. M., P. Canepari, G. Cornaglia, R. Fontana, and G. Satta. 1987. Bacteriostatic and bactericidal activities of β-lactams against *Streptococcus* (*Enterococcus*) *faecium* are associated with saturation of different penicillin-binding proteins. Antimicrob. Agents Chemother. 31:1618–1626.

21. Massidda, O., G. M. Rossolini, and G. Satta. 1991. The *Aeromonas hydrophila cphA* gene: molecular heterogeneity among class B metallo-β-lactamases. J. Bacteriol. 173:4311–4317.

22. Neu, H. C., A. Novelli, and C. Nai-Xun. 1989. In vitro activity and β-lactamase stability of a new carbapenem, SM-7338.

23. Nikaido, H. 1985. Role of permeability barriers in resistance to β-lactam antibiotics. Pharmacol. Ther. 27:197–231.

24. Nikaido, H. 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. 33:1831–1836.

25. Nikaido, H., W. Liu, and E. Y. Rosenberg. 1990. Outer membrane permeability and β-lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. Antimicrob. Agents Chemother. 34:337–342.

26. Nikaido, H., E. Y. Rosenberg, and J. Foulds. 1983. Porin channels in *Escherichia coli*: studies with β-lactams in intact cells. J. Bacteriol. 153:232–240.

27. Raimondi, A., A. Traverso, and H. Nikaido. 1991. Imipenem- and meropenem-resistant mutants of *Enterobacter cloacae* and *Proteus rettgeri* lack porin. Antimicrob. Agents Chemother. 35:1174–1180.

28. Richmond, M. H., D. C. Clark, and S. Wotton. 1976. Indirect method for assessing the penetration of beta-lactamase-nonsusceptible penicillins and cephalosporins in *Escherichia coli* strains. Antimicrob. Agents Chemother. 10:215–218.

29. Sahm, D. F., and J. A. Washington II. 1991. Susceptibility tests: microdilution and macrodilution broth procedures, p. 1105–1116. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.

30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed, p. 1.82–1.84. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

31. Satta, G., G. Cornaglia, P. Canepari, and R. Pompei. 1988. Evaluation of bactericidal activity of cefotaxime and other beta-lactams by a novel method. Drugs 35(Suppl. 2):35–40.

32. Spratt, B. G. 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72:341–352.

33. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 17:45–50.

34. Sumita, Y., M. Fukasawa, and T. Okida. 1989. Comparison of two carbapenems, SM-7338 and imipenem: affinities for penicillin-binding proteins and morphological changes. J. Antibiot. 43:314–320.

35. Trias, J., J. Dufresne, R. C. Levesque, and H. Nikaido. 1989. Decreased outer membrane permeability in imipenem-resistant mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 33:1201–1206.

36. Trias, J., and H. Nikaido. 1990. Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. J. Biol. Chem. 265:15680–15684.

37. Vu, H., and H. Nikaido. 1985. Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum β-lactams. Antimicrob. Agents Chemother. 27:393–398.

38. Wandersman, C., F. Moreno, and M. Schwartz. 1980. Pleiotropic mutations rendering *Escherichia coli* K-12 resistant to bacteriophage TP1. J. Bacteriol. 143:1374–1383.

39. Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi. 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 35:147–151.

40. Yoshimura, F., and H. Nikaido. 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. 152:636–642.

41. Yoshimura, F., and H. Nikaido. 1985. Diffusion of β-lactam antibiotics through the porin channels of *Escherichia coli* K-12. Antimicrob. Agents Chemother. 27:84–92.

42. Zimmermann, W., and A. Rosselet. 1977. The function of the outer membrane of *Escherichia coli* as a permeability barrier to β-lactam antibiotics. Antimicrob. Agents Chemother. 12:368–372.

Antimicrob. Agents Chemother. 33:1009–1018.