Interactions of Biapenem with Active-Site Serine and Metallo-β-Lactamases

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**Biapenem, formerly LJC 10,627 or L-627, a carbapenem antibiotic, was studied in its interactions with 12
β-lactamases belonging to the four molecular classes proposed by R. P. Ambler (Philos. Trans. R. Soc. Lond.
Biol. Sci. 289:321–331, 1980). Kinetic parameters were determined. Biapenem was readily inactivated by
metallo-β-lactamases but behaved as a transient inhibitor of the active-site serine enzymes tested, although
with different acylation efficiency values. Class A and class D β-lactamases were unable to confer in vitro
resistance toward this carbapenem antibiotic. Surprisingly, the same situation was found in the case of class
B enzymes from *Aeromonas hydrophila* AE036 and *Bacillus cereus* 5/B/6 when expressed in *Escherichia coli* strains.**

It has been clearly shown that bacterial resistance to β-lactam antibiotics is often a multifactorial event in which outer membrane impermeability and β-lactamase production by gram-negative bacteria play central roles (39).

β-Lactamase degradation of β-lactam antibiotics represents one of the most important biochemical mechanisms of resistance to these molecules in bacteria (19). These enzymes, which are bacterial hydrolases (EC 3.5.2.6), have been classified into four different molecular classes on the basis of their primary structures and catalytic mechanisms (1). Enzymes of classes A, C, and D exert their catalytic activity by a reactive serine residue in the active site, while class B β-lactamases are metalloproteins which require a divalent transition metal ion for their activity, most often Zn^{2+}.

Antibiotic pressure has often selected bacteria able to elaborate two or more specific chromosomally encoded or plasmid-mediated β-lactamases which are active against a wide range of β-lactam compounds.

An expanded spectrum of β-lactams has been developed over the past 20 years in order to overcome bacterial resistance to molecules previously developed for clinical use. Carbapenem antibiotics are reported to be very stable in the presence of active-site serine β-lactamases, with the exception of class C enzymes produced by members of the family *Enterobacteriaceae* which exhibit slow antibiotic hydrolysis (25). This phenomenon, combined with a reduced permeability of the bacterial outer membrane, leads to resistance in hyperproducing strains of *Enterobacter cloacae* and *Pseudomonas aeruginosa*. These novel antibiotics also show a broad range of activity against gram-negative and gram-positive bacteria (22, 30), with the exception of *Xanthomonas maltophilia*, which produces a chromosomally encoded class B β-lactamase (4, 36)

which is able to inactivate all β-lactam antibiotics except aztreonam (14). Moreover, carbapenem antibiotics have variable stability to hydrolysis by mammalian dehydropeptidase-I (18, 20, 21, 34). For this reason, imipenem, the first carbapenem molecule to be introduced in the clinical setting, is administered in combination with cilastatin, a dehydropeptidase-I inhibitor. Despite the antimicrobial efficacy of imipenem, it is only relatively recently that reports concerning the kinetic interaction of this molecule with β-lactamases have been published (6, 19, 29, 31).

Today, strains resistant to these drugs are isolated daily throughout the world. This has stimulated the search for new carbapenem derivatives characterized by optimal binding to penicillin-binding proteins, good permeation, and insensitivity to the action of β-lactamases.

Several recent observations have prompted us to investigate in detail the kinetics of interaction between the novel carbapenem antibiotic biapenem (8, 27, 41) and active-site serine and metallo-β-lactamases. These observations include the discovery of a plasmid-mediated metallo-β-lactamase in *P. aeruginosa* (40); the emergence of strains of *Bacteroides* spp. producing class B enzymes (10, 35, 42); the appearance of other mechanisms of resistance to imipenem, including a decline in production of the D2 outer membrane protein in *Pseudomonas* spp. (26); and the combined derepression of class C β-lactamase production and impaired outer membrane permeability or β-lactamase production in certain strains of the *Enterobacteriaceae* (25, 33).

MATERIALS AND METHODS

Bacterial strains. *Citrobacter diversus* ULA-27, *Proteus vulgaris* POP9, *Acinetobacter calcoaceticus* ULA-513, *Pseudomonas stutzeri* ULA-506, *Morganella morganii* MDM, and *X. maltophilia* ULA-511 were all clinical isolates. *Mycobacterium fortuitum* D316 was obtained from an American Type Culture Collection strain after nitrosoguanidine treatment (12). An *Escherichia coli* clone containing the plasmid pJT4 encoding the TEM-1 β-lactamase was a kind gift of Tony Farmer (Smith Kline-Beecham Pharmaceuticals, Brentford, Middlesex, United Kingdom). A clone of *E. coli* HB101, containing the plasmid pWRTH012, which was resistant to 30 μg of chloramphenicol per ml, was used to produce the *Bacillus cereus* 5/B/6 metallo-β-lactamase. The *Aeromonas hydrophila* AE036

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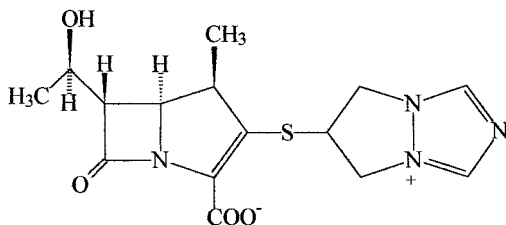
FIG. 1. Structure of biapenem (LJC 10,627 or L-627).

metallo-β-lactamase was produced from *E. coli* DH5α, containing the plasmid pAA20R, which was resistant to 50 μg of tetracycline per ml (28). *E. coli* HB101 containing the plasmid pMON301 coding for the OXA-1 β-lactamase was a kind gift of G. Cornaglia (University of Verona, Verona, Italy). *E. cloacae* P99 was kindly provided by R. L. Then (Hoffman-La Roche, Basel, Switzerland). *E. coli* HB101 containing the plasmid bladiv, encoding the β-lactamase from *C. diversus* ULA-27, was from our laboratory.

Antibiotics. Biapenem was kindly provided by Cyanamid (Catania, Italy). Imipenem was obtained from Merck Sharp & Dohme (Rome, Italy). Cefazolin was obtained from Eli Lilly & Co. (Indianapolis, Ind.). Tetracycline and chloramphenicol were purchased from Sigma (Milan, Italy). Nitrocefin was purchased from Unipath (Milan, Italy). The structure of biapenem (LJC 10,627 or L-627) is shown in Fig. 1.

Purification of enzymes. The production and purification of the β-lactamases from *C. diversus* ULA-27 and *M. fortuitum* D316 were performed as described elsewhere (2, 3). The class A β-lactamase produced by *P. stutzeri* ULA-506 was purified according to the protocol reported by Franceschini et al. (16). The enzymes from *P. vulgaris* POP9 and *E. cloacae* P99 were purified by using a phenylboronic acid-Sepharose column as described by Cartwright and Waley (7). *A. hydrophila* AE036 (from *E. coli* DH5α containing the plasmid pAA20R) and *X. maltophilia* ULA-511 metallo-β-lactamases were purified as previously described (14). The *B. cereus* 5/B/6 metallo-β-lactamase (produced by *E. coli* HB101 containing the plasmid pWRTH012) was purified as described by Felici and Amicosante (13). The TEM-1 β-lactamase was obtained as reported by Fisher et al. (15). The OXA-1 β-lactamase was purified to homogeneity as previously described (24). *M. morganii* MDM and *A. calcoaceticus* ULA-513, two clinical isolates, and the purification procedure of the respective β-lactamases are described elsewhere (unpublished data). Protein concentrations were estimated by the method of Bradford (5), using bovine serum albumin (BSA) as the standard.

Assay conditions for kinetic experiments. The metallo-β-lactamases from *A. hydrophila* AE036 and *X. maltophilia* ULA-511 were assayed in the presence of 30 mM sodium cacodylate buffer, pH 6.5, containing 0.1 mM ZnCl₂. *B. cereus* 5/B/6 metalloenzyme was assayed using 10 mM sodium cacodylate buffer, pH 6.0, containing 0.5 M NaCl and 0.1 mM ZnCl₂. The assays were performed at 30°C. The class A, class C, and class D enzymes used in this study were assayed in the presence of 25 mM phosphate buffer, pH 7.0, containing 0.1 M KCl, at 30°C. Enzyme concentrations below 0.1 mg/ml were assayed in the presence of 0.1 mg

of BSA per ml for enzyme stability. Table 1 summarizes the experimental conditions used in the assays.

Determination of kinetic parameters for class B β-lactamases. The enzyme activity was determined by measuring the absorbance variation resulting from the hydrolysis of biapenem ($\lambda = 293$ nm; $\Delta\epsilon_M^{293} = -7600 \pm 200$ M^{-1} cm^{-1}) in a Perkin-Elmer Lambda-2 UV/VIS spectrophotometer (Perkin-Elmer, Rome, Italy) connected to an Epson PSE 30 microcomputer via RS232C interface. Cells with 0.2- to 1.0-cm pathlengths were used, depending on the antibiotic solution concentration used in the spectrophotometric assays. K_m, k_{cat}, and k_{cat}/K_m ratio values were determined either under initial-rate conditions, using the Hanes-Woolf linearization of the Michaelis-Menten equation (37), with *B. cereus* 5/B/6 and *A. hydrophila* AE036 metallo-β-lactamases, or by analyzing the complete time course of the hydrolysis of biapenem in the case of *X. maltophilia* ULA-511, as described by De Meester et al. (11). For all data, standard deviations were calculated by the Enzfitter program (23) and were less than 10%. The total reaction volume was 0.6 ml in all cases.

Determination of kinetics of enzyme reactivation (k_{+3} values). The k_{+3} values were obtained by monitoring the reactivation of the enzyme in the presence of 100 μM nitrocefin, at 30°C. Each enzyme was first completely inactivated, and the reaction mixture was then diluted to reduce the inactivator concentration to a negligible value. The activity was then monitored after 2 h, and a limit k_{+3} value was determined.

In order to have a more precise indication of enzyme reactivation, the following two active-site serine β-lactamases were used as prototypes: *E. coli* TEM-1 (class A) and *E. cloacae* P99 (class C) enzymes. The β-lactamases (16 nM) were incubated with biapenem at enzyme/biapenem ratios of 1:100, 1:500, and 1:1,000 until complete inactivation was observed. The reaction mixture was diluted to reduce the inactivator concentration to a negligible value, and the activity was then monitored until complete reactivation of the enzymes by determining the v_0 values by reference to the hydrolysis of 100 μM nitrocefin, at 30°C. In the case of complete reactivation:

$$\frac{V_f - V_t}{V_f - V_0} = \exp^{-k_{+3}t}$$

where v_0, v_t, and v_f are the rates of the reporter substrate hydrolysis at times zero and t and after complete reactivation, respectively.

Determination of kinetic parameters of transient inactivation with class A, class C, and class D enzymes. With biapenem, a stable acylenzyme (EC* [see below]) was found to accumulate, and its interaction with active-site serine β-lactamases was studied on the basis of the following model:

$$E + C \underset{}{\overset{K}{\rightleftharpoons}} E \cdot C \xrightarrow{k_{+2}} EC^* \xrightarrow{k_{+3}} E + P$$
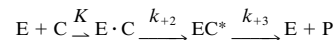
where E, C, E · C, and P represent the enzyme, inhibitor, Henri-Michaelis complex, and hydrolysis product, respectively. k_{+2} and k_{+3} are the first-order acylation and deacylation rate constants, respectively, and K represents the dissociation constant of the Henri-Michaelis complex. The values of the first-order rate constant (k_i) characterizing the rate of EC* accumulation were obtained by monitoring the hydrolysis of nitrocefin ($\lambda = 482$ nm; $\Delta\epsilon_M^{482} = 15,000$ M^{-1} cm^{-1}) or cefazolin ($\lambda = 260$ nm; $\Delta\epsilon_M^{260} = -7,400$ M^{-1} cm^{-1}) utilized as

TABLE 1. Experimental conditions for determination of kinetic parameters

Source of β-lactamase and type	Molecular class[a]	Enzyme quantity (ng)	Biapenem concn (μM)	Method used[b]
E. coli HB101(pWRTH012), BcII	B	10–30	50–1,000	1
X. maltophilia ULA-511, L1	B	5–10	100–300	2
E. coli DH5α(pAA20R), A2	B	5–10	50–1,500	1
C. diversus ULA-27	A	10–30	50–500	3
M. fortuitum D316	A	50–100	100–800	3
P. vulgaris POP9	A	100–200	25–200	3
P. stutzeri ULA-506	A	100–200	1–50	3
E. coli HB101(pJT4), TEM-1	A	50–100	10–100	3
A. calcoaceticus ULA-513	—[c]	100–200	10–200	3
E. cloacae P99	C	10–30	0.25–10	3
M. morganii MDM	C	20–50	2–25	3
E. coli HB101(pMON301), OXA-1	D	10–30	1–20	3

[a] According to the system of R. P. Ambler (1).

[b] Method 1: The kinetic parameters (k_{cat} and K_m values) were determined by calculating the initial-rate value of hydrolysis (v_0) in the presence of various biapenem concentrations (S_0) and treating the obtained data by Hanes-Woolf linearization of the Michaelis-Menten equation (37). Method 2: In this case, k_{cat} and K_m values were calculated by analyzing the complete curve of biapenem hydrolysis, according to the method of De Meester et al. (11). Method 3: K and k_{+2} values of inactivation were determined by calculating the pseudo-first-order rate of inactivation (k_i) and treating the data according to the method of Frère et al. (17). For more details, see Materials and Methods.

[c] —, unclassified.

TABLE 2. Comparison of kinetic parameters for class B β-lactamases determined with biapenem and with imipenem[a]

Class B β-lactamase	Biapenem			Imipenem[b]		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
B. cereus 5/B/6	>1,500	>100	8.62×10^{3c}	>1000	>100	1.20×10^5
A. hydrophila AE036	>1,500	>100	5.00×10^{5c}	80 ± 8	168	2.10×10^6
X. maltophilia ULA-511	56 ± 1	65	1.16×10^6	90 ± 8	65	7.30×10^5

[a] For assay conditions, see Materials and Methods.
[b] Results for imipenem are as previously reported by Felici et al. (14).
[c] Only the k_{cat}/K_m ratio was determined, because v_0 remained proportional to S_0 up to the highest substrate concentration which could be used.

reporter substrates (11). Less than 20% of the reporter substrate used was hydrolyzed during each assay. Reaction volumes were 0.6 ml.

Determination of MICs. The determination of MICs for selected organisms was performed by the conventional broth macrodilution procedure, as recommended by the National Committee for Clinical Laboratory Standards reference manual (32).

RESULTS

Kinetic parameters. (i) Metallo-β-lactamases. Table 2 lists the kinetic parameters calculated for the metallo-β-lactamases tested in this study with biapenem and compares them with those previously reported for imipenem (14). *B. cereus* 5/B/6 metallo-β-lactamase showed a K_m value for biapenem of the same order of magnitude as that determined for imipenem. However, the lower (about 14-fold) k_{cat}/K_m ratio value indicated a reduced catalytic efficiency of *B. cereus* 5/B/6 metalloenzyme for hydrolyzing biapenem with respect to imipenem. Biapenem behaved as a good substrate for *X. maltophilia* ULA-511 class B β-lactamase, with a physiologic efficiency of the same order as that observed with imipenem. The *A. hydrophila* AE036 metalloenzyme, which can be considered a rather specific carbapenemase (13, 14), showed a reduced affinity for biapenem and had a k_{cat}/K_m ratio value that was four to five times lower than that determined for imipenem, indicating a slight influence of molecular substitutions on enzyme activity.

(ii) Class A, class C, and class D β-lactamases. Biapenem behaved as an inhibitor of the active-site serine β-lactamases tested in this study. After correction for the protection by the reporter substrate, the individual parameters k_{+2} and K were derived from the dependence of k_i upon biapenem concentration by using the following equation (17):

$$k_i = k_{+3} + \frac{k_{+2} \cdot [C]}{[C] + K\left(\dfrac{K_m^s + [S]}{K_m^s}\right)}$$

where $[S]$ and K_m^s represent the concentration and K_m value for the reporter substrate, respectively, and $[C]$ represents the inhibitor concentration.

Table 3 summarizes the values of K, k_{+2}, k_{+3}, and k_{+2}/K calculated for the active-site serine β-lactamases tested in the presence of biapenem. For all data, the standard deviations were less than 10% of the mean of five different measurements. Among the class A β-lactamases, the enzyme from *P. stutzeri* ULA-506 exhibited a very low K value and a good acylation efficiency. The β-lactamases from *C. diversus* ULA-27 and *M. fortuitum* D316 exhibited the lowest k_{+2}/K ratio values, which were accompanied by a relatively low affinity for biapenem. The class C β-lactamases from *M. morganii* MDM and *E. cloacae* P99 exhibited low K values but similar k_{+2}/K ratio values. The inactivation by biapenem of the unclassified (probably a class C; unpublished data) enzyme produced by *A. calcoaceticus* ULA-513 was also studied. For this β-lactamase, biapenem behaved as an inhibitor and its acylation efficiency was similar to that determined for some class A β-lactamases.

Determination of k_{+3} values. Figure 2 shows the accumulation of product with time when the inactivated TEM-1 and *E. cloacae* P99 β-lactamases were diluted in the presence of 100 μM nitrocefin as substrate. From the curves of enzyme reactivation, the following k_{+3} values were determined, as described in Materials and Methods: $(1.00 \pm 0.1) \times 10^{-3}$ s^{-1} for the TEM-1 enzyme (Fig. 2A) and $(3.87 \pm 0.1) \times 10^{-4}$ s^{-1} for the β-lactamase produced by *E. cloacae* P99 (Fig. 2B). Each calculated value represents the mean from three different experiments.

Determination of MICs. Table 4 summarizes the in vitro activity of imipenem and biapenem against strains harboring some of the β-lactamases tested in kinetic experiments. *X. maltophilia* ULA-511 was omitted because of its resistance to carbapenem antibiotics. As it can be noted, class A and class C β-lactamases did not seem to confer resistance toward both imipenem and biapenem, because the determined MICs

TABLE 3. Kinetic parameters calculated with active-site serine β-lactamases, using biapenem as a transient inactivator[a]

Source of β-lactamase and type	K (μM)	k_{+2} (s^{-1})	k_{+3} (s^{-1})	k_{+2}/K (M^{-1} s^{-1})	Molecular class[b]
C. diversus ULA-27	147 ± 10	1.10×10^{-2}	$<1.50 \times 10^{-2}$	7.48×10^1	A
P. stutzeri ULA-506	1.7 ± 0.1	5.40×10^{-2}	$<6.30 \times 10^{-4}$	3.18×10^4	A
E. coli(pJT4), TEM-1	30 ± 2	3.80×10^{-2}	1.00×10^{-3}	1.27×10^3	A
M. fortuitum D316	267 ± 5	3.50×10^{-2}	$<2.20 \times 10^{-2}$	1.31×10^2	A
P. vulgaris POP9	80 ± 2	5.05×10^{-2}	$<9.20 \times 10^{-2}$	6.31×10^2	A
E. coli(pMON301), OXA-1	2.5 ± 0.1	1.20×10^{-2}	$<9.10 \times 10^{-3}$	4.80×10^3	D
M. morganii MDM	5.0 ± 0.1	1.10×10^{-1}	$<4.00 \times 10^{-4}$	2.20×10^4	C
E. cloacae P99	0.8 ± 0.01	2.45×10^{-2}	3.87×10^{-4}	3.06×10^4	C
A. calcoaceticus ULA-513	90 ± 5	4.00×10^{-2}	$<8.20 \times 10^{-2}$	4.44×10^2	—[c]

[a] For more details see Materials and Methods.
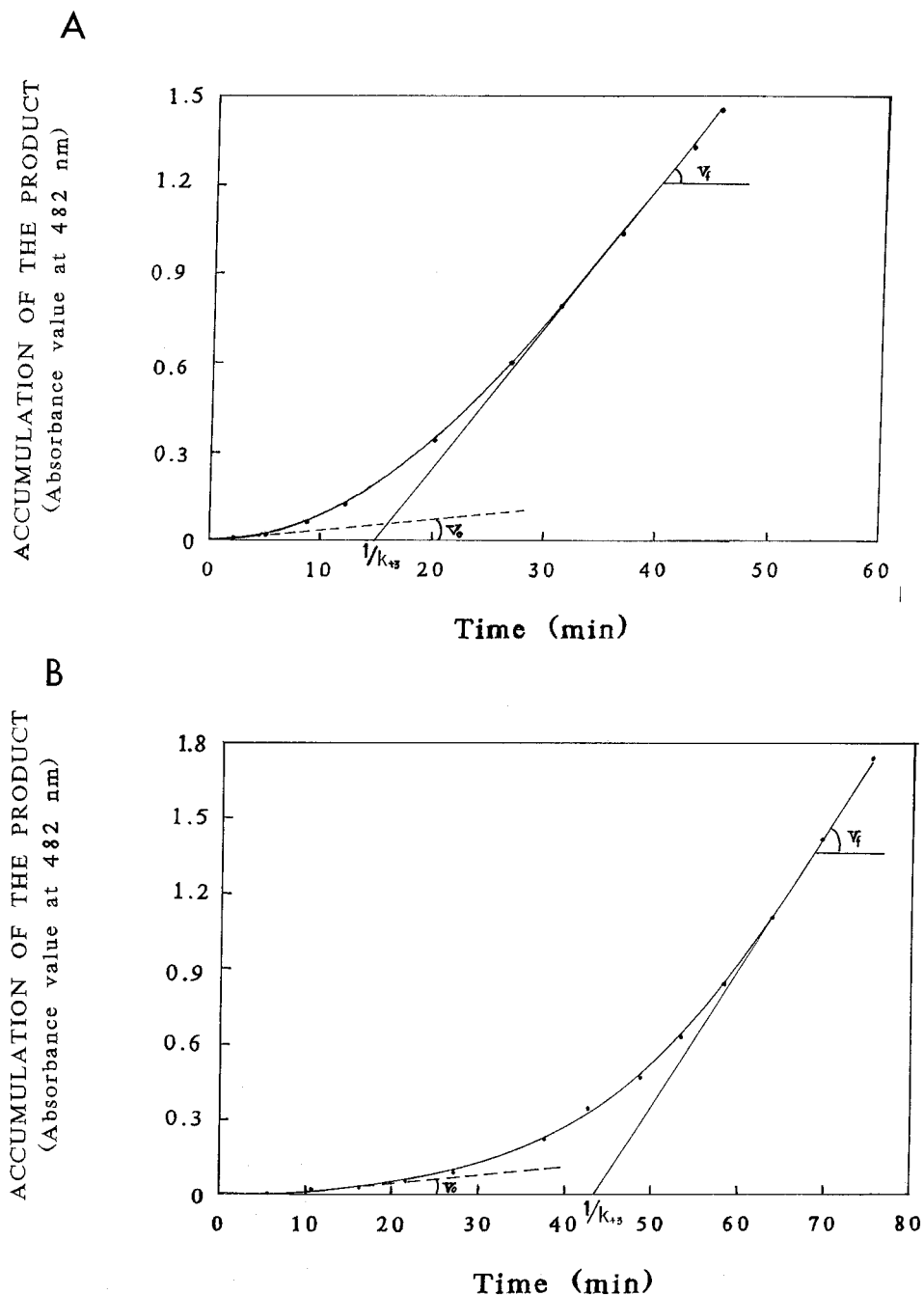[b] According to the system of R. P. Ambler (1).
[c] —, unclassified.

A

B

FIG. 2. Restoration of β-lactamase activity with time after incubation with biapenem as inactivator. In each case, an enzyme/biapenem concentration ratio of 1:500 was used. The k_{+3} value was determined by extrapolating the curve to the time axis. For more details see Materials and Methods. (A) *E. coli*(pJT4) TEM-1 β-lactamase; (B) *E. cloacae* P99 enzyme.

ranged between 0.06 and 8 μg/ml. In particular, when imipenem was used, slightly higher MICs were found with *E. coli* HB101 carrying the plasmid pJT4, probably due to a hyperproduction of the TEM-1 β-lactamase by this strain. Metallo-β-lactamases, produced by *E. coli* strains carrying the plasmids encoding these zinc enzymes, conferred resistance to imipenem when a higher concentration of inoculum was used. Surprisingly, the same enzymes did not allow bacteria to be resistant to biapenem.

DISCUSSION

In this paper we have evaluated the interaction between biapenem and β-lactamases belonging to the four molecular classes described by Ambler (1) and the effective contribution of these enzymes to the resistance mechanism of clinical isolates and laboratory strains.

A comparative analysis, using three class B enzymes, of the kinetic parameters obtained for some carbapenems indicated

TABLE 4. MICs of biapenem and imipenem, using two
different inoculum concentrations

Organism	MIC (μg/ml)			
	Imipenem (CFU/ml)		Biapenem (CFU/ml)	
	10^5	10^8	10^5	10^8
C. diversus ULA-27	<0.125	0.25	<0.0625	8
E. coli HB101(bladiv)	0.5	0.5	<0.0625	0.125
E. coli HB101(pJT4)	0.25	0.5	<0.0625	0.0625
P. stutzeri ULA-506	0.25	1	0.25	0.5
E. coli HB101(pMON301)	<0.125	0.25	0.125	0.125
A. calcoaceticus ULA-513	<0.125	0.5	0.25	1
E. cloacae P99	<0.125	0.25	0.125	0.25
E. coli DH5α(pAA20R)	0.5	128	0.125	0.25
E. coli HB101(pWRTH012)	4	>128	0.125	0.5

that the purified metallo-β-lactamase from *X. maltophilia* ULA-511 was the most active toward imipenem and biapenem. Although biapenem was hydrolyzed by the *B. cereus* 5/B/6 and *A. hydrophila* AE036 metalloenzymes, it exhibited a low affinity and a reduced catalytic efficiency. We extended the comparison to activity in vitro, using *E. coli* strains carrying the genes coding for the above-mentioned class B β-lactamases. Biapenem was more effective than imipenem against these strains, although the kinetic parameters determined appeared to be of the same order of magnitude.

The behavior was more heterogeneous when purified active-site serine β-lactamases were used. In fact, the enzymes from *M. morganii* MDM and *E. cloacae* P99 formed a relatively stable acylenzyme with a k_{+2}/K value of about 2×10^4 to 3×10^4 M^{-1} s^{-1}, whereas the chromosomal cephalosporinase (probably a class C enzyme) produced by *A. calcoaceticus* ULA-513 had a k_{+2}/K value that was 40-fold lower.

In general, class A β-lactamases had k_{+2}/K values lower than those calculated for class C enzymes, with the noticeable exception of the class A β-lactamase produced by *P. stutzeri* ULA-506. Biapenem behaved as a competitive inhibitor of this enzyme, and the k_{+3} value calculated indicated the formation of a relatively stable acylenzyme. Our data are, therefore, in good agreement with those previously reported (19, 29, 31).

Analysis of the k_{+3} values calculated for all active-site serine β-lactamases tested suggested that the half-life of the acylenzyme was shorter than the generation time of the bacterium. The situation was somewhat improved with class C β-lactamases, in which a doubling of the k_{+3} values was observed. On the basis of these observations, it would appear that biapenem behaves as a very poor substrate or as a transient inhibitor of these enzymes, which is similar to the case for imipenem.

The in vitro activity of biapenem against bacterial strains producing the enzymes investigated led to different conclusions and confirmed the previously reported antimicrobial efficacy of this antibiotic (8, 9, 27). The major antibacterial activity of biapenem could be related to the higher k_{cat}/K_m ratio values for imipenem exhibited by *B. cereus* and *A. hydrophila* metallo-β-lactamases. The differences in MIC data found between imipenem and biapenem for *C. diversus* ULA-27 cannot be explained on the basis of the kinetic parameters determined. In fact, as previously reported (3), imipenem was hydrolyzed with a low k_{cat}/K_m value (4.10×10^2 M^{-1} s^{-1}). On the contrary, biapenem was not hydrolyzed by this β-lactamase; from these findings the differences observed in MICs could be attributed to a greater ability of imipenem to permeate, with respect to biapenem.

In conclusion, our study confirms the excellent stability of biapenem to β-lactamases and its powerful antibiotic activity in vitro. Interestingly, the Zn^{2+}–β-lactamases from *B. cereus* 5/B/6 and *A. hydrophila* AE036 expressed in *E. coli* strains confer resistance to imipenem but not to biapenem, even when a high concentration of inoculum is used. As shown by Ubukata et al. (38) and by Yang et al. (41), there are not significant differences in binding to *E. coli* penicillin-binding proteins; if anything, imipenem binds better to PBP 1b as a secondary target, and permeability is actually a bit less for biapenem into *Serratia marcescens*. The possibility exists that the enzyme from *A. hydrophila* AE036 is not able to reach maximal activity because of a high K_m value for biapenem with respect to imipenem.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. Lond. B Biol. Sci. **289:**321–331.
2. **Amicosante, G., N. Franceschini, B. Segatore, A. Oratore, L. Fattorini, G. Orefici, J. Van Beeumen, and J.-M. Frère.** 1990. Characterization of a β-lactamase produced in *Mycobacterium fortuitum* D316. Biochem. J. **271:**729–734.
3. **Amicosante, G., A. Oratore, N. Franceschini, M. Maccarrone, R. Strom, M. Galleni, and J.-M. Frère.** 1988. *Citrobacter diversus* ULA-27 β-lactamases. Improved purification and general properties. Biochem. J. **254:**885–890.
4. **Bicknell, R., E. L. Emanuel, J. Gagnon, and S. G. Waley.** 1985. The production and molecular properties of the zinc β-lactamase from *Pseudomonas maltophilia* IID 1275. Biochem. J. **229:**791–797.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Bush, K., K. Tanaka, D. P. Bonner, and R. B. Sykes.** 1985. Resistance caused by decreased penetration of β-lactam antibiotics into *Enterobacter cloacae*. Antimicrob. Agents Chemother. **27:**555–560.
7. **Cartwright, S. J., and S. G. Waley.** 1984. Purification of β-lactamases by affinity chromatography on phenylboronic acid-agarose. Biochem. J. **221:**505–512.
8. **Catchpole, C. R., R. Wise, D. Thornber, and J. M. Andrews.** 1992. In vitro activity of L-627, a new carbapenem. Antimicrob. Agents Chemother. **36:**1928–1934.
9. **Chen, H. Y., and D. Livermore.** 1994. In-vitro activity of biapenem, compared with imipenem and meropenem, against Pseudomonas aeruginosa strains and mutants with known resistance mechanisms. J. Antimicrob. Chemother. **33:**949–958.
10. **Cuchural, G. J., Jr., M. H. Malamy, and F. P. Tally.** 1986. β-Lactamase-mediated imipenem resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **30:**645–648.
11. **De Meester, F., B. Joris, G. Reckinger, C. Bellefroid-Bourgignon, J.-M. Frère, and S. G. Waley.** 1987. Automated analysis of enzyme inactivation phenomena. Biochem. Pharmacol. **36:**2393–2403.
12. **Fattorini, L., D. Fiorentino, G. Amicosante, N. Franceschini, A. Oratore, and G. Orefici.** 1989. Beta-lactamase production and biological characteristics in nitrosoguanidine induced *Mycobacterium fortuitum* mutants. Acta Leprolog. **1**(Suppl.):44–47.
13. **Felici, A., and G. Amicosante.** 1995. Kinetic analysis of extension of substrate specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* metallo-β-lactamases. Antimicrob. Agents Chemother. **39:**192–199.
14. **Felici, A., G. Amicosante, A. Oratore, R. Strom, P. Ledent, B. Joris, L. Fanuel, and J.-M. Frère.** 1993. An overview of the kinetic parameters of class B β-lactamases. Biochem. J. **291:**151–155.
15. **Fisher, J., J. G. Belasco, S. Khosla, and J. R. Knowles.** 1980. β-Lactamase proceeds via an acyl-enzyme intermediate. Interaction of the *Escherichia coli* RTEM enzyme with cefoxitin. Biochemistry **19:**2895–2901.
16. **Franceschini, N., M. Galleni, J.-M. Frère, A. Oratore, and G. Amicosante.** 1993. A class A β-lactamase from *Pseudomonas stutzeri* that is highly active

against monobactams and cefotaxime. Biochem. J. **292:**697–700.

17. **Frère, J.-M., C. Dormans, C. Duyckaerts, and J. De Graeve.** 1982. Interaction of β-iodopenicillanate with the β-lactamases of *Streptomyces albus* G and *Actinomadura* R39. Biochem. J. **207:**437–444.

18. **Fukasawa, M., Y. Sumita, E. T. Harabe, T. Tanio, H. Nouda, T. Kohzuki, T. Okuda, H. Matsumura, and M. Sunagawa.** 1992. Stability of meropenem and effect of 1β-methyl substitution on its stability in the presence of renal dehydropeptidase I. Antimicrob. Agents Chemother. **36:**1577–1579.

19. **Galleni, M., G. Amicosante, and J.-M. Frère.** 1988. A survey of the kinetic parameters of class C β-lactamases. Cephalosporins and other β-lactam compounds. Biochem. J. **255:**123–129.

20. **Hikida, M., K. Kawashima, K. Nishiki, Y. Furukawa, K. Nishizawa, I. Saito, and S. Kuwao.** 1992. Renal dehydropeptidase-I stability of LJC 10,627, a new carbapenem antibiotic. Antimicrob. Agents Chemother. **36:**481–483.

21. **Kropp, H., J. G. Sundelof, R. Hajdu, and F. M. Kahan.** 1982. Metabolism of thienamycin and related carbapenem antibiotics by the renal dipeptidase, dehydropeptidase-I. Antimicrob. Agents Chemother. **22:**62–70.

22. **Kropp, H., J. G. Sundelof, J. S. Kahan, F. M. Kahan, and J. Birnbaum.** 1980. MK0787 (*N*-formimidoyl thienamycin): evaluation of in vitro and in vivo activities. Antimicrob. Agents Chemother. **17:**993–1000.

23. **Leatherbarrow, R. J.** 1987. Enzfitter: a new non linear regression data analysis program for the IBM PC/P52. Elsevier Biosoft, Cambridge.

24. **Ledent, P., X. Raquet, B. Joris, J. Van Beeumen, and J.-M. Frère.** 1993. A comparative study of class D β-lactamases. Biochem. J. **292:**555–562.

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

26. **Livermore, D. M.** 1992. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2046–2048.

27. **Malanoski, G. J., L. Collins, C. Wennersten, R. Moellering, Jr., and G. M. Eliopoulos.** 1993. In vitro activity of biapenem against clinical isolates of gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. **37:**2009–2016.

28. **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:**4611–4617.

29. **Matagne, A., J. Lamotte-Brasseur, and J.-M. Frère.** 1993. Interaction between active-site serine β-lactamases and so-called β-lactamase stable antibiotics. Eur. J. Biochem. **217:**61–67.

30. **Mitsuashi, S.** 1983. *In vitro* and *in vivo* antibacterial activity of imipenem against clinical isolates of bacteria. J. Antimicrob. Chemother. **22:**62–70.

31. **Monks, J., and S. G. Waley.** 1988. Imipenem as substrate and inhibitor of β-lactamases. Biochem. J. **253:**323–328.

32. **National Committee for Clinical Laboratory Standards.** 1990. Reference methods for the determination of minimum inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.

33. **Nordman, P., S. Mariotte, T. Naas, R. Labia, and M. H. Nicholas.** 1993. Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*. Antimicrob. Agents Chemother. **37:**939–946.

34. **Oh, C. H., and J. H. Cho.** 1994. Studies on the synthesis and antibacterial activity of new zwitterionic carbapenems. J. Antibiot. **47:**126–128.

35. **Rasmussen, B. A., Y. Gluzman, and F. P. Tally.** 1990. Cloning and sequencing of the class B β-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **34:**1590–1592.

36. **Saino, Y., F. Kobayashi, M. Inoue, and S. Mitsuashi.** 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. **22:**564–570.

37. **Segel, I. H.** 1976. Biochemical calculations. John Wiley & Sons, N.Y.

38. **Ubukata, K., M. Hikida, M. Yoshida, K. Nishiki, Y. Furukawa, K. Tashiro, M. Konno, and S. Mitsuhashi.** 1990. In vitro activity of LJC10,627, a new carbapenem antibiotic with high stability to dehydropeptidase I. Antimicrob. Agents Chemother. **34:**994–1000.

39. **Waley, S. G.** 1992. β-Lactamase: mechanism of action, p. 198–228. *In* M. I. Page (ed.), The chemistry of beta-lactams. Chapman & Hall, London.

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

41. **Yang, Y., N. Bhachech, and K. Bush.** 1995. Biochemical comparison of imipenem, meropenem and biapenem: permeability, binding to penicillin-binding proteins, and stability to hydrolysis by β-lactamases. J. Antimicrob. Chemother. **35:**75–84.

42. **Yang, Y., B. A. Rasmussen, and K. Bush.** 1992. Biochemical characterization of the metallo β-lactamase CcrA from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **36:**1155–1157.