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High Specificity of *cphA*-Encoded Metallo-β-Lactamase from *Aeromonas hydrophila* AE036 for Carbapenems and Its Contribution to β-Lactam Resistance

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The *Aeromonas hydrophila* AE036 chromosome contains a *cphA* gene encoding a metallo-β-lactamase highly active against carbapenem antibiotics. This enzyme was induced in strain AE036 to the same extent by both benzylpenicillin and imipenem. When the *cphA* gene was inserted into plasmid pACYC184, used to transform *Escherichia coli* DH5α, the MICs of imipenem, meropenem, and penem HRE664 for recombinant clone DH5α(pAA20R), expressing the *Aeromonas* metallo-β-lactamase, were significantly increased, but those of penicillins and cephalosporins were not. When the metallo-β-lactamase purified from *E. coli* DH5α(pAA20R) was assayed with several β-lactam substrates, it hydrolyzed carbapenems but not penicillins or cephalosporins efficiently. These results demonstrate that this metallo-β-lactamase possesses an unusual spectrum of activity compared with all the other class B enzymes identified so far, being active on penems and carbapenems only. This enzyme may thus contribute to the development of resistance to penems and carbapenems but not other β-lactams.

Limited diffusion of β-lactam antibiotics through the outer membrane of gram-negative bacteria and the presence of β-lactamases in the periplasm are two of the main reasons for the failure of treatment for infections caused by these microorganisms (4, 10, 13, 19). In addition, more than one β-lactamase type may be produced by these organisms after antibiotic induction, so it may be difficult to assess the precise role of each enzyme in β-lactam resistance. In particular, among gram-negative rods, strains of *Aeromonas* spp. have been reported to produce up to three different β-lactamases (16). Some authors used the catalytic properties of a β-lactamase with respect to a number of β-lactam compounds to correlate the theoretically predictable MICs and the experimentally observed MICs (6, 13, 17).

In a previous paper, Iaconis and Sanders (7) reported some properties of two inducible β-lactamases produced by *Aeromonas* spp.; in particular, *Aeromonas hydrophila* AER19M produces two enzymes, called A1 and A2. The former was described as a cephalosporinase with a pI of 7.0 and a molecular mass of 42,500 Da, whereas A2 (pI 8.0) was described as a β-lactamase highly active against penicillins and carbapenems but sensitive to EDTA. Recently, a metallo-β-lactamase encoded by *A. hydrophila* AE036 *cphA* was cloned in *Escherichia coli* DH5α, and the gene was sequenced by Massidda et al. (11). In the present work, we evaluated the contribution of this enzyme to the resistance pattern showed by *E. coli* DH5α(pAA20R), which was used to express the metallo-β-lactamase to eliminate the hydrolytic contribution of the other *Aeromonas* β-lactamase present in the original strain. The properties of the *cphA*-encoded enzyme were also compared with those of a similar enzyme (A2) previously characterized by other authors (7).

MATERIALS AND METHODS

Bacterial strains. The strains of *A. hydrophila* (AE036) and *E. coli* [DH5α and DH5α(pAA20R)] used here were the same as those used in a previous work (11). Plasmid pAA20R has a 2.0-kb *Eco*RI *Aeromonas* fragment containing the *cphA* gene and inserted into pACYC184, which does not have an ampicillin resistance marker for selection. The chromosomal AmpC β-lactamase in *E. coli* DH5α and *E. coli* DH5α (pAA20R) was undetectable.

Induction kinetics for *A. hydrophila* AE036 β-lactamases. *A. hydrophila* AE036 was grown aerobically overnight in brain heart infusion broth with shaking (180 rpm) at 37°C, the culture was diluted 10-fold with fresh medium, and aliquots of 100 ml were reincubated. After 2 h, the inducer (benzylpenicillin or imipenem) was added, and the resulting β-lactamase production was tested at different times (see Fig. 1). In brief, the culture was centrifuged at 10,000 × *g*, resuspended in 5 ml of 25 mM phosphate buffer (pH 7.4), and disrupted by ultrasonic treatment with a Lab Line ultrasonic disintegrator. The suspension was clarified at 105,000 × *g* in a Beckman L8 70 ultracentrifuge; the supernatant was dialyzed for 4 h against 25 mM cacodylate buffer (pH 7.4) containing 50 μM $ZnCl_2$ and used to determine activity against imipenem and nitrocefin with or without 20 mM EDTA. The protein contents were determined by the procedure of Bradford (3) with bovine serum albumin as the standard.

MIC determinations. MICs for either *E. coli* DH5α or *E. coli* DH5α(pAA20R) producing the metallo-β-lactamase were determined by the double-dilution technique with Mueller-Hinton broth and a bacterial inoculum of 5×10^4 or 5×10^8 CFU/ml, respectively, as previously described (2).

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All the drugs tested in these experiments were kindly donated by the following manufacturers: Merck Sharp & Dohme, Rome, Italy (imipenem); ICI, Cheshire, England (meropenem); Hoechst, Milan, Italy (cefotaxime and HRE664); Squibb, Princeton, N.J. (aztreonam); and Smith-Kline-Beecham, Betchworth, England (carbenicillin). Benzylpenicillin and cephaloridin were supplied by Sigma, St. Louis, Mo. Nitrocefin was purchased from Unipath S.p.A., Milan, Italy.

Enzyme purification. The metallo-β-lactamase was purified as follows. *E. coli* DH5α(pAA20R) containing the *cphA* gene was grown overnight in brain heart infusion broth at 37°C with shaking as reported above. Bacterial cells were collected by centrifugation and disrupted by ultrasonic treatment, and the clear lysates were loaded onto a Sephadex G-75 column equilibrated with 50 mM cacodylate buffer (pH 7.4) containing 50 μM $ZnCl_2$. The active fractions were assayed with imipenem as the substrate and pooled. Subsequently, 25 ml of this pool was diluted 1:1 with double-distilled water and loaded into a Rotofor apparatus (Bio-Rad, Richmond, Calif.). The sample was mixed with 2% Ampholine to generate a pH gradient ranging from 3 to 10. Separation was carried out at 4°C for 4 h at 1,500 V and a constant power of 12 W. The metallo-β-lactamase was found in the fractions with pHs ranging from 7.3 to 8.2. Fractions with pHs ranging from 7.8 to 8.2 were used to determine kinetic parameters. This enzyme preparation contained 138 U of specific activity per mg when imipenem was used as the substrate and showed a purification factor of about 600 and a yield relative to the total amount of metallo-β-lactamase of 19%. One unit was defined as 1 μmol of imipenem hydrolyzed per min by 0.01 mg of protein at 30°C.

M_r and isoelectric point determinations. Polyacrylamide gel electrophoresis was done as described by Laemmli (8). Proteins (standards and metallo-β-lactamase) were stained with Coomassie blue R-250. Isoelectric focusing (IEF) was performed with clarified cell extracts or pure metallo-β-lactamase as previously reported (14). Nitrocefin was used as the chromogenic substrate to reveal the β-lactamase activity in the focused gel as previously described (12). When necessary, β-lactamase activity was detected by the iodometric technique with imipenem as the substrate as previously described (11).

Treatment with EDTA and pCMB. For evaluation of the effect of EDTA on β-lactamase activity, experiments were performed at 30°C by use of 50 mM cacodylate buffer (pH 7.4) with or without 50 μM $ZnCl_2$ and with 100 and 200 μM imipenem, respectively, as the substrate. For this purpose, we used either purified enzyme (50 nM) or crude extracts of *A. hydrophila* AE036 (100 μl) obtained after induction with benzylpenicillin. The interaction was studied by incubating the enzyme samples with 20 mM EDTA and determining the residual activity at different incubation times: 0, 5, 15, 30, 60, and 90 min.

Samples of purified enzyme (50 nM) were also incubated with 0.5 mM *p*-hydroxymercuribenzoate (pCMB) for 10 min in 50 mM cacodylate buffer (pH 7.4) with 50 μM $ZnCl_2$, and the residual activity was measured in the presence of 100 and 200 μM imipenem.

Kinetic parameter determinations. The activity of the purified metallo-β-lactamase was monitored spectrophotometrically by use of a Perkin-Elmer lambda-2 instrument and 50 mM cacodylate buffer (pH 7.4) containing 50 μM $ZnCl_2$ at 30°C. Under these conditions, the hydrolysis of each substrate without the enzyme was lower than 1%, although the hydrolysis was not negligible when Tris buffer was used, as we previously reported (15). Hydrolysis rates were determined with imipenem (50 to 200 μM; λ = 299 nm), cephaloridine (80 to 240 μM; λ = 260 nm), benzylpenicillin (250 to 1,000 μM; λ = 230 nm), meropenem (100 to 300 μM; λ = 299 nm), HRE664 (10 to 100 μM; λ = 265 nm), aztreonam (100 to 1,000 μM; λ = 318 nm), carbenicillin (200 to 800 μM; λ = 235 nm), cefotaxime (30 to 120 μM; λ = 260 nm), and nitrocefin (50 to 150 μM; λ = 482 nm). In the presence of higher substrate concentrations, 0.2-cm-path-length quartz cuvettes were used. Catalytic rate constant (k_{cat}) and K_m values were obtained by applying the integrated Hanes equation to the initial rates of hydrolysis. In separate experiments, we also determined the enzymatic activity in crude extracts of *A. hydrophila* AE036 to obtain information on basal activity or activity after induction with imipenem, cephaloridine, benzylpenicillin, and nitrocefin (see Table 2).

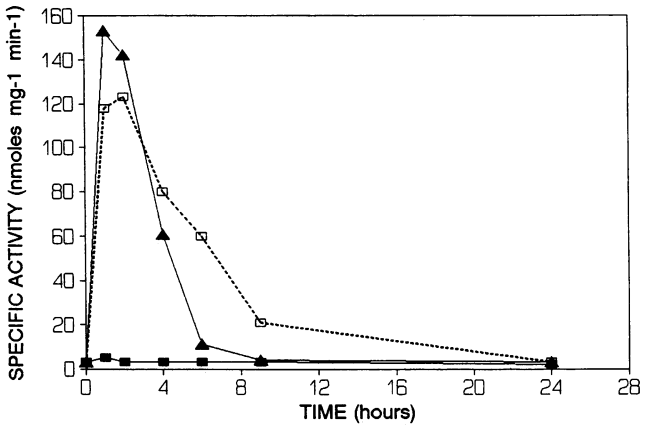

FIG. 1. Time course of metallo-β-lactamase production in *A. hydrophila* AE036. Symbols: ■, control (without an inducer); □, imipenem (5 μg/ml) as the inducer; ▲, benzylpenicillin (100 μg/ml) as the inducer. The activity was monitored at various times with 100 and 200 μM imipenem as the substrate. For experimental details, see the text.

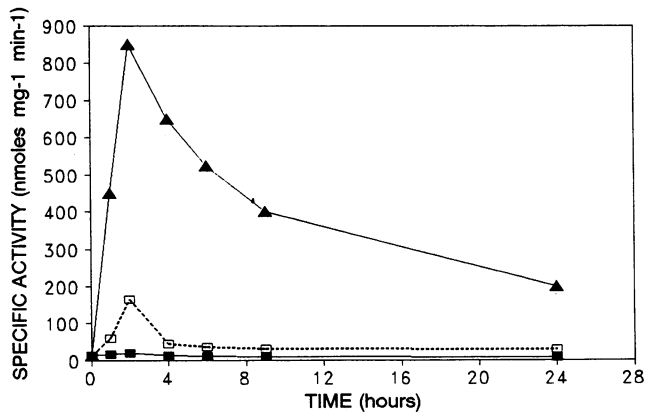

FIG. 2. Time course of nitrocefin-hydrolyzing β-lactamase production in *A. hydrophila* AE036. Symbols: ■, control (without an inducer); □, imipenem (5 μg/ml) as the inducer; ▲, benzylpenicillin (100 μg/ml) as the inducer. The activity was monitored as reported in Fig. 1 with 80 and 100 μM nitrocefin as the substrate.

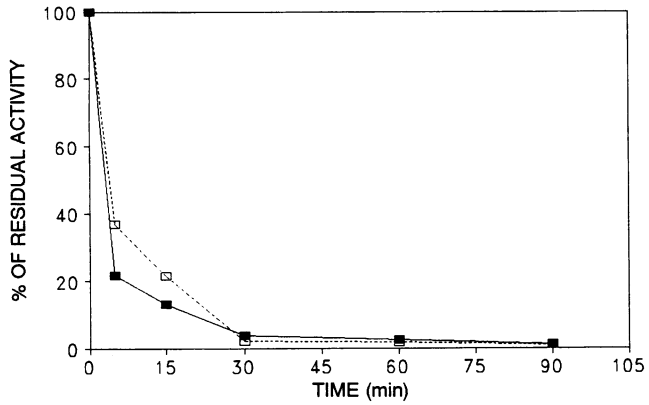

FIG. 3. Time course of EDTA inhibition of metallo-β-lactamase activity determined by use of purified enzyme obtained from *E. coli* DH5α and crude extracts obtained after induction from *A. hydrophila* AE036. Symbols, ■, crude extracts after induction; □, purified enzyme. The activity was monitored in both cases with imipenem (100 and 200 μM) as the substrate. For experimental details, see the text.

RESULTS

β-Lactamase induction. The basal activity of β-lactamases in *A. hydrophila* AE036 was found to be negligible with imipenem but easily detectable with nitrocefin. Moreover, as reported in Fig. 1 and 2, both imipenem and benzylpenicillin acted as good β-lactamase inducers in the tested strain. After induction, two enzymes, with pIs of 6.6 and 7.0, were easily detectable with nitrocefin; a third enzyme, with a pI of 8.0 and active against imipenem but with a poor affinity for nitrocefin, was identified by IEF analysis using imipenem as the substrate (data not shown) or by a spectrophotometric assay. Enzyme production reached a maximum during the first 2 h of bacterial growth after induction. We were unable to detect any metallo-β-lactamase activity with nitrocefin by use of IEF analysis of the crude extracts and the purified enzyme, in keeping with the previous report by Massidda et al. (11); the isoelectric point was determined with 20 μg of a pure enzyme preparation run on the same gel. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we found an M_r of about 28 kDa for the pure enzyme and, by IEF analysis, we found an isoelectric point of 8.0 ± 0.1, in agreement with the data reported elsewhere for this enzyme (5, 11).

Interaction with non-β-lactam compounds. Both crude extracts and the pure enzyme were strongly inhibited when incubated with 20 mM EDTA. The loss of activity reached a maximum after 30 min of treatment, as reported in Fig. 3. The small differences in the inhibition data reported for the pure enzyme and crude extracts are probably due to the different amounts of metallo-β-lactamase in the samples.

The interaction with pCMB revealed a significant inhibition of the pure metallo-β-lactamase after 10 min of incubation (17% residual activity). The A2 protein produced by *A. hydrophila* AER19M was inhibited to a lesser extent, although these data were obtained under different experimental conditions, as reported in Table 1.

TABLE 2. Basal activity and activity after induction of β-lactamases in crude extracts of *A. hydrophila* AE036

Antibiotic	Activity (nmol min^{-1} mg^{-1} of protein)			
	Control	Control + 20 mM EDTA	After induction[a]	After induction[a] + 20 mM EDTA
Imipenem	1.1	1.0	63.6	2.8
Cephaloridine	0.5	0.01	10.45	6.7
Benzylpenicillin	0.01	0.01	44.7	42.4
Nitrocefin	228	228	4,420	4,420

[a] The inducer was 100 μg of benzylpenicillin per ml.

Substrate hydrolysis determinations. We used imipenem, cephaloridine, benzylpenicillin, and nitrocefin in kinetic assays to evaluate the contribution to β-lactam hydrolysis of the β-lactamases, whether constitutively produced or induced in *A. hydrophila* AE036. Imipenem is reported to be a very good substrate for metallo-β-lactamases, whereas benzylpenicillin and cephaloridine are reported to be good substrates for serine β-lactamases of classes A and C, respectively.

As reported in Table 2, the basal activity was low against either imipenem or cephaloridine, although benzylpenicillin was a surprisingly poor substrate. After induction, the activity of crude extracts increased for all the compounds, particularly for benzylpenicillin (4,400-fold increase). Incubation with 20 mM EDTA indicated that benzylpenicillin was not a substrate for the metallo-β-lactamase and that it was probably hydrolyzed by the other enzymes induced in strain AE036. To confirm these findings, we used a purified preparation of the metallo-β-lactamase to determine the kinetic parameters for the set of β-lactams tested, as reported in Table 3. These data confirmed the results obtained with the crude preparations of the induced enzyme. Benzylpenicillin was a very poor substrate, and k_{cat} values for nitrocefin and cephaloridine were negligible compared with those for imipenem; penem HRE664 showed the highest

TABLE 1. Molecular parameters of the metallo-β-lactamase from *A. hydrophila* AE036 (*cphA* gene) in comparison with those of the A2 protein produced by *A. hydrophila* AER19M

Molecular parameter	Result for:	
	AE036 metallo-β-lactamase	A2 protein[a]
Molecular mass	28,000	31,500
Isoelectric point	8.0	8.0
Inhibition by EDTA	+	+
Sensitivity to pCMB	High (residual activity, <20%)	Low (residual activity, 83%)
Nitrocefin hydrolysis	Low	High
Aztreonam affinity	Very low (>1 mM)	Intermediate (≈50 μM)

[a] The data for the *A. hydrophila* AER19M enzyme were obtained under different experimental conditions (0.1 M phosphate buffer, pH 7.0, nitrocephin as the substrate).

TABLE 3. Kinetic parameters of the metallo-β-lactamase from *A. hydrophila* AE036 (*cphA* gene) in comparison with those of the A2 protein produced by *A. hydrophila* AER19M

Antibiotic	AE036 metallo-β-lactamase				A2 protein[a]	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (10^6) (h^{-1} s^{-1})	Relative k_{cat} (%)	Relative V_{max} (%)	K_m (μM)
Nitrocefin	0.4	96	0.004	100	100	30.6
Benzylpenicillin	3	630	0.0047	750	170	124
Carbenicillin	10	500	0.02	2,500	ND[b]	ND
Cephaloridine	0.3	200	0.0015	75	ND	ND
Cefotaxime	0.1	34	0.0029	25	0.5	15
Aztreonam	ND	>1000	ND	ND	3.2	53.3
HRE664	2.5	1.9	1.32	6,250	ND	ND
Imipenem	140	86	1.6	35,000	68.5	30
Meropenem	53	250	0.21	13,250	ND	ND

[a] The data for the *A. hydrophila* AER19M enzyme were obtained under different experimental conditions (0.1 M phosphate buffer, pH 7.0, nitrocefin as the substrate).
[b] ND, not determined.

affinity for the metallo-β-enzyme, while aztreonam was poorly recognized.

In vitro susceptibility. *E. coli* DH5α(pAA20R) containing the *cphA* gene was used to evaluate the contribution of the metallo-β-lactamase to β-lactam resistance for selected compounds. As Table 4 shows, this enzyme apparently confers resistance only to carbapenems (imipenem and meropenem) or penem HRE664. As reported by Massidda et al. (11), the MICs of carbapenems for *A. hydrophila* AE036 were similar when the same inoculum was used. Benzylpenicillin and cephaloridine, easily hydrolyzed by most β-lactamases, were scarcely influenced under our experimental conditions.

DISCUSSION

Metallo-β-lactamases represent a growing threat in resistance phenomena because of their broad spectrum of activity against β-lactam substrates, including the so-called "stable compounds" (9). The recent appearance of a plasmid-mediated enzyme belonging to class B is now causing problems in the treatment of infectious diseases mediated by organisms able to express these class B enzymes (18). Recently, Massidda et al. (11) reported the sequencing of a gene encoding an *A. hydrophila* metallo-β-lactamase showing strong hydrolytic activity against imipenem and meropenem. The reported amino acid sequence of the protein showed partial homology to that of the metallo-β-lactamases produced by *Bacillus cereus* and *Bacteroides fragilis*. The authors also concluded that the enzyme produced in *Aeromonas* spp. is less closely related to these enzymes, suggesting a new variant among class B β-lactamases. Recently, Bakken et al. (1) and Iaconis and Sanders (7) published some interesting papers concerning *Aeromonas* sp. β-lactam resistance caused by two inducible enzymes subsequently purified and characterized. These enzymes, called A1 and A2, appear to be a cephalosporinase and a penicillin- and carbapenem-hydrolyzing β-lactamase, respectively (7). Moreover, the results reported in this study show that *A. hydrophila* AE036 possesses two inducible enzymes, as revealed by IEF analysis, and that its crude extracts hydrolyze carbapenems. Benzylpenicillin and imipenem were good inducers of both β-lactamases, confirming the ability of imipenem to cause induction in *Aeromonas* spp. (9). The substrate profile of the purified metallo-β-lactamase also proved to be interesting. Carbapenems and penem HRE664 were found to be good substrates, whereas benzylpenicillin and cephalosporins were poorly hydrolyzed. Wild-type *A. hydrophila* AE036 was able to hydrolyze benzylpenicillin and cephaloridine by means of a second serine enzyme, most probably a cephalosporinase with a pI of 7.0. When we consider the kinetic parameters of the metallo-β-lactamase, we observe a correlation between the lack of resistance under any conditions and the lack of hydrolysis of the tested compound. As reported in Table 4, strain DH5α(pAA20R) was also susceptible to carbapenems and HRE664 when we used an inoculum of 10^4 CFU/ml, but the effect of the metallo-β-lactamase really became important at 10^8 CFU/ml.

Finally, the purified enzyme was compared with the A2 enzyme, previously described by others (7). As indicated in Tables 1 and 3, there are some differences between the two proteins, but probably only sequencing of the gene encoding the A2 enzyme can help us to decide whether there are two distinct β-lactamases in *Aeromonas* spp. In conclusion, in this paper we describe a metallo-β-lactamase that is produced by *A. hydrophila* and that hydrolyzes benzilpenicillin and cephalosporins at a low level and most probably does not confer resistance to these drugs. It will be very important for future studies to determine which factors are involved in the narrow substrate specificity of this protein.

TABLE 4. MICs determined for *E. coli* DH5α with or without the metallo-β-lactamase gene

Antibiotic	MIC for *E. coli* DH5α at the indicated CFU/ml:			
	Without the *cphA* gene		With the *cphA* gene	
	10^4	10^8	10^4	10^8
Imipenem	0.25	0.5	0.5	128
Meropenem	0.0078	0.5	0.06	64
HRE664	0.5	1	16	128
Aztreonam	0.125	128	0.125	128
Benzylpenicillin	32	64	32	128
Carbenicillin	2	64	4	128
Cefotaxime	0.015	2	0.03	4
Cephaloridine	1	16	2	16

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