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The *Aeromonas hydrophila cphA* Gene: Molecular Heterogeneity among Class B Metallo-β-Lactamases

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An *Aeromonas hydrophila* gene, named *cphA*, coding for a carbapenem-hydrolyzing metallo-β-lactamase, was cloned in *Escherichia coli* by screening an *Aeromonas* genomic library for clones able to grow on imipenem-containing medium. From sequencing data, the cloned *cphA* gene appeared able to code for a polypeptide of 254 amino acids whose sequence includes a potential N-terminal leader sequence for targeting the protein to the periplasmic space. These data were in agreement with the molecular mass of the original *Aeromonas* enzyme and of the recombinant enzyme produced in *E. coli*, evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude β-lactamase preparations followed by renaturation treatment for proteins separated in the gel and localization of protein bands showing carbapenem-hydrolyzing β-lactamase activity by a modified iodometric technique. The deduced amino acid sequence of the CphA enzyme showed regions of partial homology with both the β-lactamase II of *Bacillus cereus* and the CfiA β-lactamase of *Bacteroides fragilis*. Sequence homologies were more pronounced in the regions encompassing the amino acid residues known in the enzyme of *B. cereus* to function as ligand-binding residues for the metal cofactor. The CphA enzyme, however, appeared to share a lower degree of similarity with the two other enzymes, which, in turn, seemed more closely related to each other. These results, therefore, suggest the existence of at least two molecular subclasses within molecular class B metallo-β-lactamases.

In recent classification schemes for β-lactamases, a distinct grouping has been reserved for those enzymes requiring a metal cofactor for activity (metallo-β-lactamases) (1, 4, 5). The prototype for this group of enzymes is the β-lactamase II of *Bacillus cereus*, which has long been studied as a model of metallo-β-lactamases and for which molecular sequence data, along with enzymologic and crystallographic data, are available (3, 12, 17, 27). In the molecular classification of β-lactamases, a class B was devised for this enzyme (1).

Interest in this group of enzymes has grown recently since an increasing number of metallo-β-lactamases have been described in several gram-negative species which share the important characteristic of being able to hydrolyze carbapenem compounds (2, 7, 9, 22, 25, 26, 31). Carbapenems are new β-lactam antibiotics of great therapeutic potential, since they are not hydrolyzed by most bacterial β-lactamases and show a broad spectrum of activity.

Information currently available on carbapenem-hydrolyzing (CH) metallo-β-lactamases is limited, in most cases, to a few biochemical features and suggests the existence of different molecular species within this group of enzymes (5). Molecular sequence data are presently available only for the CfiA β-lactamase of *Bacteroides fragilis* TAL2480 (28) and for the β-lactamase II of *B. cereus* 569/H (12) and 5/B/6 (17). A strong sequence similarity exists between these two enzymes (28), suggesting that the CfiA enzyme also belongs to molecular class B. More information on molecular sequence data of other CH metallo-β-lactamases is needed for a better understanding of this group of enzymes, including their biochemical properties and evolutionary relationships.

Production of CH metallo-β-lactamases has been reported among members of the genus *Aeromonas* (2, 26). In this report, we describe the molecular cloning in *Escherichia coli* and the characterization of an *Aeromonas hydrophila* gene coding for an inducible CH metallo-β-lactamase, named CphA, which resembles the A2 β-lactamase recently purified (13).

The deduced amino acid sequence of the CphA enzyme showed regions of partial homology with both the β-lactamase II of *B. cereus* 569/H (12) and the CfiA β-lactamase of *B. fragilis* TAL2480 (28), sequence homologies being more pronounced in the regions encompassing the amino acid residues known in the *B. cereus* enzyme to be involved in binding of the metal cofactor. The CphA enzyme, however, appeared to share a lower degree of similarity with the two other enzymes, which, in turn, seemed to be more similar to each other. These results, therefore, suggest the existence of at least two molecular subclasses within class B β-lactamases.

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MATERIALS AND METHODS

Bacterial strains. *A. hydrophila* AE036, from which the *cphA* gene was derived, had been isolated from stools of a healthy child and identified according to the *Manual of Clinical Microbiology* (29). *E. coli* DH5α (23) was used as the host for genetic vectors and recombinant plasmids.

Genetic vectors. The Bluescript SK plasmid (Stratagene, La Jolla, Calif.) was used for construction of the *A. hydrophila* genomic library and for some subcloning procedures. Plasmids pGEM5Zf(−) (Promega Biotec, Madison, Wis.) and pACYC184 (6) were also used for other subcloning procedures.

Culture media and growth conditions. LB (23), brain heart infusion (Difco Laboratories, Detroit, Mich.), and Mueller-

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Hinton (Difco) media were used as specified. Bacteria were grown aerobically (with shaking for liquid cultures) at 37°C.

Antibiotics and testing of susceptibility to antimicrobial agents. Imipenem (IMP) was obtained from Merck Sharp & Dohme (Rome, Italy), and meropenem (MRP) was obtained from ICI Pharma S.p.A. (Milan, Italy). Carbenicillin (CB) and penicillin G were from a commercial source. Tetracycline and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, Mo.). MICs were determined by an agar dilution technique (30), using Mueller-Hinton agar and a bacterial inoculum of 10^6 CFU, unless otherwise specified.

β-Lactamase assays. β-Lactamase activity was assayed in crude cell extracts prepared as described by Shannon et al. (26). Cells for extract preparation were grown in brain heart infusion broth. In induction experiments, penicillin G (100 μg/ml) was used as an inducer. β-Lactamase activity against IMP and MRP in the crude extracts was assayed by a spectrophotometric method as previously described (22). One unit of CH β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 nmol of substrate per min at 25°C in 10 mM sodium phosphate buffer, pH 7.0. The CH β-lactamase specific activity of the crude preparations was expressed in units per minute per milligram of protein.

Protein determination. Protein concentrations were determined by using a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used as a standard.

Detection of CH β-lactamases by SDS-PAGE. Crude β-lactamase extracts were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by a method similar to that described by Laemmli (15). The stacking and separating gels were 5 and 15% polyacrylamide at pH 6.8 and 8.8, respectively. The gels were run at 10 mA of constant current for approximately 18 h in a continuous SDS-Tris-glycine buffering system (pH 8.3). After electrophoresis, the gels were either stained with Coomassie brilliant blue R250 or incubated for 4 h at 37°C in phosphate buffer containing 1% (vol/vol) deionized Triton X-100 and 0.1 mM $ZnSO_4$ to obtain renaturation of β-lactamases. In polyacrylamide gels subjected to renaturation treatment, the CH β-lactamase activity was localized by a modified iodometric technique (14), which was performed as follows. After the renaturation step was completed, the gel was overlaid with 1% agarose gel containing 0.2% (wt/vol) soluble starch and 1% (wt/vol) IMP in phosphate buffer and then submerged in Lugol solution. After a 5-min incubation at room temperature, the excess iodine solution was discarded and CH β-lactamase activity was documented by the appearance of a sharp zone of discoloration in the dark background after incubation at 37°C.

IEF. Isoelectric focusing (IEF) was performed in 5% polyacrylamide gel containing pH 3 to 10 ampholyte (Pharmacia, LKB, Uppsala, Sweden) in a 2117 Multiphor unit (LKB). Gels were focused at 9°C and 25 W for 90 min. CH β-lactamase activities were revealed by the iodometric method described above.

Recombinant DNA methodology. Basic recombinant DNA techniques were essentially as described by Sambrook et al. (23). *Aeromonas* genomic DNA was extracted as previously described (21). DNA restriction fragments were purified from agarose gels by Geneclean (BIO 101 Inc., La Jolla, Calif.). Competent *E. coli* cells for library construction and subcloning procedures were prepared according to Hanahan (10), using the protocol that includes $RbCl_2$ among cations (12). The genomic library of AE036 was constructed in the *E. coli* plasmid vector Bluescript SK. For library construction, *Aeromonas* genomic DNA was partially digested with *Sau*3A restriction endonuclease, and restriction fragments 3 to 8 kb in length were purified by preparatory agarose gel electrophoresis; these fragments were then ligated to *Bam*HI-linearized and dephosphorylated plasmid Bluescript SK, and the ligation mixture transformed in *E. coli* DH5α competent cells. Transformants were selected on LB agar plates containing CB at 100 μg/ml (LB-CB); the estimated number of recombinant clones present in the library was >95%, as evaluated by plating the library on LB-CB agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). For Southern blot analysis, DNA digests were resolved on 0.8% agarose gels and transferred to nylon membranes (Hybond-N, Amersham plc, Little Chalfont, England) by capillary blotting according to the supplier's instructions. Filters were hybridized with ^{32}P-labeled probes as specified by the supplier. Probes were labeled with [α-^{32}P]dCTP by the random priming method (8). Restriction endonucleases were from Boehringer GmbH (Mannheim, Germany) or New England BioLabs (Beverly, Mass.). T4 DNA ligase, alkaline phosphatase, and reagents for random priming labeling reactions were from Boehringer. [α-^{32}P]dCTP (10 mCi/ml; specific activity, 800 Ci/mmol) was purchased from Amersham.

DNA sequencing. All sequences were determined on denatured double-stranded DNA templates by the dideoxy-chain termination method (24), using a Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP (10 mCi/ml; specific activity, 1,000 Ci/mmol; Amersham). In some cases, dITP was used in place of dGTP for a more reliable reading through regions of high G+C content. Sequencing primers were from Stratagene (T3, T7, SK, and KS sequencing primers) or Promega Biotec (M13 forward and M13 reverse sequencing primers) or were synthesized on a DNA-SM DNA synthesizer (Beckman Instruments Co., Palo Alto, Calif.).

Sequence analysis and comparisons. Computer analysis of nucleotide sequences for possible coding regions was performed by using the Microgenie sequence analysis software (Beckman) running on an IBM PC AT. Sequences were aligned with the CLUSTAL program for multiple sequence alignment (11) running on a VAX 750 machine (Digital Equipment Corp.). The minimum value for conservative amino acid substitutions according to the log-odds matrix of Dayhoff was fixed at 8 unless otherwise specified.

Nucleotide sequence accession number. The *cphA* sequence

TABLE 1. CH β-lactamase activity in crude enzyme extracts of *A. hydrophila* AE036, *E. coli* DH5α(pAS50), and *E. coli* DH5α(Bluescript)

Strain	Sp act (U/min/mg of protein)[a]		% Activity inhibited by EDTA[b]
	Uninduced	Induced	
AE036	2.5	375	100
DH5α(pAS50)	ND[c]	740	100
DH5α(Bluescript)	ND	2	ND

[a] One unit is defined as the amount of enzyme which hydrolyzes 1 nmol of IMP per min at 25°C in 10 mM $NaPO_4$ buffer (pH 7.0)–0.1 mM $ZnSO_4$. The specific activity was approximately twofold lower against MRP.

[b] Crude enzyme extracts were treated with EDTA (final concentration, 2 mM) at room temperature. Residual β-lactamase activity was assayed spectrophotometrically. Activity (98%) was recovered after dialysis, followed by addition of $ZnSO_4$ (final concentration, 1 mM).

[c] ND, not determined.

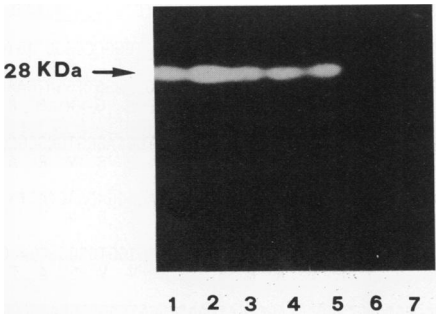

FIG. 1. Analysis of CH β-lactamase activity of crude extracts by SDS-PAGE followed by gel renaturation. Lanes: 1, *A. hydrophila* AE036; 2, *E. coli*(pAA20R); 3, *E. coli*(pAA40H); 4, *E. coli* (pAS20R); 5, *E. coli*(pAS50); 6, *E. coli*(pACYC184); 7, *E. coli* (Bluescript).

has been submitted to the EMBL/GenBank data library and assigned accession number X57102.

RESULTS

Characteristics of the CH β-lactamase activity of *A. hydrophila* AE036. *A. hydrophila* AE036 was selected as the potential source of an *Aeromonas* CH metallo-β-lactamase gene since its crude β-lactamase preparation showed high hydrolyzing activity against IMP and MRP in a spectrophotometric assay, and this activity was inhibited by EDTA (Table 1) and restored by the addition of Zn^{2+} ions (data not shown). CH β-lactamase activity of AE036 was inducible (Table 1). As previously reported for other *A. hydrophila* strains producing CH β-lactamase activity (26), the enzyme was not able to confer an IMP-resistant phenotype on the strain, the MIC for AE036 being relatively low (2 μg/ml). However, a strong inoculum size effect (MIC = 128 μg/ml) was observed when the bacterial inoculum used was raised to 10^9 CFU.

SDS-PAGE analysis of the crude β-lactamase preparation, followed by gel renaturation treatment, showed the presence of a single band of CH activity, with an apparent molecular mass of 28 kDa (Fig. 1). IEF analysis of the same extracts showed a single CH β-lactamase band with an isoelectric point (pI) of 8.0 (data not shown). These data suggested that the CH β-lactamase activity of AE036 was probably due to a single enzyme constituted by a single polypeptide species of this size.

Molecular cloning of a gene of *A. hydrophila* AE036 coding for a CH β-lactamase in *E. coli*. To clone the gene coding for the CH β-lactamase of AE036, a genomic library of AE036 constructed in the *E. coli* plasmid vector Bluescript SK was screened for the ability to grow on LB agar plates containing IMP. Screening was initially performed by plating the library directly on LB agar containing IMP at 1 or 5 μg/ml. However, this strategy failed to reveal any clone able to grow in the IMP-containing medium. Reasoning that the inoculum size could be critical for growth on IMP-containing medium, we then performed the screening by first plating the library on LB-CB agar plates at a colony density of ~200 colonies per plate and then, when the colonies had reached at least 2 mm in diameter, replica plating them on LB agar plates containing IMP at 5 μg/ml (LB-IMP). Using this approach, a single clone able to grow on LB-IMP medium was found among 3.5×10^3 recombinant clones examined. This clone was found to harbor a recombinant plasmid (pAS50) containing a 5-kb DNA insert, a restriction map of which is shown in Fig. 2. The crude β-lactamase preparation from this clone appeared to possess a strong hydrolyzing activity against IMP and MRP (Table 1), and this activity was affected by the presence of EDTA and $ZnSO_4$ in the same manner as that of AE036 (Table 1 and data not shown).

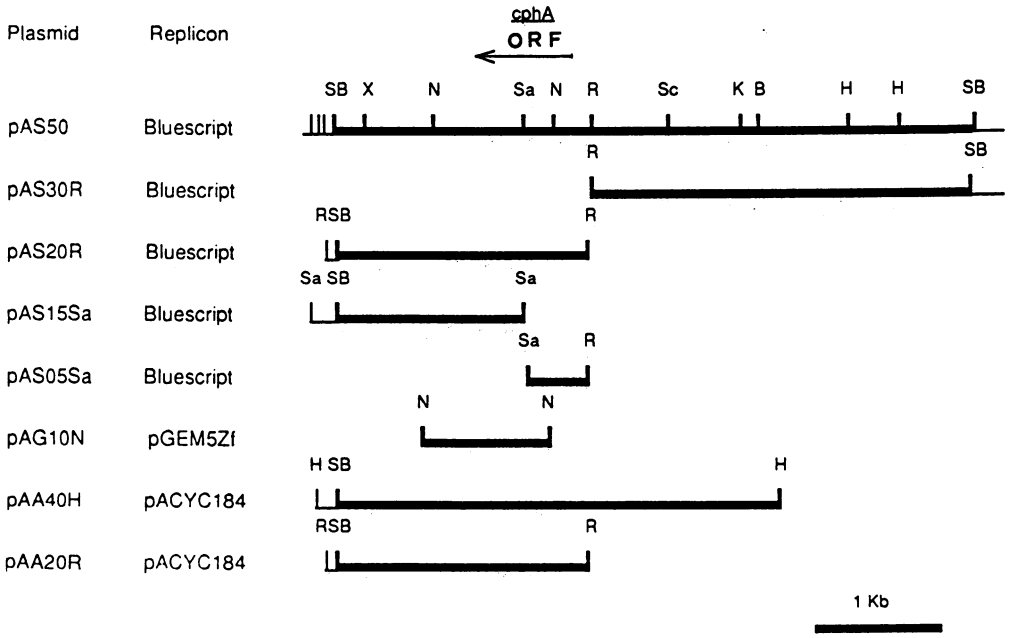

FIG. 2. Restriction endonuclease map of the *Aeromonas* DNA insert of pAS50 and subcloning strategy. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI; Sa, *Sal*I; Sc, *Sac*I; SB, *Sau*3A-*Bam*HI junction; X, *Xho*I. The location of the putative *cphA* ORF identified on the basis of sequencing data is shown above the map.

TABLE 2. Functional analysis of subclones

Recombinant plasmid	Growth on LB-IMP[a]	CH β-lactamase activity[b]	Detection of 28-kDa CH protein[c]
pAS50	+	+	+
pAS30R	−	−	ND[d]
pAS20R[e]	+	+	+
pAS15Sa[e]	−	−	ND
pAS05Sa	−	−	ND
pAG10N[e]	−	−	ND
pAA40H[e]	+	+	+
pAA20R[e]	+	+	+

[a] After replica plating of colonies (~2-mm diameter) grown in LB medium supplemented with the appropriate antibiotic for selection of the plasmid marker.
[b] Determined spectrophotometrically.
[c] Detected by SDS-PAGE followed by gel renaturation.
[d] ND, not determined.
[e] The results of functional analysis were independent of insert orientation.

SDS-PAGE analysis, followed by gel renaturation treatment and IEF analysis of the crude extract of this clone, showed the presence of an IMP-hydrolyzing protein with a molecular mass and pI comparable to those observed for the CH β-lactamase of AE036 (Fig. 1 and data not shown). The ability to produce the same CH β-lactamase activity could be transferred to a new *E. coli* host by transforming it with pAS50. All of these data suggested that pAS50 actually contained an *Aeromonas* CH β-lactamase-encoding gene (named *cphA*) that could be expressed also in *E. coli*. As observed with AE036, the *E. coli* recombinant clone expressing the *cphA* gene did not show an IMP-resistant phenotype unless a large inoculum size was used in the assay.

To confirm that the DNA insert of plasmid pAS50 was derived from the chromosome of AE036, chromosomal DNA of AE036 was digested with several restriction endonucleases, including *Eco*RI, *Bam*HI, *Xho*I, *Hin*dIII, *Apa*I, *Pst*I, *Xba*I, *Sac*I, and *Sal*I, and restriction fragments were analyzed by Southern blot using the pAS50 insert as a hybridization probe. In every case, the hybridization profiles obtained were consistent with the assumption that the DNA insert of pAS50 was a single chromosomal fragment of *A. hydrophila* AE036 (data not shown).

Localization of the *cphA* gene in the cloned DNA fragment. To determine the approximate location of the *cphA* gene in the cloned DNA fragment, several subclones were generated (Fig. 2); for each subclone, the ability to grow on LB-IMP medium (after replica plating) was assayed, and the presence of CH β-lactamase activity in crude β-lactamase preparations was determined spectrophotometrically (Table 2). When present, the CH activity was also analyzed, in some instances, by SDS-PAGE analysis followed by gel renaturation treatment (Fig. 1 and Table 2). The results of this analysis indicated that the *cphA* gene was apparently located within the 2-kb *Eco*RI-*Sau*3AI *Aeromonas* DNA insert of plasmid pAS20R, probably encompassing the *Sal*I site and at least one of the *Nco*I sites.

Nucleotide sequence of the *cphA* gene and deduced amino acid sequence of CphA β-lactamase. The nucleotide sequence of the 2-kb *Aeromonas* DNA insert of plasmid pAS20R was determined, and a computer analysis was performed to seek possible coding regions. A single open reading frame (ORF) was identified whose size was compatible with the results of SDS-PAGE analysis and whose location was compatible with the results of functional analysis of different subclones (Fig. 2). The nucleotide sequence of the putative *cphA* ORF along with its flanking regions is shown in Fig. 3. This ORF is 764 nucleotides long, beginning at an ATG codon (nucleotides 148 to 150) and terminating at an ochre codon (nucleotides 910 to 912), and has the potential to code for a polypeptide of 254 amino acids with a predicted molecular mass of 28,019 Da. The amino acid sequence of the NH_2-terminal region of the polypeptide shows features resembling those of prokaryotic signal sequences for protein export to the periplasmic space (19). A putative ribosomal binding site, resembling those of *E. coli*, is located just upstream of the ATG codon at position 148. The ORF is preceded by potential promoter sequences and followed by two short inverted repeats which could function as a rho-independent transcription terminator.

All *E. coli* strains harboring recombinant plasmids which

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   1 GAATTCCACCTGACTTTCCCCTGACTTCACCTTCTCTTCACACAAGCCCATTTTCCCTTC
  61 ACAAATCCCCCGTATTTTCTGCCCACTGATAAATGGGCCCGGCTTGTCACAGGGGTGGCG
 121 AGGGGCCGGTTGGAGCGAGGGAGCAAGATGATGAAAGGTTGGATGAAGTGTGGATTGGCC
                              M  M  K  G  W  M  K  C  G  L  A
 181 GGCGCCGTGGTGCTGATGGCGAGTTTCTGGGGTGGCAGCGTGCGGGCGGCGGGGATGTCG
      G  A  V  V  L  M  A  S  F  W  G  G  S  V  R  A  A  G  M  S
 241 CTGACGCAGGTGAGCGGCCCTGTGTATGTGGTAGAGGACAACTACTACGTGCAGGAAAAT
      L  T  Q  V  S  G  P  V  Y  V  V  E  D  N  Y  Y  V  Q  E  N
 301 TCCATGGTCTATTTCGGGGCCAAGGGCGTGACTGTGGTGGGGGCGACCTGGACGCCGGAC
      S  M  V  Y  F  G  A  K  G  V  T  V  V  G  A  T  W  T  P  D
 361 ACCGCCCGCGAGCTGCACAAGCTGATCAAACGGGTCAGCCGCAAGCCGGTGCTGGAGGTG
      T  A  R  E  L  H  K  L  I  K  R  V  S  R  K  P  V  L  E  V
 421 ATCAACACCAACTACCACACCGACCGGGCTGGCGGTAACGCCTACTGGAAGTCCATCGGT
      I  N  T  N  Y  H  T  D  R  A  G  G  N  A  Y  W  K  S  I  G
 481 GCCAAGGTGGTGTCGACCCGCCAGACCCGGGATCTGATGAAGAGCGACTGGGCCGAGATT
      A  K  V  V  S  T  R  Q  T  R  D  L  M  K  S  D  W  A  E  I
 541 GTTGCCTTTACCCGCAAGGGGCTGCCGGAGTACCCGGATCTGCCGCTGGTGCTGCCCAAC
      V  A  F  T  R  K  G  L  P  E  Y  P  D  L  P  L  V  L  P  N
 601 GTGGTGCACGATGGCGACTTCACGCTGCAAGAGGGCAAGGTGCGCGCCTTCTACGCGGGC
      V  V  H  D  G  D  F  T  L  Q  E  G  K  V  R  A  F  Y  A  G
 661 CCGGCCCATACGCCGGACGGCATCTTTGTCTACTTCCCCGACGAGCAGGTGCTCTATGGC
      P  A  H  T  P  D  G  I  F  V  Y  F  P  D  E  Q  V  L  Y  G
 721 AACTGCATTCTCAAGGAGAAGCTGGGCAACCTGAGCTTTGCCGATGTGAAGGCCTATCCA
      N  C  I  L  K  E  K  L  G  N  L  S  F  A  D  V  K  A  Y  P
 781 CAGACGCTTGAGCGGCTGAAAGCGATGAAGCTGCCGATCAAGACGGTGATCGGCGGTCAC
      Q  T  L  E  R  L  K  A  M  K  L  P  I  K  T  V  I  G  G  H
 841 GACTCACCGCTGCACGGCCCCGAGCTGATTGATCACTACGAAGCGCTGATCAAGGCCGCA
      D  S  P  L  H  G  P  E  L  I  D  H  Y  E  A  L  I  K  A  A
 901 CCCCAGTCATAAGCCGTCCGACGGCAGCCGGATGACGGCTGGGCGCACGGTAAAAGAGGT
      P  Q  S  *
 961 GCCAGGCCGGGCGTTGCTGGCAGGGGGACGCCCTTGCCCCCTGTTTCGGGCTGTTCAAGG
1021 GCGGCCCCCTTTCATAAGATGGGCCTCTCTTCACGCAGTTAGCGGGCCTTTCGTGATGCA
1981 CGCAATTCAAGACGAACAACCCGATTCACTTGGCCGCCTCAAGCAGGCTTTCATCACCGG
1141 CATGTGGGCGTCGCTGGCCA
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FIG. 3. Nucleotide sequence of the *cphA* ORF and flanking regions. Number 1 represents the first base of the cloned insert of plasmid pAS20R, at the *Eco*RI site present in *Aeromonas* DNA upstream the *cphA* gene. Putative sequences involved in transcription control and a putative ribosomal binding sequence are underlined or overlined. The initiation and termination codons are boxed. The deduced amino acid sequence of the CphA protein is also shown.

included this ORF and flanking sequences were able to grow on LB-IMP, and their crude β-lactamase preparations showed strong CH activity, independently of the insert orientation and of the presence of *E. coli* promoter sequences located near the insert junctions. On the other hand, strains containing recombinant plasmids which did not encompass the entire ORF were not able to grow on LB-IMP and did not express CH β-lactamase activity. These data are in agreement with the hypothesis that this ORF encodes the *Aeromonas* CphA β-lactamase, and they also suggest that the *Aeromonas* promoter sequences located upstream of the *cphA* gene can be functional in *E. coli*.

Inducibility of the *cphA* gene in *E. coli*. The possible clustering, near the *cphA* gene, of *Aeromonas* genetic elements involved in the regulation of *cphA* gene expression that might also be functional in *E. coli* when carried along with the gene in the same multicopy plasmid vector was studied by using two *E. coli* strains harboring recombinant plasmids pAA40H and pAA20R, respectively (Fig. 2), which do not contain an ampicillin-resistant marker for selection. These plasmids contain an *Aeromonas* DNA insert that encompasses the *cphA* gene flanked by a 1-kb 3′-flanking region and a 0.15- or 2.0-kb 5′-flanking region, respectively. For both strains, the CH activities of the crude β-lactamase extracts, assayed spectrophotometrically, were found to be similar under induced or uninduced conditions (data not shown), and the original regulation of *cphA* gene expression was not present.

Comparison of the CphA enzyme with other CH β-lactamases. The deduced amino acid sequence of the CphA enzyme was compared with those of the β-lactamase II of *B. cereus* 569/H (12) and of the CfiA β-lactamase of *B. fragilis* TAL2480 (28). The CphA enzyme showed a significant degree of sequence homology with the two other enzymes, particularly in some regions (Fig. 4). The overall amino acid identity was 24.7% when compared with the *B. cereus* enzyme (Fig. 4a) and 25% when compared with the *B. fragilis* enzyme (Fig. 4b), while the degree of similarity increased to 61.6 and 66.2%, respectively, after allowing for conservative substitutions.

The results of multiple sequence alignment analysis (Fig. 5) showed that at least five regions of homology can be identified in the three different proteins. Three of these regions encompass the residues that function as ligand-binding residues in β-lactamase II, while two shorter regions are located closer to the N and C terminus, respectively, of the protein.

The results of the alignment analysis also indicated that the CphA enzyme shares a lower degree of similarity with the other two enzymes, which, in turn, appear to be more closely related to each other (Fig. 5). It should be noted that comparison of the amino acid sequences of β-lactamase II and CfiA β-lactamase, performed according to the same criterion, gave an overall amino acid identity of 30.3% and a degree of similarity of 71.9%.

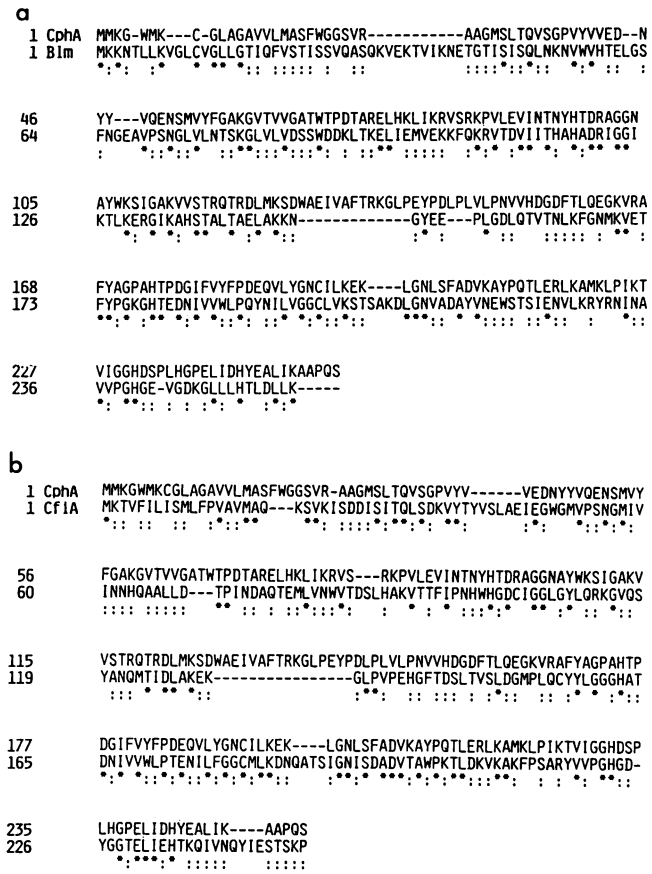

FIG. 4. Comparison of the deduced amino acid sequence of the CphA β-lactamase from *A. hydrophila* AE036 with that of the β-lactamase II (Blm) from *B. cereus* 569/H (a) and of the CphA β-lactamase from *A. hydrophila* AE036 with that of the CfiA β-lactamase from *B. fragilis* TAL2480 (b). The CphA sequence is listed above each pair of lines. Numbers located at the left correspond to the residue numbers of the respective proteins. Aligned identical residues are indicated by asterisks; conservative amino acid substitutions are indicated by colons.

DISCUSSION

From a molecular standpoint, the CphA metallo-β-lactamase of *A. hydrophila* AE036 appears to be quite different from the β-lactamase II of *B. cereus* 569/H and the CfiA β-lactamase of *B. fragilis* TAL2480. From sequencing data, in fact, the degree of homology of the CphA enzyme with the two other β-lactamases appeared to be lower than that observed between the two latter enzymes, although regions of similarity are clearly shared by the three sequences. In particular, of the four residues that are known to bind the metal cofactor in β-lactamase II (27), and which are conserved and can be perfectly aligned in CfiA β-lactamase, only three (the two histidine residues at positions 118 and 240 and the cysteine residue at position 198) can be aligned with homologous residues in the CphA enzyme in a corresponding region of homology, while the histidine residue at position 116, which has been shown by X-ray crystallography to give only a minor contribution to the binding of the metal cofactor in β-lactamase II (27), is substituted by an asparagine residue (a conservative substitution) in the CphA enzyme. Moreover, close to the glutamic acid residue at position 87 of β-lactamase II, which has been identified as essential for enzyme function (18), a region of extensive homology can be identified in the CfiA but not the CphA protein (28; Fig. 4a). In addition, while the amino acid sequence of β-lactamase II can be aligned without gaps with that of the CfiA β-lactamase over the entire region encompassing the four ligand-binding residues, this is not the case when these two sequences are aligned with that of the CphA enzyme (Fig. 5). These results, therefore, suggest that there

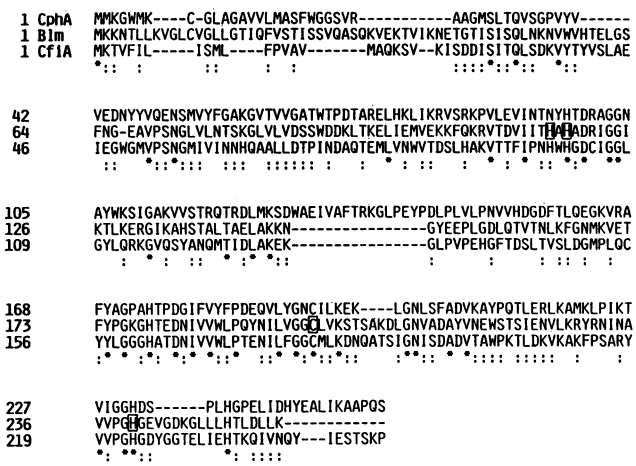

FIG. 5. Comparison of the deduced amino acid sequence of the CphA β-lactamase from *A. hydrophila* AE036 with that of the CfiA β-lactamase from *B. fragilis* TAL2480 and that of the β-lactamase II (Blm) from *B. cereus* 569/H. Identical residues are indicated by asterisks; conservative amino acid substitutions are indicated by colons. Amino acids in the Blm enzyme known to be involved in zinc ion binding are boxed.

are at least two distinct molecular subclasses within class B metallo-β-lactamases.

Interestingly, production of the CphA enzyme is not able to confer a carbapenem-resistant phenotype on the original *Aeromonas* strain unless a very large bacterial inoculum is used, and the same is true for the *E. coli* strains in which the cloned gene is constitutively expressed. The failure to isolate any CphA-producing recombinant clone when the *Aeromonas* genomic library was directly plated on IMP-containing medium is apparently related to this fact. Only when screening of the library was performed by replica plating of large colonies, previously grown on CB-containing medium, was it successful in isolating a recombinant clone that produced the enzyme. This screening criterion might therefore prove useful for cloning genes coding for similar enzymes.

SDS-PAGE followed by gel renaturation treatment that allows the detection of enzymatic activity directly in the gel is a technique that has been used by other authors to detect cell wall hydrolases of bacteria (16, 20), but it has not previously been used for β-lactamases. This technique could reliably be used to detect the activity of the CphA β-lactamase, and it was found to be very useful both for estimating the molecular mass of the polypeptide constituting the original enzyme and for comparing it with the recombinant one expressed in *E. coli*. The molecular mass of the polypeptide constituting the CphA enzyme, estimated by this technique, was apparently in agreement with the calculated mass and similar to that reported by Iaconis and Sanders for the A2 β-lactamase of *A. hydrophila* AER19 (13). This technique may prove useful for rapid characterization of β-lactamase activity in crude preparations in addition to the classical IEF analysis.

The use of a modified iodometric technique using a carbapenem substrate to detect CphA β-lactamase activity following both IEF and renaturing SDS-PAGE was adopted since the CphA enzyme showed very low activity against the chromogenic cephalosporin nitrocefin, which did not permit the reliable detection of the enzymatic activity in gels. This detection technique, which was specific only for the CH activities, could enable the rapid and specific evaluation of similar activities in crude β-lactamase preparations by these methods.

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REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. London Sect. B Biol. Sci. **289**:321–331.
2. **Bakken, J. S., C. C. Sanders, R. B. Clark, and M. Hori.** 1988. β-Lactam resistance in *Aeromonas* spp. caused by inducible β-lactamases active against penicillins, cephalosporins, and carbapenems. Antimicrob. Agents Chemother. **32**:1314–1319.
3. **Bicknell, R., and S. G. Waley.** 1985. Cryoenzymology of *Bacillus cereus* β-lactamase II. Biochemistry **24**:6876–6887.
4. **Bush, K.** 1989. Classification of β-lactamases: groups 1, 2a, 2b, and 2b′. Antimicrob. Agents Chemother. **33**:264–270.
5. **Bush, K.** 1989. Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob. Agents Chemother. **33**:271–276.
6. **Chang, A. C. Y., and S. N. Cohen.** 1977. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
7. **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.
8. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
9. **Fujii, T., K. Sato, K. Miyata, M. Inoue, and S. Mitsuhashi.** 1986. Biochemical properties of β-lactamase produced by *Legionella gormanii*. Antimicrob. Agents Chemother. **29**:925–926.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
11. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73**:237–244.
12. **Hussain, M., A. Carlino, M. J. Madonna, and J. O. Lampen.** 1985. Cloning and sequencing of the metallothioprotein β-lactamase II gene of *Bacillus cereus* 569/H in *Escherichia coli*. J. Bacteriol. **164**:223–229.
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. **Joergensen, J. H., S. A. Crawford, and G. A. Alexander.** 1977. Rapid penicillinase paper strip test for detection of β-lactamases producing *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **11**:1087–1088.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Leclerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can. J. Microbiol. **35**:749–753.
17. **Lim, H. M., J. J. Pène, and R. W. Shaw.** 1988. Cloning, nucleotide sequence, and expression of the *Bacillus cereus* 5/B/6 β-lactamase II structural gene. J. Bacteriol. **170**:2873–2878.
18. **Little, C., E. L. Emanuel, J. Gagnon, and S. G. Waley.** 1986. Identification of an essential glutamic acid residue in β-lactamase II from *Bacillus cereus*. Biochem. J. **233**:465–469.
19. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39**:615–618.
20. **Potvin, C., D. Leclerc, G. Tremblay, A. Asselain, and G. Bellemare.** 1988. Cloning, sequencing and expression of a *Bacillus* bacteriolytic enzyme in *Escherichia coli*. Mol. Gen. Genet. **214**:241–248.
21. **Priefer, U., R. Simon, and A. Puhler.** 1984. Cloning with cosmid, p. 190–201. *In* A. Puhler and K. N. Timmis (ed.), Advanced molecular genetics. Springer-Verlag KG, Berlin.

22. **Saino, Y., F. Kobayashi, M. Inoue, and S. Mitsuhashi.** 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. **22**:564–570.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
25. **Sato, K., T. Fujii, R. Okamoto, M. Inoue, and S. Mitsuhashi.** 1985. Biochemical properties of β-lactamase produced by *Flavobacterium odoratum*. Antimicrob. Agents Chemother. **27**: 612–614.
26. **Shannon, K., A. King, and I. Phillips.** 1986. β-Lactamases with high activity against imipenem and Sch 34343 from *Aeromonas hydrophila*. J. Antimicrob. Chemother. **17**:45–50.
27. **Sutton, B. J., P. J. Artymiuk, A. E. Cordero-Borboa, C. Little, D. C. Phillips, and S. G. Waley.** 1987. An X-ray-crystallographic study of β-lactamase II from *Bacillus cereus* at 0.35 nm resolution. Biochem. J. **248**:181–188.
28. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* β-lactamase II. J. Bacteriol. **172**:2584–2593.
29. **von Graevenitz, A.** 1985. *Aeromonas* and *Plesiomonas*, p. 278–281. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
30. **Washington, J. A., II.** 1985. Susceptibility tests: agar dilution, p. 967–971. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
31. **Yang, Y., P. Wu, and D. M. Livermore.** 1990. Biochemical characterization of a β-lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. Antimicrob. Agents Chemother. **34**:755–758.