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Distribution of *cphA* or Related Carbapenemase-Encoding Genes and Production of Carbapenemase Activity in Members of the Genus *Aeromonas*

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The prevalence of the *cphA* gene or related carbapenemase-encoding genes was investigated in 114 *Aeromonas* strains belonging to the six species of major clinical interest. A species-related distribution of *cphA*-related sequences was observed. Similar sequences were found in *A. hydrophila*, *A. veronii* bv. *sobria*, *A. veronii* bv. *veronii*, and *A. jandaei*, but not in *A. caviae*, *A. trota*, or *A. schubertii*. However, a single *A. caviae* strain (of 62 tested) was found carrying *cphA*-related sequences, suggesting the possibility of the horizontal transfer of this gene to species which normally do not carry it. Production of carbapenemase activity was detectable in 83% of the hybridization-positive strains but in none of the hybridization-negative ones. When it was present, carbapenemase activity was always inhibitable by EDTA. Either carbapenemase-producing or not, *Aeromonas* strains appeared to be susceptible to imipenem when in vitro susceptibility testing was performed with inocula of conventional size (10^5 CFU), for which MICs were always ≤1 μg/ml. With a larger inoculum (10^8 CFU), the MICs for carbapenemase-negative strains always remained ≤1 μg/ml, while those for carbapenemase-producing strains were always ≥4 μg/ml, being usually higher than the breakpoint for susceptibility. The present results indicate that the production of metallocarbapenemase activity, apparently encoded by *cphA* homologs, is widespread among some of the *Aeromonas* species of clinical interest (*A. hydrophila*, *A. veronii* bv. *sobria*, *A. veronii* bv. *veronii*, and *A. jandaei*) and that imipenem MICs for carbapenemase-producing strains are subjected to a relevant inoculum size effect.

Carbapenem compounds are β-lactam antibiotics of great therapeutic potential owing to their broad spectra of activity and resistance to most bacterial β-lactamases. However, enzymes able to inactivate the carbapenem molecule by hydrolysis of the β-lactam ring (carbapenemases) are produced by some microbial species and can cause microbial resistance to these compounds (11, 12, 18). Most of the carbapenemases thus far described are metalloenzymes which use a zinc ion-containing active site (11), and all of the metallocarbapenemases thus far characterized at the sequence level belong to the same molecular class (class B), suggesting a common phylogeny (6, 7, 14, 17, 25).

Members of the genus *Aeromonas*, which in the last decade have been the objects of increasing interest as human and animal pathogens (9), are among the few microorganisms that can produce metallocarbapenemase activities (1, 8, 24). The CphA metallo-β-lactamase of *A. hydrophila* is one of the most active carbapenemases known, showing also, in comparison with other class B enzymes, a very specific substrate profile (5, 6, 23). The production of carbapenemase activity has previously been investigated in *Aeromonas* strains belonging to different species (1, 8, 24). However, recent developments in the field of *Aeromonas* taxonomy have led to a redefinition of some of the old species and to the description of new species (9) in which production of carbapenemase activity has not been studied, so that current knowledge on carbapenemase production in members of the genus *Aeromonas* is still incomplete. Moreover, the genetic bases for the production of metallocarbapenemase activity have been studied only in *A. hydrophila* (14).

In the work described here we evaluated the prevalence of the *cphA* or related genes and the production of carbapenemase activity in several *Aeromonas* strains belonging to the six recognized species of major clinical interest. The results indicated a species-related distribution of *cphA* homologs, which are present in *A. hydrophila*, *A. veronii* (both *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii*), and *A. jandaei*, but not in *A. caviae*, *A. trota*, or *A. schubertii*. The horizontal transfer of the *cphA* locus to species which normally do not carry it, however, is apparently possible since *cphA*-related sequences were also found in one *A. caviae* strain. Production of carbapenemase activity is restricted to strains carrying *cphA*-related sequences, although not all of them are apparently able to express the enzymatic activity. In vitro susceptibility testing showed that, whether they were carbapenemase-producing or not, all strains appear to be susceptible to imipenem when inocula of conventional sizes are used. However, unlike carbapenemase-negative strains, for carbapenemase-producing strains MICs are usually higher than the breakpoint for susceptibility when larger inocula are used for MIC determinations.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the study were clinical isolates and were identified as described by Janda (9) and Carnahan et al. (2). *A. hydrophila* ATCC 7966^T, *A. veronii* bv. *sobria* ATCC 9071^T, *A. veronii* bv. *veronii* ATCC 35624^T, *A. jandaei* ATCC 49568^T, *A. caviae* ATCC 15468^T, *A. trota* ATCC 49657^T, and *A. schubertii* ATCC 43700^T were used as the reference strains for the different *Aeromonas* species. *A. hydrophila* AE036 (14) was used as a reference

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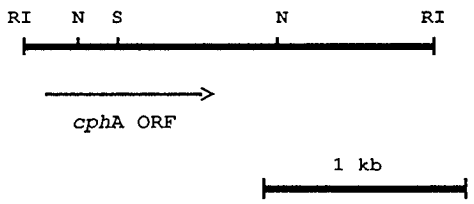

FIG. 1. Design of the *cphA*-specific probe used for colony hybridization assays. The probe was the 2-kb insert of plasmid pAA20R and corresponds to an *Eco*RI (RI) genomic fragment derived from *A. hydrophila* AE036, which encompasses the *cphA* gene and flanking sequences (14). N, *Nco*I; S, *Sal*I; ORF, open reading frame.

strain carrying the *cphA* gene. Bacterial strains were stored at −80°C in brain heart infusion broth containing 20% (vol/vol) glycerol until used.

Filter hybridization techniques. For colony hybridization, bacteria were grown for 16 h at 37°C on nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) placed on Columbia blood agar plates. Bacterial colonies were lysed in situ by placing the filter (side on which bacteria were grown up) for 7 min on filter paper soaked with a solution containing 1.5 M NaCl and 0.5 M NaOH. The filter was then neutralized by placing it twice for 5 min on filter paper soaked with a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.2). The filter was then deproteinated for 1 h at 37°C in a solution of 1× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.05% (wt/vol) sodium dodecyl sulfate (SDS) and 50 μg of proteinase K per ml, air dried, and baked at 80°C for 90 min under vacuum. Prehybridization was for 2 h at 65°C in 5× SSC–5× Denhardt's solution containing 0.5% SDS and 100 μg of sonicated and denatured salmon sperm DNA per ml (21). Hybridization was done for 16 h at 65°C in the same solution used for prehybridization. Final washings were performed at 65°C in 1× SSC containing 0.1% SDS. The hybridization probe was labeled with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) by the random priming technique (4) with a commercial kit (Boehringer GmbH, Mannheim, Germany). All colony hybridization experiments were performed in duplicate, and *A. hydrophila* AE036 (14) was always included as a reference strain carrying the *cphA* gene.

Assay for production of carbapenemase activity. Carbapenemase activity was assayed in crude cell extracts prepared from cultures induced with penicillin G (100 μg/ml) as described previously (23). Sodium phosphate buffer (10 mM; pH 7.0) was used for cell resuspension and was maintained as the final buffer. Cultures were collected 2 h after induction. Imipenem (100 μM) was used as the substrate, and its hydrolysis was monitored by a spectrophotometric method as described previously (20). One unit of carbapenemase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per min at 25°C in 10 mM sodium phosphate buffer (pH 7.0). EDTA inhibition assays were performed by determining the residual carbapenem-hydrolyzing activity after incubation of the crude extract (100 μl) for 30 min at 25°C in the presence of 10 mM EDTA. Incubation was performed in a 1-ml final volume in EDTA-containing sodium phosphate buffer (pH 7.0). Imipenem at 100 μM was used as the substrate for the measurement of residual activity. In all cases a control assay without EDTA was run in parallel. The protein concentration of the extracts was determined by using a commercial kit (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.). Each induction experiment was performed in duplicate.

In vitro susceptibility tests. The MIC of imipenem for the *Aeromonas* strains was determined by an agar dilution technique (19) with Mueller-Hinton agar and inocula of 10^5 and 10^8 CFU per spot on the plate. All MIC determinations were performed in duplicate.

RESULTS

Prevalence of *cphA*-related sequences among members of the genus *Aeromonas*. The presence of *cphA*-related sequences was analyzed in 114 *Aeromonas* strains of different species by colony hybridization with the DNA probe shown in Fig. 1.

Results of the analysis showed that sequences recognized by the probe were present in all of the *A. hydrophila*, *A. veronii* (both *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii*), and *A. jandaei* strains tested. For most *A. hydrophila* strains (18 of 21 strains) the hybridization signal was comparable to that obtained with *A. hydrophila* AE036, which is the strain from which the *cphA* gene was cloned (14), while 3 strains showed a markedly weaker hybridization signal. Hybridization signals weaker than those observed with AE036 were also observed with all of the strains belonging to the two other species (Table 1). The *cphA* probe did not hybridize with any of the *A. trota* strains tested or with the *A. schubertii* type strain (Table 1). Among *A. caviae*, only 1 of 62 strains was recognized by the probe, showing a hybridization signal comparable to that obtained with AE036 (Table 1). It should be noted that the probe used included not only the β-lactamase gene but also flanking sequences (Fig. 1) and that the weaker hybridization signals observed with some strains (Table 1) could be due either to the lack of part of the genomic region recognized by the probe or to a certain degree of sequence divergence within the same region.

TABLE 1. Results of colony hybridization with the *cphA*-specific probe

Species (no. of strains)	(%) No. of strains recognized by the probe
A. hydrophila (21)	21 (100)[a]
A. veronii bv. *sobria* (20)	20 (100)[b]
A. veronii bv. *veronii* (3)	3 (100)[c]
A. jandaei (3)	3 (100)[d]
A. caviae (62)	1 (1.6)[e]
A. trota (4)	0 (0)
A. schubertii (1)	0 (0)

[a] Three *A. hydrophila* strains showed a hybridization signal markedly weaker than that observed with AE036. The ATCC 7966^T reference strain and all other *A. hydrophila* strains showed a hybridization signal comparable to that of AE036.
[b] All of the *A. veronii* bv. *sobria* strains, including the ATCC 9071^T reference strain, showed a hybridization signal weaker than that observed with AE036.
[c] All of the *A. veronii* bv. *veronii* strains, including the ATCC 35624^T reference strain, showed a hybridization signal weaker than that observed with AE036.
[d] All of the *A. jandaei* strains, including the ATCC 49568^T reference strain, showed a hybridization signal weaker than that observed with AE036.
[e] The hybridization signal showed by this strain was comparable to that observed with AE036. All other strains, including the ATCC 15468^T reference strain, did not show any hybridization signal with the probe.

Production of carbapenemase activity in strains recognized or not recognized by the *cphA*-specific probe. The production of carbapenemase activity was assayed in the 114 *Aeromonas* strains previously analyzed for the presence of *cphA*-related sequences. Since previously described *Aeromonas* carbapenemases are inducible enzymes (1, 14, 24), the production of carbapenemase activity was always assayed following induction with penicillin G, which is apparently a suitable inducer for these enzymes (23, 24).

Production of carbapenemase activity was detected in 83% of the hybridization-positive strains but in none of the hybridization-negative ones (Table 2). The hybridization-positive strains unable to produce carbapenemase activity included 5 (24%) of the 21 *A. hydrophila* strains and 3 (15%) of the 20 *A. veronii* bv. *sobria* strains tested (Table 2). Interestingly, all three *A. hydrophila* strains showing a markedly weaker hybridization signal with the probe (Table 1) did not produce carbapenemase activity.

In all cases carbapenemase activity was inhibited by 10 mM EDTA, suggesting that metalloenzymes were always responsible for it.

In vitro susceptibilities of *Aeromonas* strains to imipenem on the basis of carbapenemase production. The in vitro susceptibilities of the 114 *Aeromonas* strains to imipenem were determined.

The results of the experiments showed that, with an inoculum of 10^5 CFU, the MIC of imipenem for both carbapenemase-producing and carbapenemase-non-producing strains was always ≤1 μg/ml (Table 3), i.e., lower than the breakpoint for susceptibility (which is 8 μg/ml [15]). With a larger inoculum (10^8 CFU), the MICs of imipenem for carbapenemase-

TABLE 2. Production of carbapenemase activity by hybridization-positive and hybridization-negative *Aeromonas* strains

Species (no. of strains)	Hybridization pattern	No. (%) of strains[a]	
		Producing CA	Not producing CA
A. hydrophila (21)	Positive	16[b]	5 (24)[c]
A. veronii bv. *sobria* (20)	Positive	17[d]	3 (15)
A. veronii bv. *veronii* (3)	Positive	3	0
A. jandaei (3)	Positive	3	0
A. caviae (1)	Positive	1	0
A. caviae (61)	Negative	0	61
A. trota (4)	Negative	0	4
A. schubertii (1)	Negative	0	1

[a] The production of carbapenemase activity (CA) was assayed as described in Materials and Methods. A strain was defined as a producer of carbapenemase activity if crude extracts showed imipenem-hydrolyzing activity in duplicate experiments. In carbapenemase activity-positive strains, the carbapenemase activity was always >10 and usually >100 mU/mg of total protein. When it was present, carbapenemase activity was always inhibited by 10 mM EDTA.

[b] Including the ATCC 7966^T reference strain.

[c] All three *A. hydrophila* strains showing a much weaker hybridization signal (Table 1) were included in this group.

[d] Including the ATCC 9071^T reference strain.

negative strains always remained ≤1 μg/ml, while those for carbapenemase-producing strains were always ≥4 μg/ml and were usually higher than the breakpoint for susceptibility (Table 3).

DISCUSSION

The results of the present study suggest a species-related distribution of *cphA* homologs in members of the genus *Aeromonas*. In fact, sequences recognized by a probe containing the entire *cphA* gene and flanking regions from *A. hydrophila* AE036 (14) were found in *A. hydrophila*, *A. veronii* (both *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii*), and *A. jandaei*, but not in *A. caviae* (except for one strain), *A. trota*, or *A. schubertii*. It should be noted that with some strains (three *A. hydrophila* and all of the *A. veronii* bv. *sobria*, *A. veronii* bv. *veronii*, and *A. jandaei* strains studied) the intensity of the hybridization signal was lower than that obtained with AE036 and most other *A. hydrophila* strains. The markedly weaker hybridization signals observed with the three *A. hydrophila* strains were likely due to the occurrence of a deletion of most of the chromosomal region recognized by the probe in these strains, as also suggested by their inabilities to express carbapenemase activity. On the other hand, the weaker hybridization signals observed with the *A. veronii* and *A. jandaei* strains were more likely due to the occurrence of some sequence divergence within the *cphA* locus in members of the species listed above, as also suggested by their ability to express a metallocarbapenemase activity. However, since the probe used in the present study included not only the β-lactamase gene but also some flanking sequences, it cannot definitely be excluded that the weaker hybridization results obtained with the strains listed above could be due to hybridization to the flanking sequences and may have nothing to do with the β-lactamase.

TABLE 3. In vitro susceptibilities of the 114 *Aeromonas* strains to imipenem

Species (no. of strains)	Carbapenemase activity	MIC (μg/ml) for an inoculum of:			
		10^5 CFU		10^8 CFU	
		Range	90%[a]	Range	90%
A. hydrophila (16)	+	≤0.25–1	0.5	4–>128	>128
A. hydrophila (5)	−	≤0.25–1		1–1	
A. veronii bv. *sobria* (17)	+	≤0.25–1	0.5	4–>128	>128
A. veronii bv. *sobria* (3)	−	≤0.25–0.5		1–1	
A. veronii bv. *veronii* (3)	+	≤0.25–1		4–>128	
A. jandaei (3)	+	≤0.25–0.5		128–>128	
A. caviae (1)	+	≤0.25		4	
A. caviae (61)	−	≤0.25–0.5	≤0.25	≤0.25–1	1
A. trota (4)	−	≤0.25–≤0.25		≤0.25–0.5	
A. schubertii (1)	−	≤0.25		≤0.25	

[a] 90%, MICs at which 90% of strains are inhibited.

According to current knowledge on *Aeromonas* phylogenesis (13), the most plausible hypothesis to explain a similar distribution of *cphA* homologs in members of the genus *Aeromonas* is that an ancestral *cphA* gene was present in the *Aeromonas* ancestor and that gene loss has subsequently occurred during the evolution of some lineages. Data from hybridization experiments also suggest that the *cphA* homologs carried by *Aeromonas* strains of different species could be quite conserved, although not identical, at the sequence level.

Notwithstanding the species-related pattern of distribution, *cphA*-related sequences were also found in one *A. caviae* strain. This finding likely reflects a horizontal transfer of genetic material and suggests that transfer of the *cphA* gene to species which normally do not carry it can occur. Since the hybridization signal given by the *A. caviae* strain was comparable to that observed with *A. hydrophila* strains, it seems likely that the gene was derived from *A. hydrophila*. This hypothesis is consistent with the view that *A. hydrophila* and *A. caviae* are closely related species from a phylogenetic standpoint (13).

Concerning the relationship between the presence of *cphA*-related sequences and the production of carbapenemase activity, only strains carrying such sequences were found to be able to express a similar enzymatic activity, which apparently was always class B enzymatic activity. These data suggest that carbapenemase production is restricted to *Aeromonas* strains carrying the genes described above and also that the carbapenemase activity detected in such strains is actually encoded by their *cphA* homologs. Not all strains carrying *cphA*-related sequences, however, were able to produce carbapenemase activity. In fact, five *A. hydrophila* strains (24% of the strains of this species analyzed) and three *A. veronii* bv. *sobria* strains (15% of the strains of this species analyzed) were found to be unable to produce any carbapenemase activity. Of such strains, three were the *A. hydrophila* strains showing a markedly reduced hybridization signal with the *cphA*-specific probe. In these cases a gross deletion of the *cphA* locus could have occurred and could have been responsible for the CphA$^-$ phenotype. In the other hybridization-positive but carbapenemase-negative strains the hybridization signal was comparable to that observed with carbapenemase-producing strains of the same species, suggesting that other mechanisms could also be responsible for the apparent silencing of the *cphA* allele. We are investigating the strains described above to understand the genetic bases of their CphA$^-$ phenotypes.

In comparison with the situation in other species which can produce metallocarbapenemase activities, the situation observed in the genus *Aeromonas* appears to be variable. In particular, for the *cphA*-positive *Aeromonas* species the situation is similar to that of *Xanthomonas maltophilia* and *Bacillus cereus*, in which the carriage and expression of carbapenemase-

encoding genes are universal within species (11), although the occurrence of some hybridization-positive strains which are unable to produce carbapenemase activity suggests that either the *cphA* gene or other genes involved in its regulation are somewhat prone to undergoing silencing mutational events. For *A. caviae*, on the other hand, the situation appears to be similar to those for *Bacteroides fragilis*, *Serratia marcescens*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa*, in which carbapenemase-encoding sequences are found only in a minority of strains (11).

Whether they were carbapenemase producing or not, *Aeromonas* strains appeared to be susceptible to imipenem when the in vitro susceptibility test was performed with inocula of conventional sizes. These data are in agreement with those previously reported by Shannon et al. (24) and Bakken et al. (1). With an increase in the inoculum size, however, a clear distinction in imipenem susceptibility was observed between carbapenemase-producing and carbapenemase-non-producing strains. Under these conditions, in fact, only the latter strains remained susceptible to imipenem, with MICs always being far below the breakpoint for susceptibility, while the former were usually resistant or, at most, the MICs for the strains were close to the breakpoint for susceptibility. For this reason, and since the microbial population present in vivo is often likely to be larger than that used in conventional in vitro susceptibility testing, in clinical practice it would be advisable to consider a priori as imipenem resistant all *Aeromonas* strains belonging to *cphA*-positive species. In addition, given the possibility of horizontal gene transfer, it is advisable to always perform susceptibility testing with large inocula or to analyze all strains of *cphA*-negative species with a *cphA*-specific probe. Since imipenem was the sole carbapenem tested in the present study, the considerations given above could not apply to other carbapenems such as meropenem which, in comparison with imipenem, usually has lower MICs for *Aeromonas* strains of *cphA*-positive species (10, 16) and also appears to be hydrolyzed somewhat less efficiently by the CphA enzyme (23). However, the facts that the CphA enzyme is also active against meropenem (14, 23), that the mutation leading to constitutive high-level expression of the *Aeromonas* carbapenemase results in resistance to both imipenem and meropenem (22), and that the inoculum size effect is observed for both imipenem and meropenem with clinical *A. hydrophila* and *A. veronii* bv. *sobria* isolates (3) strongly suggest that large inocula of carbapenemase-producing *Aeromonas* strains could also demonstrate a resistance phenotype to meropenem and, possibly, other carbapenem compounds.

It should be noted that, in comparison with the homologous enzymes produced by *X. maltophilia*, *B. cereus*, and *B. fragilis*, the CphA metallocarbapenemase purified from *A. hydrophila* AE036 hydrolyzes carbapenems with approximately the same efficiency but shows a considerably lower efficiency (usually 10- to 100-fold) for penicillins and cephalosporins (5, 6). It has also been observed that *Escherichia coli* expressing the *A. hydrophila cphA* gene is resistant only to carbapenems and only when high inocula (10^8 CFU) are used for MIC determinations (23). The current results suggest that a similar behavior is common to all of the *Aeromonas* strains that produce carbapenemase activity.

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