

Inducible Oxacillin-Hydrolyzing Penicillinase in *Aeromonas hydrophila* Isolated from Fish

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An inducible penicillinase was shown to be present in a strain of *Aeromonas hydrophila* subsp. *hydrophila* isolated from freshwater fish. Enzyme induction was observed with benzylpenicillin or 6-aminopenicillanic acid, and the enzyme was cell bound. The penicillinase was purified 50-fold from a crude cell extract. The molecular weight was estimated to be 23,000 by gel filtration. The pH and temperature optima for the enzyme activity were 8.0 and 35°C, respectively. The penicillinase showed a unique substrate profile by hydrolyzing oxacillin about twice as rapidly as benzylpenicillin. The enzyme activity was weakly inhibited by sodium chloride but was not affected by *p*-chloromercuribenzoate. The property of penicillinase production by the *A. hydrophila* strain could not be transferred to *Escherichia coli* and also could not be eliminated from the bacteria by ethidium bromide treatment.

Beta-lactamase (EC 3.5.2.6, penicillin β -lactam amidohydrolase) is a hydrolytic enzyme produced by species representing many bacterial genera. Although a physiological role for the enzyme in bacterial metabolism has not been determined, the enzyme plays its most important role in the drug resistance of many pathogenic bacteria to beta-lactam antibiotics. Many beta-lactamases are thought to be species specific. In addition to these enzymes, beta-lactamases mediated by a gene(s) borne on R plasmids are widely distributed among gram-negative bacteria.

Since the discovery of R plasmids specifying ampicillin resistance (1), we have studied the penicillinases mediated by R plasmids as well as the species-specific beta-lactamases of various gram-negative bacteria (10, 14, 18-20, 22, 23). One of the purposes of these studies has been to elucidate the phylogenetic relationship between the plasmid- and the chromosome-mediated beta-lactamases. The penicillinases mediated by R plasmids have been classified for convenience into two groups, i.e., type I and II, according to their enzymological and immunological properties (10, 18, 23). Our previous studies demonstrated a striking similarity in enzymological and immunological properties between the species-specific penicillinases of the *Klebsiella* group and type I R plasmid penicillinases (18, 20). However, we could not find a species-specific penicillinase similar to type II penicillinase, which has a unique substrate specificity able to hydrolyze methicillin and oxacillin and its derivatives.

Recently, we have extended our survey to gram-negative bacteria that are not of human origin. One of them is *Aeromonas hydrophila*, isolated from freshwater fishes by Aoki and Egusa (4). This paper presents data concerning an inducible penicillinase of *A. hydrophila* that shows an unusual substrate specificity able to hydrolyze the semisynthetic penicillins, which are resistant to many beta-lactamases.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *A. hydrophila* subsp. *hydrophila* 67-P-24 and Y-62 were isolated by T. Aoki and S. Egusa from freshwater fishes in Japan (4) and kindly made available to us. The two strains had previously been reported by the original collectors to be *Aeromonas liquefaciens*. The strains were again characterized in this laboratory by means of their biological and biochemical properties, as interpreted by Schubert (21). The strains grew in nutrient broth at 37°C but could not grow in nutrient broth containing 7.5% (wt/vol) sodium chloride at 37°C. They could grow with ammonium sulfate and glucose as the sole source of nitrogen and carbon and also could utilize *L*-arginine, *L*-asparagine, or *L*-histidine as the sole source of carbon. The strains fermented glucose and glycerol with the production of gas and were positive to the Voges-Proskauer test; they were tested for production of 2,3-butanediol dehydrogenase and were found to be positive. Both strains are resistant to ampicillin but susceptible to chloramphenicol, tetracycline, streptomycin, and kanamycin.

Nutrient broth and heart infusion broth were used for bacterial cultivation in liquid medium, and heart infusion agar was used for the determination of bacterial resistance to antibiotics. The media used

were all products of Eiken Chemical Co., Tokyo, Japan. Bacterial cultivation was performed at 30°C.

Beta-lactam antibiotics. Penicillins and cephalosporins were kindly provided by the following pharmaceutical companies: benzylpenicillin, ampicillin, phenethicillin, methicillin, and 6-amino-penicillanic acid, Meiji Seika Co., Tokyo, Japan; cloxacillin and oxacillin, Bristol Laboratories, Inc., Syracuse, N.Y.; cephalothin, Shionogi Pharmaceutical Co., Osaka, Japan; cephaloridine, Torii Pharmaceutical Co., Tokyo, Japan; carbenicillin, Fujisawa Pharmaceutical Co., Osaka, Japan.

Determination of penicillinase activity. Penicillinase activity was determined iodometrically at 30°C in 0.1 M phosphate buffer (pH 7.5) by a modification of Perret's method (16). The microiodometric assay method devised by Novick (15) was used with slight modification for kinetic measurements of the enzyme reaction. Penicillinase activity was expressed in units; one unit of the enzyme activity was defined as the activity that hydrolyzes 1 μ mol of benzylpenicillin per min under the conditions described above.

Since ampicillin is more readily hydrolyzed than benzylpenicillin, it was used in the experiments for determination of optimum pH, optimum temperature, molecular weight, and effect of inhibitors.

Determinations of protein. The concentration of protein in column eluates was followed by measuring the absorbancy at 280 nm. Bovine serum albumin was used as the protein standard. For more accurate estimations, the method of Lowry et al. (13) was used.

Estimation of molecular weight. The molecular weight of penicillinase was estimated by gel filtration through a Sephadex G-75 column (1.2 by 75 cm) according to the method of Andrews (2). Ovalbumin, α -chymotrypsinogen, and cytochrome *c* were used as the internal standard proteins. All the standard proteins were the products of C. F. Boehringer & Soehne GmbH, Mannheim, Germany.

Polyacrylamide gel electrophoresis. Analytical disc polyacrylamide gel electrophoresis was performed with 7.5% gel (0.5 by 7 cm) and tris(hydroxymethyl)aminomethane-glycine buffer at pH 8.7. Electrophoresis was carried out at a constant current of 3 mA/tube at 5°C for 2 h, and the gel was stained with 0.25% Coomassie brilliant blue. The stained protein band, which corresponded to penicillinase, was identified by the position of penicillinase activity in a non-stained gel. For detection of penicillinase activity in the gel, the non-stained gel was cut into 2-mm slices, and the enzyme was extracted from the slices with 0.1 M phosphate buffer (pH 7.5).

Assay of bacterial resistance to penicillins and cephaloridine. The determination of levels of bacterial resistance to antibiotics was performed according to procedures described previously (10), except that the temperature of bacterial growth was 30°C.

Transfer test. Transfer of ampicillin resistance by cell-to-cell contact from *A. hydrophila* 67-P-24 to *Escherichia coli* was examined by the method described previously (10). Briefly, *E. coli* 58-161 F⁻Nal^r (resistant to nalidixic acid) was used as a recip-

ient strain. After overnight incubation of the donor strain with the recipient cells in heart infusion broth at 30°C, portions of the mixed culture were plated on heart infusion agar containing 50 μ g of nalidixic acid and 800 or 1,600 μ g of ampicillin per ml. The plates were incubated at 30°C for 48 h. Because penicillinase from the donor cell inactivates ampicillin in the selective medium, 800 μ g of ampicillin per ml was found to be the minimal concentration for inhibiting the growth of the *E. coli* strain when mixedly plated with the donor strain.

RESULTS

A. hydrophila strains 67-P-24 and Y-62 were selected from the stock cultures of T. Aoki and S. Egusa as representatives of highly ampicillin-resistant and less resistant strains, respectively. Levels of resistance to ampicillin and other beta-lactam antibiotics in the two strains are shown in Table 1. Strain 67-P-24 is resistant to both the penicillins and cephaloridine. Strain Y-62 shows higher susceptibility to the antibiotics, especially to cephaloridine, than strain 67-P-24, although from a clinical point of view it may be considered as a strain resistant to the clinically used penicillins.

Transfer of the ampicillin resistance gene from strain 67-P-24 to *E. coli* could not be detected, although about 10⁶ recipient cells were examined. Ampicillin resistance was stable during long storage of the organism in cooked meat medium and could not be eliminated by treatment with ethidium bromide, according to the procedures described by Bouanchaud et al. (7).

Penicillinase activity in organisms growing exponentially in nutrient broth was assayed after sonically disrupting the cells. The enzyme activity of strain 67-P-24 was 0.11 units/mg (dry weight) of bacteria, and we could not detect measurable penicillinase activity in the culture of strain Y-62. When benzylpenicillin at a sub-inhibitory concentration (50 μ g/ml) was added

TABLE 1. Levels of resistance to beta-lactam antibiotics in *A. hydrophila* strains 67-P-24 and Y-62

Drug	Drug resistance (μ g/ml) ^a	
	67-P-24	Y-62
Benzylpenicillin	400	200
Ampicillin	400	50
Carbenicillin	400	25
Oxacillin	1,600	200
6-Aminopenicillanic acid	100	12.5
Cephaloridine	50	6.3

^a The level of drug resistance is expressed as the maximum concentration of drug that allows visible growth of bacteria after 18 h of incubation at 30°C.

to the logarithmic-phase culture of 67-P-24, the specific enzyme activity at 1 h after addition of the inducer was 1.7 units. Although we did not perform a quantitative assay, penicillinase activity was also found in the culture of strain Y-62 after induction with benzylpenicillin. 6-Aminopenicillanic acid, known as the most effective inducer for a cephalosporinase of *Pseudomonas aeruginosa* (17), was also an effective inducer, comparable to benzylpenicillin.

Figure 1 shows the kinetics of induced penicillinase synthesis in strain 67-P-24 growing exponentially. By addition of benzylpenicillin as inducer, a marked increase in enzyme activity occurred after a lag period of less than 15 min. The produced enzyme was rarely released into the growth medium and appeared to be cell bound, similar to other beta-lactamases of gram-negative bacteria.

From cells of strain 67-P-24 induced for 2 h

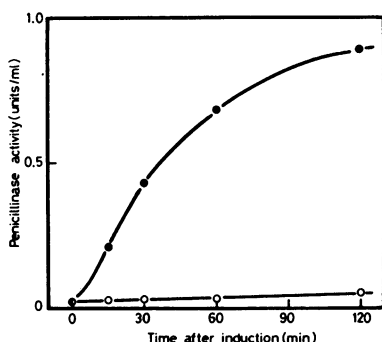


FIG. 1. Kinetics of induction of penicillinase synthesis by benzylpenicillin in *A. hydrophila* 67-P-24. A bacterial culture grown overnight in nutrient broth at 30°C was diluted fivefold with fresh broth and incubated with shaking at the same temperature. When the optical density of the culture at 610 nm reached about 0.4 (in mid-exponential growth phase), benzylpenicillin was added to the culture to a final concentration of 50 µg/ml and incubation was continued. Portions of the culture were removed at the indicated times, and penicillinase activity was assayed after sonically disrupting the cells. Symbols: ●, induced culture; ○, uninduced culture.

with benzylpenicillin (100 µg/ml) the enzyme was extracted and purified by the procedures summarized in Table 2. Although the enzyme was purified 50-fold from the crude cell extract, the purified enzyme preparation gave three minor protein bands other than the major band corresponding to penicillinase activity in an acrylamide gel.

The pH activity plot for the partially purified penicillinase, using ampicillin as a substrate, is shown in Fig. 2A. The enzyme has an optimum pH of 8.0. The temperature activity curve of the enzyme at pH 7.5 is shown in Fig. 2B, and the optimum temperature for the enzyme activity is 35°C.

The relative activities (relative V_{max}) of the enzyme from strain 67-P-24 at substrate saturation for seven penicillins and two cephalosporins are given in Table 3, together with the Michaelis constants (K_m) for some of these substrates. The K_m values were obtained from Lineweaver-Burk plots (12). The penicillinase has a unique substrate profile that shows a highly hydrolytic activity and a high affinity against oxacillin. Oxacillin is known as a semi-synthetic penicillin resistant to hydrolysis by many beta-lactamases. Two other semisynthetic penicillins, cloxacillin and methicillin, which are resistant to many known beta-lactamases, are also effectively hydrolyzed by the enzyme. Another striking characteristic of the penicillinase is that the enzyme is able to hydrolyze ampicillin at a high rate, about five times that of benzylpenicillin. The crude enzyme preparation from *A. hydrophila* Y-62 also showed a substrate profile similar to that of the enzyme of strain 67-P-24.

The molecular weight of the enzyme was estimated by gel filtration experiments on Sephadex G-75. The average of two measurements gave a molecular weight of $23,000 \pm 1,000$ for the penicillinase of strain 67-P-24.

p-Chloromercuribenzoate and sodium chloride were examined for their inhibitory effect on the activity of the enzymes from the strains

TABLE 2. Summary of purification of penicillinase from *A. hydrophila* 67-P-24

Step	Penicillinase activity recovered (units)	Penicillinase sp act (units of protein)	Purification	Recovery (%)
Ultrasonic disintegration followed streptomycin treatment and dialysis	1,500 ^a	3.67	1	100
Chromatography on carboxymethyl-Sephadex C-50 (0.2 M NaCl, stepwise)	1,033	25.0	6.8	69
Gel filtration on Sephadex G-75	1,000	90.0	24.5	67
Chromatography on carboxymethyl-Sephadex C-50 (0 to 0.4 M NaCl, gradient)	450	183	50	30

^a Extracted from cells cultured in 2 liters of medium.

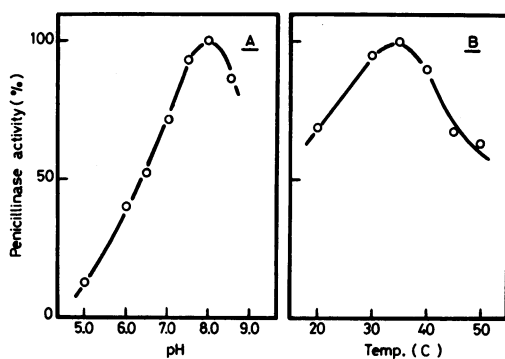


FIG. 2. Effects of pH and temperature on penicillinase activity. Effects of pH (A) and temperature (B) on the activity of the penicillinase of *A. hydrophila* 67-P-24 were measured with ampicillin as a substrate. (A) Acetate buffer (0.1 M) was used at pH 5.0, and 0.1 M phosphate buffer was used for the range pH 6.0 to 8.5. (B) The penicillinase in 0.1 M phosphate buffer, pH 7.5, containing 8 mM of the substrate was incubated for 10 min at the indicated temperature, and reaction rates were determined idiometrically.

67-P-24 and Y-62 (crude enzyme preparation) according to the procedures described previously (23). *p*-Chloromercuribenzoate did not show an inhibitory effect on either of the enzyme activities at a concentration of 1 mM. In the presence of 100 mM sodium chloride, both enzymes were 25 to 30% inhibited in their activities against ampicillin. Chloride ion is known to be a specific inhibitor for the type II penicillinase mediated by R plasmid (23).

DISCUSSION

Aoki and Egusa demonstrated that most of the *Aeromonas* strains isolated from freshwater fishes and soft-shelled turtles cultured in farm ponds in Japan were highly resistant to ampicillin (4). They reported that the ampicillin resistance in *Aeromonas* failed to transfer to *E. coli* by conjugation, although sulfanilamide, tetracycline, and chloramphenicol resistances on R plasmids were transferable from various species to *E. coli* (3, 5, 6). Therefore, they suggested that ampicillin resistance may be an innate resistance in this species.

We have also failed to demonstrate transferability of ampicillin resistance in strain 67-P-24. Furthermore, it was observed that the ampicillin resistance was not lost from the organism after long storage of the strain in cooked-meat medium or by treatment of the organism with ethidium bromide. The penicillinase characterized in this paper may be a species-specific beta-lactamase of *A. hydrophila*. Although the ampicillin resistance cannot be attributed only to

TABLE 3. Substrate profiles and kinetic properties of penicillinases from *A. hydrophila*

Substrate	Penicillinase of 67-P-24		Penicillinase of Y-62 ^a (Relative rate of hydrolysis [%]) ^b
	Relative rate of hydrolysis (%) ^b	K_m (μ M)	
Benzylpenicillin	100	14	100
Ampicillin	534	143	441
Phenethicillin	243		208
Oxacillin	212	33	
Cloxacillin	90	77	75
Methicillin	32	154	
6-Aminopenicillanic acid	48		91
Cephaloridine	95		183
Cephalothin	49		50

^a Crude extract from cells of strain Y-62.

^b The rate of hydrolysis was determined at 30°C in 0.1 M phosphate buffer (pH 7.5) with a substrate concentration of 8 mM. The rates given are expressed as the percentage of hydrolysis of benzylpenicillin.

the penicillinase, the close relationship between the levels of ampicillin resistance and the enzyme activity to ampicillin observed in strains 67-P-24 and Y-62 suggests that the enzyme plays a significant role in the high ampicillin resistance of this species. It is also interesting to note that beta-lactam antibiotics have not been used in fish culturing in Japan.

Oxacillin, its derivatives, and methicillin are known as semisynthetic penicillins that are resistant to hydrolysis by many beta-lactamases. However, these semisynthetic penicillins are subject to hydrolysis by two specific beta-lactamases, i.e., beta-lactamase II produced by *Bacillus cereus* 569/H (11) and penicillinases mediated by R plasmids RGN238 and R1818 (9, 10, 23). Recently, Dale and Smith (8) classified the oxacillin-hydrolyzing penicillinases of R plasmids into two main groups. Enzymes of the first group, including the enzyme of R plasmid RGN238, hydrolyze methicillin about three times as rapidly as benzylpenicillin and have a relatively lower molecular weight (about 24,000). Enzymes of the second group, which include the enzyme of R plasmid R1818, have relatively lower activity against methicillin and a very high molecular weight, 41,000 to 46,000. These two group-specific enzymes are produced constitutively in the host organisms. When the penicillinase of *A. hydrophila* was compared with the enzymes of R plasmids, it was found to have a substrate profile intermediate between those of the two enzymes of R plasmids and a molecular weight close to the enzymes of the first group. The most significant difference between the enzymes of *A. hydrophila* and R plasmids is in inducibility. The

penicillinase presented in this paper is, to our knowledge, the only inducible penicillinase that has been found in gram-negative bacteria.

As demonstrated by Dale and Smith (8), the oxacillin-hydrolyzing penicillinases mediated by R plasmids are not homogeneous, and it is possible to classify them into seven subgroups. Our investigation revealed the existence of an oxacillin-hydrolyzing penicillinase that may be chromosomally mediated. More detailed comparison of the penicillinase with the oxacillin-hydrolyzing penicillinases of R plasmids is a subject of deep interest in connection with the origin of the genetic determinants for the enzymes on R plasmids. A comparison of physico-chemical and immunological properties of the two enzymes is in progress.

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