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Comparison of the Substrate Specificities of the β-Lactamases from *Klebsiella aerogenes* 1082E and *Enterobacter cloacae* P99

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A potent β-lactamase (EC 3.5.2.6) produced by a strain of *Klebsiella aerogenes* (*K. pneumoniae*), 1082E, isolated from a hospital patient, has been examined. Its properties were different from those of most gram-negative β-lactamases previously reported. The enzyme has been partly purified, and its activity against a range of substrates has been compared with that of the enzyme from *Enterobacter cloacae* (*Aerobacter cloacae*) P99. The *K. aerogenes* enzyme, although predominantly a penicillinase, had a wide range of specificity. In addition to hydrolyzing the cephalosporins, it attacked the normally β-lactamase-resistant compounds methicillin and cloxacillin as well as cephalosporin analogues with the same acyl substituents. The results obtained with the *E. cloacae* enzyme confirmed its cephalosporinase activity and showed that, unlike the enzyme from *K. aerogenes*, it was relatively inactive against the penicillins.

The β-lactamases produced by gram-negative bacteria differ considerably in their substrate specificities. Although most of the enzymes hydrolyze penicillin G, cephalothin, and cephaloridine, comparatively few hydrolyze methicillin or cloxacillin.

Smith and Hamilton-Miller (9) examined β-lactamases from a range of gram-negative organisms, none of which hydrolyzed methicillin or cloxacillin to a significant extent. Sawai, Mitsuhashi, and Yamagishi (8) compared the β-lactamases of 28 strains representing 11 species of gram-negative bacteria; none significantly attacked cloxacillin. These authors reported one strain of *Escherichia coli* capable of hydrolyzing cloxacillin (8, 10). Datta and Kontomichalou (2) also studied a strain of *E. coli* that attacked cloxacillin. β-lactamase production was mediated by a resistance transfer factor in both of these *E. coli* strains.

During an examination of organisms isolated in various hospitals, a culture of *Klebsiella aerogenes* (*K. pneumoniae*) was observed which hydrolyzed a wide range of penicillins and cephalosporins, including methicillin, cloxacillin, and the cephalosporins 5/1 and 291/1 with corresponding acyl substituents (Fig. 1).

An intracellular β-lactamase from this strain of *K. aerogenes*, 1082E, has been partly purified, and its activity has been compared with that of the enzyme from *Enterobacter cloacae* (*Aerobacter cloacae*) P99, a strain originally isolated at the Hospital for Sick Children, Toronto, Canada (3). The enzyme from strain P99 had previously been partly purified to give a stable preparation of reproducible activity for use in the examination of cephalosporin analogues and in β-lactamase inhibition studies (5). Hennessey (4) showed that this strain produced much larger amounts of β-lactamase than any of the other strains of *Enterobacter* that he studied.

MATERIALS AND METHODS

Conditions for optimal production of β-lactamase. The effects upon enzyme production of shaken versus static culture and of the culture medium and its volume were investigated. Media were limited to commercially available preparations; Oxoid Nutrient Broth No. 2, Difco Brain Heart Infusion, and Difco Micro Inoculum Broth were used. Volumes of either 50 ml of medium in 250-ml conical flasks or 250 ml in 1-liter flasks were inoculated with 18-hr cultures of the strains at a level of 0.4% (v/v) and then were incubated for 18 hr at 37 C. This incubation period had previously been found to be optimal (Fig. 2).

Intracellular and extracellular yields of β-lactamase were examined by ultraviolet assay with cephaloridine as substrate (5). The amount of enzyme

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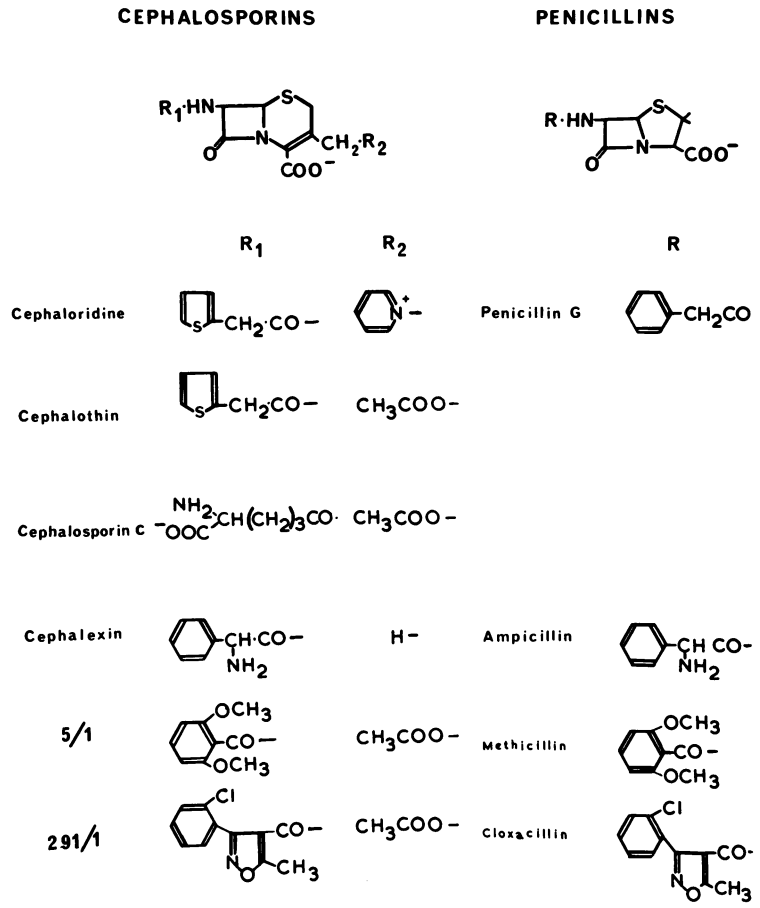

FIG. 1. *Structures of the cephalosporin and penicillin substrates.*

which hydrolyzed 1 μmole of cephaloridine/min at *p*H 7 and 37 C was regarded as 1 unit. The β-lactamase produced by *K. aerogenes* 1082E and *E. cloacae* P99 under various cultural conditions is quoted in units per liter in Table 1.

Preparation of partly purified enzymes. *K. aerogenes* 1082E and *E. cloacae* P99 cultures (five flasks, each containing 50 ml) were grown under the optimal conditions established for β-lactamase production (see below). The cells were harvested by centrifugation at 5,400 × *g* for 30 min and were resuspended in 0.1 M phosphate buffer, *p*H 7 (10 ml). The same fractionation method was used for both enzymes. The intracellular enzymes were released from the cells and partly purified by the ultrasound disruption/Sephadex G-50 method described previously (5). The resultant enzyme preparations were kept in 2-ml amounts at −25 C.

Iodometric assay of β-lactamases. Enzyme activities were assessed by the method of Perret (6) and calculated on the basis of 1 mole of hydrolyzed cephalosporin or 1 mole of hydrolyzed penicillin being equivalent to approximately 4 g atoms of iodine or 8 g atoms of iodine, respectively (1).

Microbiological assay of β-lactamase. The enzymatic destruction of substrates was recorded on a percentage basis by comparison with cephaloridine degradation. Residual antibiotic was assayed by agar diffusion with the use of the cup plate technique (7) after the enzyme reaction had been stopped by the addition of iodine (2×10^{-6} M). *Bacillus subtilis* NCIB 8533 or *Staphylococcus aureus* NCTC 7447 was used as the assay organism.

RESULTS

Optimal conditions for β-lactamase production. The optimal production of intracellular enzyme by both *K. aerogenes* 1082E and *E. cloacae* P99 occurred when the strains were grown in 50-ml amounts of Brain Heart Infusion and shaken for 18 hr at 37 C (Table 1). Previous studies had shown that the enzyme yields were optimal by 18 hr (Fig. 2).

Preparation of partly purified enzyme. The *K. aerogenes* 1082E enzyme behaved like the *E. cloacae* P99 enzyme except that it

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eluted more slowly from the Sephadex G-50 column. Disc electrophoresis indicated the presence of several protein bands in both preparations. All of the recovered enzyme activity was contained in the first of the two protein peaks in each instance. The partly purified enzymes were stable water-clear solutions and contained negligible amounts of carbohydrate and nucleic acid. Yields at the end of a three-stage process were 83% for the *K. aerogenes* enzyme and 89% for the enzyme from *E. cloacae*. The potency of the preparations was increased two- to threefold; each enzyme had a specific activity of 17 units/mg of protein.

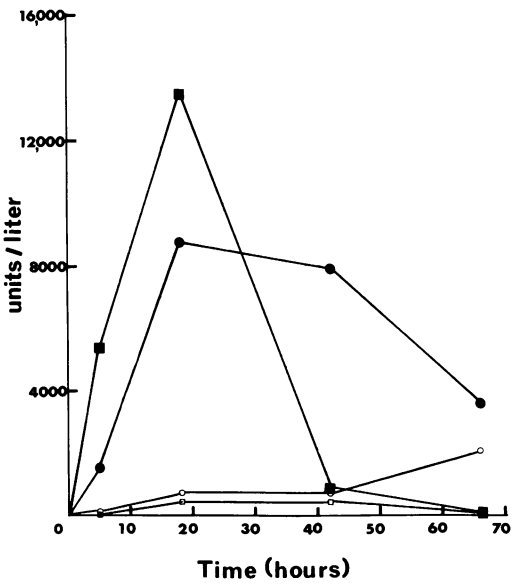

FIG. 2. *Effect of cell age on the yields of intracellular and extracellular β-lactamases. Symbols:* □, *K. aerogenes supernatant;* ○, *E. cloacae supernatant;* ■, *K. aerogenes intracellular enzyme;* ●, *E. cloacae intracellular enzyme.*

Comparison of substrate specificities of the enzymes. The two enzymes hydrolyzed cephaloridine at a similar rate at *p*H 7 when used at the same concentration (6.5 μg of protein/ml). This level was used except with some of the more susceptible substrates when the enzymes were diluted. The substrates, which ranged in molecular weight from 350 to 520, were compared on a molar basis.

The *K. aerogenes* enzyme was more active against penicillin G and ampicillin than it was against cephaloridine, especially according to the iodometric assay (Table 2). Methicillin and cloxacillin, which are normally β-lactamase-stable, were hydrolyzed by this enzyme to a greater extent than cephalosporin C or cephalexin. The normally resistant cephalosporins, 5/1 and 291/1, were attacked to a lesser extent but were more susceptible to the enzyme from *K. aerogenes* 1082E than to the enzyme from *E. cloacae* P99.

The *E. cloacae* P99 enzyme was much more active against the susceptible cephalosporins than against the penicillins, which were only degraded to the same extent as the resistant cephalosporins 5/1 and 291/1 (Table 2). Results obtained iodometrically and microbiologically were in general agreement except that the P99 enzyme appeared to have higher activity against penicillin G when assayed microbiolog-

TABLE 1. *Variations in the yields of the β-lactamases under various cultural conditions*

Cultural conditions	Medium	Culture vol (ml)	Enzyme units/liter of initial culture[a]			
			E. cloacae P99		*K. aerogenes* 1082E	
			Supernatant	Intracellular prepn	Supernatant	Intracellular prepn
Static	Nutrient Broth	50	1,085	2,890	145	1,450
		250	723	700	0	350
	Brain Heart Infusion	50	3,615	4,340	510	4,630
		250	1,085	1,160	360	1,220
	Micro Inoculum Broth	50	0	400	NT	NT
Shaken	Nutrient Broth	50	360	4,920	70	5,780
		250	540	1,450	0	3,020
	Brain Heart Infusion	50	720	9,830	435	12,730
		250	360	2,435	360	3,360
	Micro Inoculum Broth	50	0	0	NT	NT

[a] NT = not tested.

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TABLE 2. *Comparison of the substrate specificities of the K. aerogenes and E. cloacae β-lactamases*

Substrate	*K. aerogenes* 1082E enzyme		*E. cloacae* P99 enzyme	
	Iodometric assay	Microbiological assay	Iodometric assay	Microbiological assay
Cephaloridine	100	100	100	100
Cephalothin	65	75	20	60
Cephalosporin C	15	30	60	60
Cephalexin	15	10	10	20
5/1	10	25	0.5	0
291/1	5	5	0	0
Penicillin G	185	120	1.5	40
Ampicillin	190	100	0	0
Methicillin	30	30	0.5	0
Cloxacillin	25	10	0	0

ically. Both methods of assay indicated that cephalexin was 5 to 10 times more resistant to the P99 enzyme than was cephaloridine.

The greatest difference between the two enzymes with respect to substrate susceptibility was observed with ampicillin, which was highly sensitive to *K. aerogenes* 1082E β-lactamase but resistant to the enzyme from *E. cloacae* P99.

DISCUSSION

A strain of *K. aerogenes*, 1082E, isolated from a hospital patient, was found to produce a β-lactamase which had a different substrate profile from most of the gram-negative β-lactamases previously described (4, 8, 9). Since the culture was initially examined, further clinical isolates producing the same enzyme have been identified in this laboratory; the enzyme type does not appear to be a rare one. Its incidence, by comparison with that of other gram-negative β-lactamases, is being investigated.

K. aerogenes 1082E produced large amounts of β-lactamase intracellularly. This enzyme, unlike the *E. cloacae* P99 β-lactamase with which it has been compared, hydrolyzed all of the substrates included in the study. The *E. cloacae* enzyme had little or no effect on the penicillins. It attacked cephalosporin substrates with the exception of those having the same acyl substituents as methicillin and cloxacillin.

Comparison of the two enzymes showed that the cultural conditions for optimal yield were similar (Table 1). Difco Brain Heart Infusion, the richest of the three media, gave consistently higher yields than Oxoid Nutrient Broth No. 2, and Difco Micro Inoculum Broth gave negligible yields. Shaken cultures produced more enzyme than those grown statically, and 50-ml amounts of culture gave better yields than 250-ml volumes. Improvement in enzyme production appeared in each instance to be related to improved growth of the cultures. The *K. aerogenes* enzyme was less readily released from the cells than was the β-lactamase from *E. cloacae*. When partial purification of the enzymes was effected by the same method, equivalent concentrations, based on micrograms of protein per milliliter, hydrolyzed cephaloridine at similar rates at *p*H 7.

The *K. aerogenes* 1082E and *E. cloacae* P99 enzymes described represent extremes of gram-negative β-lactamase activity with respect to range of substrate specificity.

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