β-Lactamase-catalyzed hydrolysis of acyclic depsipeptides and acyl transfer to specific amino acid acceptors

(D-alanyl-D-alanine transpeptidase/β-lactamase evolution/β-lactam antibiotic)

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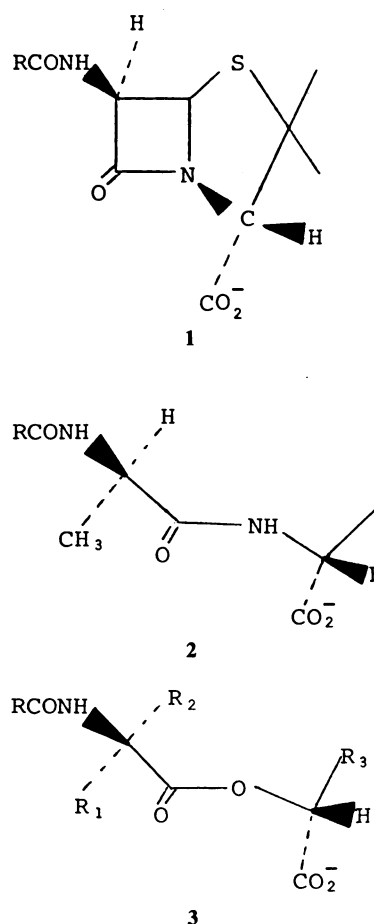Department of Chemistry, Wesleyan University, Middletown, CT 06457

ABSTRACT   β-Lactamases from all three classes, A, B, and C, catalyze the hydrolysis of specific acyclic depsipeptide ($PhCH_2CONHCR_1R_2CO_2CHR_3CO_2H$) analogs of acyl-D-alanyl-D-alanine peptides. The depsipeptides investigated, which are chemically as reactive toward nucleophiles as penicillins, are in general poor substrates, although differences between the classes of β-lactamases have been observed: the order of effectiveness seems to be C > B > A. Certain class A and C β-lactamases also catalyze phenylacetylglycyl transfer between phenylacetylglycyl depsipeptides and specific amino acid acceptors, a type of reaction hitherto identified more closely with D-alanyl-D-alanine transpeptidases than with β-lactamases. Preliminary indications of an acyl-enzyme intermediate in these reactions have been obtained. These results support the suggestion [Tipper, D. J. and Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. USA* 54, 1133–1141] that β-lactamases are evolutionary descendants of bacterial cell wall D-alanyl-D-alanine transpeptidases.

There are two groups of bacterial enzymes that interact strongly and specifically with β-lactam antibiotics, the β-lactamases on the one hand and the penicillin-binding proteins of bacterial cell walls on the other. The former group catalyze the hydrolysis of β-lactams while the latter, all of which are believed to have D-Ala-D-Ala transpeptidase or carboxypeptidase (or both) activity (and are referred to henceforth in this article as transpeptidases), are inhibited by them. It was proposed by Tipper and Strominger (1) that the affinity of β-lactams for D-Ala-D-Ala transpeptidases resulted from the structural similarity between penicillins (**1**) and D-Ala-D-Ala peptides (**2**) (Scheme 1). Further, as an extension of this idea, they suggested that β-lactamases might be evolutionary descendants of the penicillin-binding proteins, presumably selected for rapid acyl-enzyme hydrolysis to combat the effects of naturally occurring β-lactams. All subsequent structural and mechanistic investigations have in general tended to support these ideas (2–5). Hence one component of β-lactamase evolution, starting from a D-Ala-D-Ala transpeptidase precursor, would be in the direction of maintaining and perhaps enhancing the affinity of the enzyme for β-lactams and at the same time improving the mechanism of acyl-enzyme hydrolysis.

The other side of the coin in this putative sequence of β-lactamase evolution does not seem to have been as widely appreciated although it may have represented the greater challenge. This is the requirement that evolution should proceed toward an active site that no longer had affinity for, nor catalyzed reactions of, D-Ala-D-Ala peptides. This seems necessary to prevent the interference of β-lactamases in cell wall D-Ala-D-Ala metabolism. Hence it seems likely that important distinctions, and distinctions that should be of significance to antibiotic design, between the active sites of β-lac-

a	R = $PhCH_2$, R_1 = R_2 = R_3 = H	Phenylacetylglycylglycollate
b	R = $PhCH_2$, R_1 = CH_3, R_2 = R_3 = H	Phenylacetyl-D-alanylglycollate
c	R = $PhCH_2$, R_1 = R_2 = H, R_3 = CH_3	Phenylacetylglycyl-D-lactate
d	R = $PhCH_2$, R_1 = R_3 = CH_3, R_2 = H	Phenylacetyl-D-alanyl-D-lactate
e	R = $PhCH_2$, R_1 = H, R_2 = R_3 = CH_3	Phenylacetyl-L-alanyl-D-lactate
f	R = $PhCH_2$, R_1 = R_2 = H, R_3 = Ph	Phenylacetylglycyl-D-mandelate
g	R = $PhCH_2$, R_1 = CH_3, R_2 = H, R_3 = Ph	Phenylacetyl-D-alanyl-D-mandelate

Scheme 1

tamases and the transpeptidases should be found in a detailed comparison of the differences between the responses of these enzymes to D-Ala-D-Ala peptides and their analogs.

Typical β-lactamases do not catalyze the hydrolysis of linear peptides (6). Consequently, this second evolutionary goal must also have been met. An understanding of the details of this important step is one aim of the present work.

Recently, it was shown (7) that certain monocyclic β-lactams are β-lactamase substrates and that the apparent general order of β-lactamase catalytic activity—i.e., bicyclic β-lactam > monocyclic β-lactam > linear peptide—follows the susceptibility of these compounds to nucleophilic amide cleavage. This result suggested that chemically activated lin-

Biochemistry: Pratt and Govardhan

Proc. Natl. Acad. Sci. USA 81 (1984)   1303

ear peptide analogs might be β-lactamase substrates. We report in this paper that specific depsipeptides of general structure **3** are in fact substrates of certain β-lactamases and thus provide further evidence for the suggestions of Tipper and Strominger (1). We suggest that these compounds will be useful for further exploration of β-lactamase active sites, in particular with regard to their relationship to D-Ala-D-Ala transpeptidase active sites. For example, we show that certain β-lactamases can also catalyze acyl transfer from these substrates to specific amino acid acceptors, a reaction characteristic of the transpeptidases.

MATERIALS AND METHODS

Enzymes. The β-lactamases of *Bacillus cereus* (strain 569/H/9), of the TEM-2 plasmid (from *Escherichia coli*, strain W3310), of *Staphylococcus aureus* PC1, and of *Enterobacter cloacae* P99 were obtained from the Centre for Applied Microbiology and Research (Porton Down, England). The *B. cereus* enzymes (β-lactamases I and II) were separated and purified by the method of Davies *et al.* (8); the other enzymes were used as received. The *amp C* β-lactamase was isolated and purified from *E. coli* strain D21 (9) by the method of Minami *et al.* (10). β-Lactamase activity was routinely assayed against benzylpenicillin in 0.1 M phosphate buffer (pH 7.5) by the spectrophotometric method of Waley (11). The transpeptidase/carboxypeptidase of *Streptomyces* R61 was a gift of J.-M. Frère (University of Liege, Belgium).

Depsipeptides. These compounds were synthesized by the general method of Losse and Klengel (12). Details of these syntheses and the properties of the purified depsipeptides will be reported elsewhere. Phenylacetylglycyl-D-alanine was prepared by reaction of phenylacetyl chloride with glycyl-D-alanine. Available from previous studies were desthiobenzylpenicillin (7) and 6-β-bromopenicillanic acid (13).

Depsipeptides as Substrates. The susceptibility of the depsipeptides to β-lactamase-catalyzed hydrolysis was routinely tested by a proton NMR method using a Varian XL-200 instrument, as described (7), under conditions indicated in the footnote to Table 1. In all cases, from both the enzyme-catalyzed reactions and the controls, the spectra of the reaction mixture after complete reaction was that of an equimolar mixture of phenylacetylamino acid and hydroxyacid.

The hydrolysis of the D-mandelates could also be followed spectrophotometrically at 235 nm ($\Delta\varepsilon$ for **3f** and **3g** are 40 $cm^{-1} \cdot M^{-1}$ and 14 $cm^{-1} \cdot M^{-1}$, respectively), enabling quantitative kinetic measurements to be made more readily. Steady-state kinetic parameters were obtained through measurements of initial velocities at this wavelength.

Acyl Transfer to Amino Acid Acceptors. The NMR method of following the enzyme-catalyzed hydrolysis described above was also used to assess the extent of acyl transfer when an amino acid acceptor was present in the reaction mixture. Most experiments of this type were carried out using **3f** as substrate and the *En. cloacae* P99 β-lactamase. With this substrate in the presence of an amino acid acceptor, the appearance of a resonance at δ 3.94, corresponding to the glycyl methylene group of a phenylacetylglycyl amide (see below and Fig. 1), was taken as diagnostic of the presence and as a measure of the extent of transacylation.

In one case the transacylation product was isolated and characterized. The β-lactamase of *En. cloacae* P99 (1.7 nmol) was added to an aqueous solution (5 ml) containing D-phenylglycine (Sigma; 0.25 mmol), **3f** (0.15 mmol), and sodium bicarbonate (1.5 mmol) at pH 9.0. The mixture was stirred at room temperature for 24 hr. Subsequent acidification to pH 1 and chilling of the solution yielded 28 mg (57%) of a colorless solid identified after recrystallization from water as phenylacetylglycyl-D-phenylglycine: mp 165°C; proton

NMR (C^2HCl_3) δ 3.65 (s, 2, PhCH_2), 3.96 (d, J = 5 Hz, 2, glycyl CH_2), 4.48 (d, J = 7 Hz, 1, CH), 7.3 (br m, 5, ArH), 7.76 (d, J = 7 Hz, 1, NH); mass spectrum, m/e (relative intensity, %) 326 (1, M^+), 282 (19, $M - CO_2$), 191 (3), 148 (23), 135 (9), 91 (100); elemental analysis (C, H, N), $C_{18}H_{18}N_2O_4$.

RESULTS

Fig. 1 shows the proton NMR spectra of **3f** before and after treatment with the P99 β-lactamase. In the absence of enzyme, only a small amount of spontaneous hydrolysis occurs in the same time period. The β-lactamase thus appears to catalyze an esterase reaction. The other enzymes generated identical spectra. That these reactions occur at the β-lactamase active site was shown by several experiments. First, the β-lactamases of *En. cloacae*, *S. aureus*, and plasmid TEM-2 that had been inactivated by the specific serine β-lactamase inhibitor 6-β-bromopenicillanic acid (13) also retained no esterase activity. Second, the loss of the esterase activity of the *En. cloacae* β-lactamase in the presence of phenylmethylsulfonyl fluoride exactly paralleled the loss of β-lactamase activity. Finally, irreversible inactivation of *B. cereus* β-lactamase II by EDTA and iodoacetic acid (14, 15) also led to loss of its esterase activity.

A semiquantitative survey of the specificity of the esterase reaction, both with respect to substrate and to β-lactamase, is given in Table 1. Quantitative data in one particular case are given in Table 2. These results indicate that the esters examined are in general poor β-lactamase substrates. This is shown clearly in Table 2, where the steady-state parameters for one of the most favorable cases, the hydrolysis of **3f** cata-
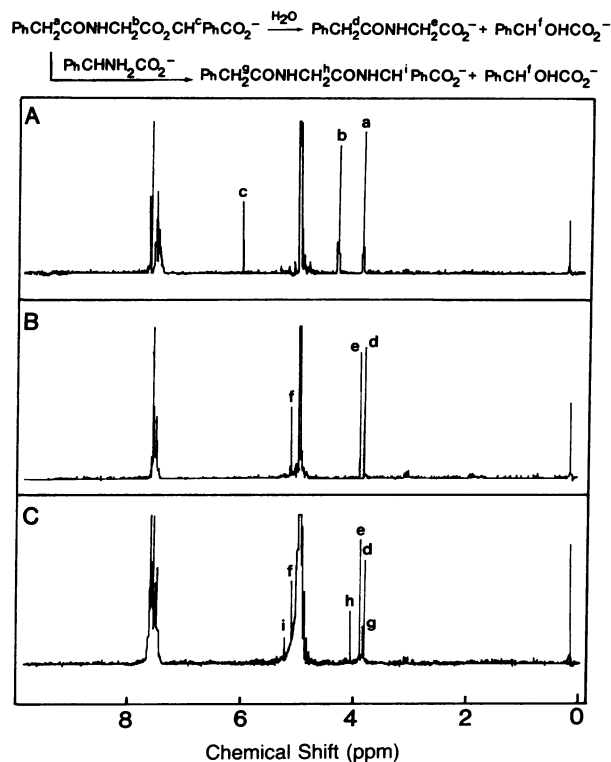

FIG. 1.   Proton NMR spectra of 10 mM **3f** in bicarbonate buffer (pH 7.5) before (*A*) and after (*B*) the addition of *En. cloacae* P99 β-lactamase; *C* is as for *B* but in the presence of 20 mM D-phenylglycine. In *B*, the products are mandelate (peak f) and phenylacetylglycine (peaks d and e); in *C*, both the transacylation product phenylacetylglycyl-D-phenylglycine (peaks g, h, and i) and the hydrolysis products can be seen.

Table 1. β-Lactamase-catalyzed hydrolysis of depsipeptides

| | Relative rate of hydrolysis in the presence of β-lactamase | | | | | |
| | Class C | | Class B | Class A | | |
Depsipeptide	P99	Amp C	BCII	TEM	SA	BCI
3a	+++	NP	+	−	++	−
3b	++	NP	−	−	±	NP
3c	++++	NP	+++	−	±	−
3d	+	NP	±	−	±	−
3e	−	NP	±	−	−	−
3f	+++	+++	+++	++	+	±
3g	+	NP	++	±	±	−

The β-lactamases of *En. cloacae* P99, *E. coli* (Amp C), the TEM plasmid (TEM), *S. aureus* PCl (SA), *B. cereus* I and II (BCI and BCII) were used. The reaction mixtures contained 10–15 mM substrate and 2.8 μM (P99), 1.0 μM (Amp C), 2.7 μM (TEM), 2.5 μM (SA), 6.1 μM (BCI), or 7.3 μM (BCII) enzyme in 0.6 ml of ^2H_2O. The buffer in each case was 50 mM NaHCO$_3$ (pH 8.0) except for BCII where it was 20 mM Tris/0.1 mM Zn^{2+}, pH 8.2. Reactions were carried out at ambient temperature. Rate symbols are as follows: ++++, *ca.* 25% complete in 1 hr, complete in 2 days; +++, *ca.* 10% complete in 1 hr, complete in 2 days; ++, no obvious reaction in 1 hr but complete in 2 days; +, no obvious reaction in 1 hr and not quite complete in 2 days; ±, only slight reaction above background hydrolysis in 2 days (background <50% hydrolysis in 2 days); −, no reaction above background in 2 days; NP, experiment not performed.

lyzed by the P99 β-lactamase, are compared with those of the same enzyme with a good substrate, benzylpenicillin. Even here the catalytic efficiency (k_{cat}/K_m) of the enzyme as an esterase is less than its efficiency as a β-lactamase by some three orders of magnitude. For the other β-lactamases tested, the difference is probably greater. It does seem, however, for the P99 enzyme that the difference is mainly in the K_m parameter. The value of k_{cat} for 3f is in fact comparable with what might be expected on the basis of its chemical reactivity in comparison with that of penicillin (second-order rate constants for reaction of 3f and benzylpenicillin with hydroxide ion were found to be 0.36 and 0.10 $sec^{-1}·M^{-1}$, respectively, at 25°C).

The P99 β-lactamase was also found to catalyze the hydrolysis of the monocyclic β-lactam desthiobenzylpenicillin, as did other β-lactamases (7), but not that of the comparable acyclic peptide phenylacetylglycyl-D-alanine. The spectrophotometric assay showed that the R61 transpeptidase catalyzed the hydrolysis of 3f and 3g.

Fig. 1 also shows the final NMR spectrum after the P99 β-lactamase-catalyzed reaction of 3f in the presence of D-phenylglycine, the amino acid analog of D-mandelate. The spectrum contains not only peaks of the hydrolysis products but also those corresponding to the aminolysis product, phenylacetylglycyl-D-phenylglycine, which was isolated and characterized (*Materials and Methods*). In the absence of enzyme no spontaneous aminolysis was observed under the same conditions. The β-lactamase thus catalyzes acyl (phenylacetylglycyl) transfer not only to water but also, in a com-

Table 2. Steady-state kinetic parameters for the β-Lactamase of *En. cloacae* P99

Substrate	K_m, mM	$k_{cat}*$, sec^{-1}	k_{cat}/K_m, $sec^{-1}·M^{-1}$
Benzylpenicillin	0.015 ± 0.002	50 ± 1	(3.33 ± 0.45) × 10^6
3f	70 ± 17	270 ± 60	3800 ± 1300
3g	≥30†	≥23	700 ± 50

*Calculated with the assumption of 39,000 as the enzyme molecular weight (16).
†Limited by solubility.

Table 3. Acyl transfer to amine acceptors

| | | Acceptor | | |
Substrate	Enzyme	Substance	Conc., mM	% trans-acylation
3f	P99	D-Phenylglycine	20	26 ± 6 (4)
3f	P99	D-Phenylglycine*	20	>60†
3f	P99	L-Phenylglycine	20	9
3f	P99	D-Alanine	100	24 ± 7 (4)
3f	P99	D-Alanine*	100	>60†
3f	P99	*meso*-Diaminopimelic acid	100	25
3f	P99	Glycine	100	28
3f	P99	Glycinamide	100	0
3f	P99	(D-Ala)$_3$	100	0
3c	P99	D-Phenylglycine	20	28, 33‡
3c	P99	D-Alanine	100	23, 17‡
3f	TEM	D-Phenylglycine	20	7
3f	BCII	D-Phenylglycine	20	0
3f	Amp C	D-Phenylglycine	20	32

Concentrations of enzymes and substrates and reaction conditions were as for Table 1 unless otherwise noted. Numbers in parentheses represent numbers of experiments.
*Reaction carried out in 50 mM carbonate buffer (pH 9).
†This figure represents a lower limit obtained by assuming that none of the observed hydrolysis product arises from the spontaneous background reaction; this assumption is not valid but accurate allowance for background hydrolysis cannot be done with the data available.
‡Two independent experiments.

peting reaction, to D-phenylglycine. Some idea of the specificity of the acyl transfer with respect to amine acceptors is given in Table 3. Furthermore under conditions in which the NMR data indicate that the majority (>60%) of the reaction product arose by amine acylation (0.1 M D-alanine, pH 9.0), the presence of 0.1 M D-alanine increased the initial rate of reaction, followed spectrophotometrically, by a factor of about 2.2.

DISCUSSION

Although simple D-Ala-D-Ala peptides **2** are not substrates of typical β-lactamases, their depsipeptide analogs **3** are now shown to be, at least for certain β-lactamases (Table 1). This result is in accord with the suggestion in ref. 7 that one reason for the impotence of β-lactamases against peptides is the chemical inertness of the latter; the depsipeptides **3** are of comparable susceptibility to nucleophilic attack by hydroxide ion as penicillins. Because we have observed that the depsipeptides **3** are also substrates of the R61 D-Ala-D-Ala transpeptidase [note also the work of Rasmussen and Strominger (17)], these substrates should provide excellent probes of the similarities and differences between the β-lactamase and D-Ala-D-Ala transpeptidase active sites.

Clear stereochemical preferences by the β-lactamases can be seen among the depsipeptides. Most of these can be understood in terms of structural comparisons between them and penicillins (Scheme 1). Since **3e** is not a substrate of any of the β-lactamases, it seems likely that their active sites cannot accept an L-amino acid in the penultimate position. This is also true of the D-Ala-D-Ala transpeptidases (2).

In all cases for which data are available for comparison (10 cases in all, covering five of the β-lactamases examined), the glycyl depsipeptides (**3**, $R_1 = R_2 = H$) appear to be better β-lactamase substrates than the D-alanyl depsipeptides (**3**, $R_1 = CH_3$, $R_2 = H$). One quantitative comparison is shown in Table 2, where it is seen that k_{cat}/K_m for the P99 β-lactamase is some 5 times greater for **3f** than for **3g**. In contrast, Ghuysen *et al.* (2) report that the *Streptomyces* transpeptidases

catalyze the hydrolysis of diacetyl-L-lysyl-D-alanyl-D-alanine much more efficiently than that of diacetyl-L-lysylglycyl-D-alanine. We find also that the *Streptomyces* R61 transpeptidase is more effective against **3g** than **3f**; the initial rate of hydrolysis of 10 mM **3g** was approximately four times that of 10 mM **3f** under the same conditions (pH 7.5, 25°C).

The transacylation reaction to amine acceptors has been, to date at least, much more typical of D-Ala-D-Ala transpeptidases than of β-lactamases. Class A and B β-lactamases have not been observed to catalyze penicilloyl transfer to nucleophiles other than water (18–20) despite the strong evidence for penicilloyl enzyme intermediates with the class A β-lactamases (19, 21, 22). Recently Knott-Hunziker *et al.* (20) have demonstrated the capture by small alcohols of what may be a penicilloyl-enzyme intermediate formed during the turnover of penicillins by two class C β-lactamases; small amines were, however, still ineffective as acceptors. It seems likely that penicilloyl-enzyme intermediates in β-lactamase catalysis are in general well shielded from non-specific nucleophiles. On the other hand, the D-Ala-D-Ala transpeptidases are well known to catalyze acyl transfer from specific peptide substrates to amino acid acceptors (2, 3, 23). However, these enzymes, like the β-lactamases, do not catalyze acyl transfer from penicillins to amino acid acceptors— i.e., penicilloylation of such acceptors—although a well-defined penicilloyl-enzyme is formed on their interaction with penicillins (2, 23). It would be reasonable to conclude from these facts that the thiazolidinyl group of a penicilloyl-enzyme (β-lactamase or D-Ala-D-Ala transpeptidase) intermediate, which, unlike the terminal residue from peptide cleavage is not released from the substrate, blocks acyl transfer to amino acid acceptors and may well occupy the acceptor site.

The partitioning of the phenylacetylglycyl group of **3f** between water and amine acceptors is suggestive of, but in itself by no means proves, the existence of an acyl-enzyme intermediate in the P99 β-lactamase-catalyzed hydrolysis of the depsipeptides. Evidence for this position does come, however, from the equal amounts of D-phenylglycine acylation found (Table 3) for the substrates **3c** and **3f**, which is indicative of a common intermediate. The same result is obtained for D-alanine. Since the rate of disappearance of **3f** in the presence of the P99 enzyme also seems to be accelerated by amine acceptors, the rate of nucleophilic cleavage of an acyl-enzyme may be rate-determining to turnover.

A final point of interest arising from Table 1 is the variation in response to a given depsipeptide among the β-lactamases. In general it appears that the class A β-lactamases (TEM, PC1, and BCI) are much poorer esterases than the class B β-lactamase (BCII) and the P99 enzyme (which may be a class C β-lactamase*). These differences presumably reflect the separate evolution of these nonhomologous (24, 25) proteins. Possibly correlated with this result is the generally higher activity of the latter two groups against cephalosporins, which suggests a less penicillin-specific active site. It is also of interest that the class B β-lactamase, BCII, does not appear to catalyze acyl transfer to an amino acid acceptor; the extracellular D-Ala-D-Ala carboxypeptidase of *Streptomyces albus* G, which also contains an essential zinc ion, also does not catalyze comparable acyl transfers from peptide substrates (26).

The results reported in this paper are interpreted to sup-

port the proposal (1) that β-lactamases and D-Ala-D-Ala transpeptidases are evolutionarily related. We have now completed the quartet of substrate overlaps—β-lactamases *can* catalyze the hydrolysis of acyclic D-Ala-D-Ala analogs. Furthermore we see indications that the extended amine acceptor binding site of transpeptidases may to some extent be present on serine β-lactamases as well. The evolutionary path from a transpeptidase to a β-lactamase seems to involve on the one hand development of a new mode of access of water to the highly shielded (in transpeptidases) acyl-enzyme intermediate and on the other loss of the D-Ala-D-Ala transpeptidase activity. The latter may have been achieved to some extent by exploiting the difference in reactivity of peptides and β-lactams but differences in shape and flexibility are probably also important, as indicated by the generally poor ability of the depsipeptides to act as β-lactamase substrates.

It is hoped that these studies and those in progress will lead to a clearer picture of the similarities and differences between the active sites of all of the penicillin-binding proteins including the β-lactamases. A firm reference point may soon be available in the x-ray crystal structure of the *Streptomyces* R61 D-Ala-D-Ala transpeptidase/carboxypeptidase (28). Even now it seems that enzymes with a complete spectrum of specificities are emerging, ranging from pure transpeptidases with essentially no carboxypeptidase or β-lactamase activity (29), through transpeptidase/carboxypeptidases with weak β-lactamase activity (30), to β-lactamases with some affinity for acyclic substrates (as seen in class B and C β-lactamases) and to almost pure β-lactamases (as in the class A β-lactamases). There may well be other "missing links" that can handle both types of substrates equally well. That there might be at least three separate evolutionary paths (corresponding to the A, B, and C classes of β-lactamases), originating from three separate ancestral transpeptidases, must be kept in mind.

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*The Ambler classification (24) of the P99 enzyme does not seem to have been made yet. The enzyme is inhibited by 6-β-bromopenicillanic acid, which is suggestive of a serine β-lactamase in the class A or C category. Because it is a Sykes class Ib (4) constitutive chromosomal enzyme from a Gram-negative bacterium, it may well belong to class C (25, 27). Further to this point, as shown in Tables 1 and 3, the activity of the class C β-lactamase, the chromosomal amp C enzyme of *E. coli*, seems very similar to that of the P99 enzyme.

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