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THE β-LACTAMASE OF *STREPTOMYCES CACAOI*: INTERACTION WITH CEFOXITIN AND β-IODOPENICILLANATE

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Cefoxitin was a very poor substrate for the β-lactamase of *Streptomyces cacaoi* (k_{cat} = 2.7 × 10^{-4} s^{-1}). In the presence of nitrocefin, a good substrate, cefoxitin behaved as a transient inactivator by immobilizing a large proportion of the enzyme as the acyl enzyme intermediate. The enzyme was also inactivated by β-iodopenicillanate. In this case, the acyl enzyme rearranged into an α–β unsaturated ester and inactivation was irreversible. In contrast to the situation prevailing with the *Streptomyces albus* G β-lactamase, no turn-over of β-iodopenicillanate was observed.

KEY WORDS: β-lactamase; *Streptomyces cacaoi*; cefoxitin; β-iodopenicillanate.

Ogawara and his coworkers have reported the purification[1] of a β-lactamase excreted by *Streptomyces cacaoi* and studied the interactions between this enzyme and various β-lactams, including substrates[1] and inactivators[2,3]. We purified the β-lactamase of *Streptomyces albus* G[4,5], determined its substrate specificity profile and studied its reactions with various inactivating β-lactams[5–9]. Both enzymes exhibited similar molecular weights (30–34 000), similar turn-over numbers on benzylpenicillin (400–500 s^{-1}) and hydrolysed penicillins better than cephalosporins. Conversely, the isoelectric pH's were somewhat different and cloxacillin, methicillin and carbenicillin were better substrates of the *S. cacaoi* enzyme.

Three classes of naturally occurring β-lactamases have presently been clearly identified.[10] Enzymes belonging to classes A and C are serine enzymes and their catalytic pathways probably involve the transient formation of an acyl enzyme intermediate. No sequence homology was observed between the two classes. Class B enzymes contain an essential Zn^{++} ion. A sulphydryl β-lactamase has also been artificially manufactured[11] by genetic engineering of the pBr 322 plasmid, which naturally codes for a class A enzyme. This result clearly demonstrated that the existence of sulphydryl β-lactamases remains a distinct possibility.

In the experiments reported so far, the *Streptomyces* enzymes behaved as serine β-lactamases[1,6–9]. In this paper, we wish to describe the interaction between the *S. cacaoi* enzyme and cefoxitin (**1**) and β-iodopenicillanate (**2**) which are well-established inactivators of various serine β-lactamases[10,12–14].

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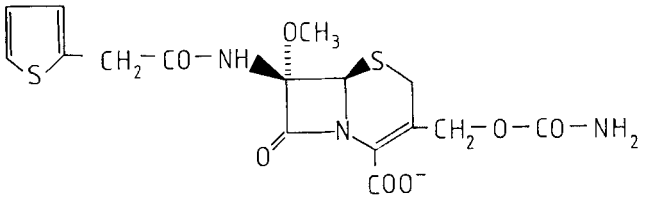

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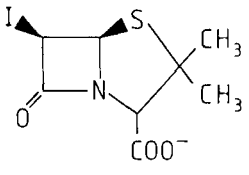

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MATERIALS AND METHODS

All spectrophotometric experiments (spectra and kinetics) were performed with the help of a Beckman DU8 recording spectrophotometer.

Cefoxitin, β-iodopenicillanate and nitrocefin were generous gifts from the Merck Institute for Therapeutic Research, Pfizer Central Research and Glaxo Research Group Ltd., respectively. *Streptomyces cacaoi* strain KCCS-0352 was a kind gift from Dr. Akio Seino, curator of the culture collection of Actinomycetes, Kaken Chemical Company Ltd., Tokyo, Japan. The β-lactamase was purified according to the procedure of Ogawara *et al.*[1] with slight modifications which will be published elsewhere. The specific activity of the enzyme was measured in 10 mM sodium cacodylate buffer, pH 6.0 using benzylpenicillin and ampicillin as substrates, by monitoring the decrease of absorbance of 0.5 mM solutions at 230 nm ($\Delta\varepsilon = -980$ and $-780\,M^{-1}cm^{-1}$ for benzylpenicillin and ampicillin, respectively). In both cases, the substrate concentration was much higher than the reported values[1] for the respective K_m's. The K_m for nitrocefin was measured by continuously monitoring the absorbance at 482 nm of a 280 μM solution and using the integrated equation

$$\frac{1}{t}\ln\frac{S_0}{S_0 - P} = \frac{1}{K_m}\left(V - \frac{P}{t}\right)^{15}.$$

In these experiments, the optical path of the cells was 4 mm.

First-order rate constants for inactivation in the presence of nitrocefin $(k_i)_{nit}$ were measured as described by Knott-Hunziker *et al.*[13] and Frère *et al.*[8]

Two buffers were used: 10 mM sodium cacodylate, pH 6.0 or 50 mM sodium phosphate, pH 7.0. All experiments were performed at 30°C.

RESULTS

Kinetic Parameters for Substrates

In 10 mM cacodylate, pH 6.0, the purified preparation exhibited specific activities of 1410 and 520 μmoles min^{-1} (mg of protein)$^{-1}$ respectively when benzylpenicillin or ampicillin were used as substrates. These values were somewhat larger than those previously reported.[1] The K_m for nitrocefin was 422 ± 25 μM [V = 610 ± 40 μmoles min^{-1} (mg protein)$^{-1}$] at pH 6.0 and 475 ± 50 μM [V = 575 ± 60 μmoles min^{-1} (mg protein)$^{-1}$] at pH 7.0 (5 determinations in each case). The kinetic parameters thus remained virtually identical in the two buffers used in the present study.

Reaction with β-Iodopenicillanate

On the basis of the following experimental data, it was concluded that no turn-over of β-iodopenicillanate occurred.

1. Complete inactivation of the enzyme did not require more than a 1/1 molar ratio of β-iodopenicillanate to enzyme (see below, titration experiments).

2. Inactivation was irreversible: enzyme (8 μg) was incubated with 100 μM β-iodopenicillanate in 200 μl of phosphate buffer, pH 7.0 and at 20°. After 10 min, it was verified that inactivation was complete. The sample was then exhaustively dialysed at 4° against cacodylate buffer containing 5% glycerol and 5% ethylene glycol. No activity was thus recovered. The dialysed sample was then incubated at 30° and tested for activity at 60-min intervals. After 3 h, no activity could be detected, indicating that less than 1% of the complex had decayed.

3. Spectrophotometric data indicated the absence of turn-over of the inactivator. Hydrolysis of β-halogenopenicillanate by β-lactamases yields 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate[9], whose absorption maximum is at 305 nm (ε_{305} = 8200 $M^{-1}cm^{-1}$) and which exhibits considerable residual absorption at 323 nm (ε_{323} = 3800 $M^{-1}cm^{-1}$). When the enzyme was inactivated by a nearly stoichiometric amount of β-iodopenicillanate, a new absorption band in the near UV was observed (see below) whose maximum was at 323 nm (ε_{323} = 10–11 000 $M^{-1}cm^{-1}$), i.e., a value close to that observed by Loosemore *et al.*[12] upon inactivation of the *B. cereus* β-lactamase I by β-bromopenicillanate, a reaction which occurred without turn-over of the inactivator. One would expect such a turn-over to shift the maximum of absorbance of the reaction mixture to lower wavelengths, as was observed with the *Streptomyces albus* G β-lactamase[9]. As shown below, the spectrum in the near UV was characteristic of the enzyme-linked chromophore and no indication was obtained of the presence of free dihydrothiazine.

Titration experiments. To 200 μl of enzyme (0.58 mg of protein ml^{-1}) in 50 mM phosphate pH 7.0, 4 aliquots of 190 μM β-iodopenicillanate were added. After each addition, the absorbance at 323 nm was continuously monitored and a 2 μl sample was removed for determination of the residual activity. Figure 1 shows the results and indicates that the addition of 13 μl of β-iodopenicillanate was sufficient to completely inactivate the enzyme. This correspnded to 2.47 nmoles, suggesting that the enzyme preparation was 72% pure. Since gel electrophoresis in the presence of

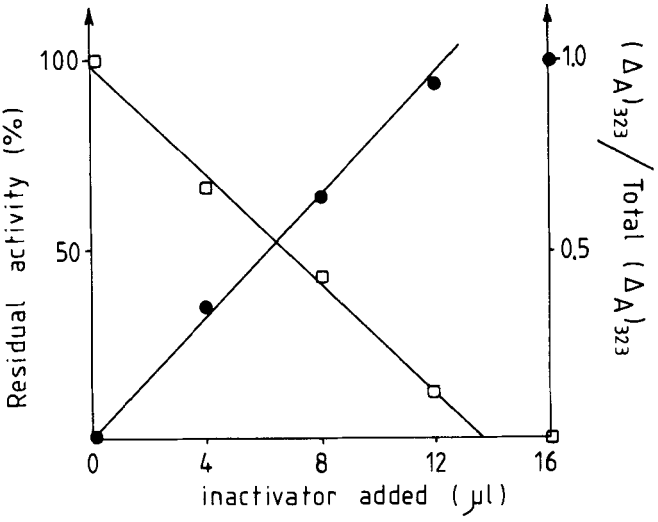


FIGURE 1 Titration of the β-lactamase with β-iodopenicillanate. Aliquots (4 μl) of 190 μM β-iodopenicillanate were added to 200 μl of enzyme (0.58 mg ml^{-1} in 50 mM phosphate pH 7.0). After each addition, the absorbance was continuously monitored at 323 nm and after the absorbance had stabilized, a 2-μl sample was removed and the residual activity determined. After the fourth addition, the activity completely disappeared and a fifth addition did not result in a further increase of A_{323}. Symbols: □, residual activity; ●, ratio of $(\Delta A)_{323}$ after stabilization to total variation observed after the fourth addition. According to the residual activity, the end-point was at 13.7 μl and at 12.4 μl on the basis of the absorbance variation.

sodium dodecylsulphate[16] did not reveal more than one band of protein whose velocity of migration agreed with the reported molecular weight of 34 000[1], the impurities had probably the same molecular weight as the enzyme or consisted of denatured enzyme molecules. In the following paragraphs, the concentrations of enzyme have been corrected on the basis of a 72% purity.

After the fourth addition of inactivator, a difference spectrum was recorded between the inactivated and the native enzyme (Figure 2). From the absorbance at 323 nm, a molar extinction coefficient of about 11 000 M^{-1} cm^{-1} was computed. Further addition of β-iodopenicillanate failed to alter the spectrum.

Kinetic model and equations. On the basis of these experiments, a simple interaction model was deduced. This model is represented by Eq. (1) where E is the enzyme, EC^* the acyl enzyme and EC^i the rearranged, chromophoric complex. K is the dissociation constant of EC and k_2 and k_4 are first-order rate constants

$$E + C \overset{K}{\rightleftharpoons} EC \xrightarrow{k_2} EC^* \xrightarrow{k_4} EC^i. \qquad (1)$$

In the presence of nitrocefin (S), the disappearance of the enzymatic activity was characterized by a pseudo first-order rate constant $(k_i)_{nit}$ which was obtained as described by Frère *et al.*[9] The variation of $(k_i)_{nit}$ with $[C]$ is given by

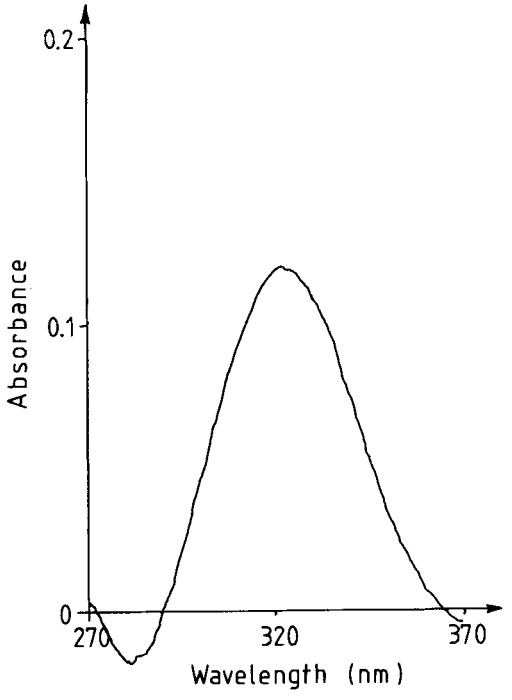


FIGURE 2 Difference spectrum between the adduct formed in the presence of β-iodopenicillanate and the free enzyme. After the fourth addition of β-iodopenicillanate in the experiment described in the legend of Figure 1, the spectrum of the mixture was recorded against a blank containing 200 μl of enzyme plus 16 μl of buffer.

$$(k_i)_{\text{nit}} = \frac{k_2[C]}{[C] + K\left(1 + \frac{[S]}{K_m}\right)}, \tag{2}$$

where K_m is that of nitrocefin.

If $[C] \ll K$, Eq. (2) simplifies to Eq. (3) and $(k_i)_{\text{nit}}$ is directly proportional to $[C]$

$$(k_i)_{\text{nit}} = \frac{k_2[C]}{K} \frac{K_m}{[S] + K_m}. \tag{3}$$

Finally, if the rate of formation of EC^* is the rate-limiting step ($k_2 \ll k_4$), the appearance of the chromophoric EC^i is also pseudo first-order and allows a direct evaluation of k_2 and K or of k_2/K.

Rate of inactivation. The enzyme (2.9 nM, final concentration) was added to 300 μl of 125 μM nitrocefin in 10 mM cacodylate, pH 6.0 or in 50 mM sodium phosphate pH 7.0 and the absorbance at 482 nm was continuously monitored. The values of $(k_i)_{\text{nit}}$, computed from the rate of decrease of the velocity of hydrolysis of nitrocefin are presented in Table I. At both pH's the values of $(k_i)_{\text{nit}}$ remained proportional to the concentration of β-iodopenicillanate. It was thus concluded that the range of inactivator concentrations utilized in our experiments was well below the value of K. From Eq. (3) and the experimental data, values of k_2/K (representing the second-order rate

TABLE I
Apparent rate constants for the inactivation of the β-lactamase by β-iodopenicillanate (*C*) in the presence of nitrocefin

pH	[*C*] (μM)	$(k_i)_{nit}$ (s^{-1})	$(k_i)_{nit}/[C]$ ($M^{-1}\,s^{-1}$)
6.0	1.56	$4.2 \pm 0.1 \times 10^{-3}$	2690 ± 70
	3.06	$7.0 \pm 0.9 \times 10^{-3}$	2290 ± 300
	6.22	$12.3 \pm 2.9 \times 10^{-3}$	1980 ± 470
	12.30	$28.0 \pm 4.5 \times 10^{-3}$	2270 ± 360
7.0	3.64	$5.1 \pm 0.2 \times 10^{-3}$	1420 ± 70
	7.02	$9.9 \pm 0.5 \times 10^{-3}$	1410 ± 70
	10.2	$14.6 \pm 0.3 \times 10^{-3}$	1430 ± 30
	13.11	$19.2 \pm 1.0 \times 10^{-3}$	1470 ± 75

constants for the formation of *EC**) of 3000 ± 400 and 1800 ± 40 $M^{-1}s^{-1}$ were computed at pH's 6 and 7, respectively.

At pH 6.0, the absorbance at 323 nm was also monitored after mixing 2.22 μM enzyme with 8.1 or 15.9 μM β-iodopenicillanate (Table II). At the lower concentration, only the first 50% of the curves were used for the computation of k_i since the concentration of enzyme could not be considered as negligible when compared to that of β-iodopenicillanate. The second-order rate constant for the formation of the chromophore which could be calculated from these data was 2460 ± 200 $M^{-1}s^{-1}$.

At the highest concentrations, the reactions were virtually completed within 60 s and it was therefore not possible to obtain accurate data at concentrations of β-iodopenicillanate exceeding 16 μM and to measure the individual values of k_2 and *K*.

Interaction with Cefoxitin

In the presence of 1 mM cefoxitin, the enzyme was completely inactivated in less than 1 min in both buffers. However, when low initial ratios (2 to 5) of inactivator to enzyme were used, a substantial proportion of the activity was recovered after 16 h. The degradation of the antibiotic could be directly observed by monitoring the decrease of A_{265} when 3.6 μM enzyme was incubated with cefoxitin concentrations ranging from 7.2 to 40 μM. (The opening of the β-lactam ring of cefoxitin is accompanied by a $\Delta\varepsilon$ of $-5100\,M^{-1}cm^{-1}$.) The rate of the reaction was however too low to be accurately measured.

Kinetic model and equations. The interaction was analysed on the basis of the model represented by Eq. (4)

$$E + C \overset{K}{\rightleftharpoons} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P, \tag{4}$$

where *P* represents degraded cefoxitin. If the rate of formation of *EC** is such that a negligible concentration of enzyme remains free at the steady-state, the disappearance of the activity in the presence of nitrocefin obeys Eq. (2) which can be linearized to Eq. (5)

$$\frac{[C]}{(k_i)_{nit}} = \frac{[C]}{k_2} + \frac{K}{k_2}\left(1 + \frac{[S]}{K_m}\right). \tag{5}$$

Estimation of k_3 rested on the determination of the residual activity after

TABLE II
Apparent rate constants for the appearance of the chromophore at 323 nm (pH = 6.0)

[C] μM*	k_i (s^{-1})	$k_i/[C]$ ($M^{-1}\,s^{-1}$)
8.1	18.5×10^{-3}	2280
15.9	42×10^{-3}	2640

*β-iodopenicillinate.

establishment of the steady-state. In the absence of nitrocefin, the concentration of free enzyme at the steady-state (E_{ss}) is given by Eq. (6)

$$\frac{E_{ss}}{E_0} = \frac{k_3 K}{k_3 K + (k_2 + k_3)\,[C]}. \tag{6}$$

A plot of E_0/E_{ss} versus [C] yields a line of slope $(k_2 + k_3)/k_3 K$. It should be noted that, when the determination of the residual activity involves the dilution of a small sample, it must be performed over a very short period of time to avoid a non-negligible recovery of activity during the estimation process.

Determination of the parameters. When 0.5 μM enzyme was incubated with 100 μM cefoxitin, about 5% of activity remained after the steady-state was established. At cefoxitin concentrations larger than 100 μM, the third step of the interaction could thus be neglected during the study of the formation of complex *EC**. The enzyme (3 nM) was incubated at 30° with 100 μM nitrocefin and cefoxitin concentrations ranging from 0.118 to 1.03 mM in 50 mM phosphate pH 7.0. Values of $(k_i)_{nit}$ were determined as above.

The data are presented in Figure 3 and values of 0.11 s^{-1} and 2.1 mM were computed for k_2 and K, respectively. However, since the slope of the line was rather shallow, these individual values should be regarded with some caution. The value of the ratio k_2/K (52 $M^{-1}s^{-1}$) which was computed from the intercept with the ordinate was, however, more accurate.

The value of k_3 was obtained by measuring the residual activity of the enzyme after establishment of the steady-state. Enzyme (2 μM) was incubated at 30° with cefoxitin concentrations ranging from 45 to 180 μM. After 30 min, the activity was determined by sampling a 25-μl aliquot, adding 250 μl of 100 μM nitrocefin and continuously recording the absorbance at 482 nm during 40–50 s. The residual activity was compared to that of a standard incubated in the absence of cefoxitin and diluted 10-fold before the addition of nitrocefin. The plot E_0/E_{ss} versus [C] (not shown) yielded a line of slope 195 mM^{-1}. Since k_3 could be neglected when compared to k_2, that slope was $k_2/k_3 K$ and the computed value of k_3 was $2.7 \times 10^{-4}\,s^{-1}$.

DISCUSSION

The β-lactamase of *Streptomyces cacaoi* was inactivated by both β-iodopenicillanate and cefoxitin. However, with this latter compound, a very slow turn-over was observed. If cefoxitin were considered as a substrate, the kinetic parameters would be $k_{cat}\,(=k_3) = 2.7 \times 10^{-4}\,s^{-1}$ and $K_m\,[=(k_3/k_2)K] = 5\,\mu M$. This contrasted with the behavior of the *S. albus* G β-lactamase with which no interaction was observed with that β-lactam.

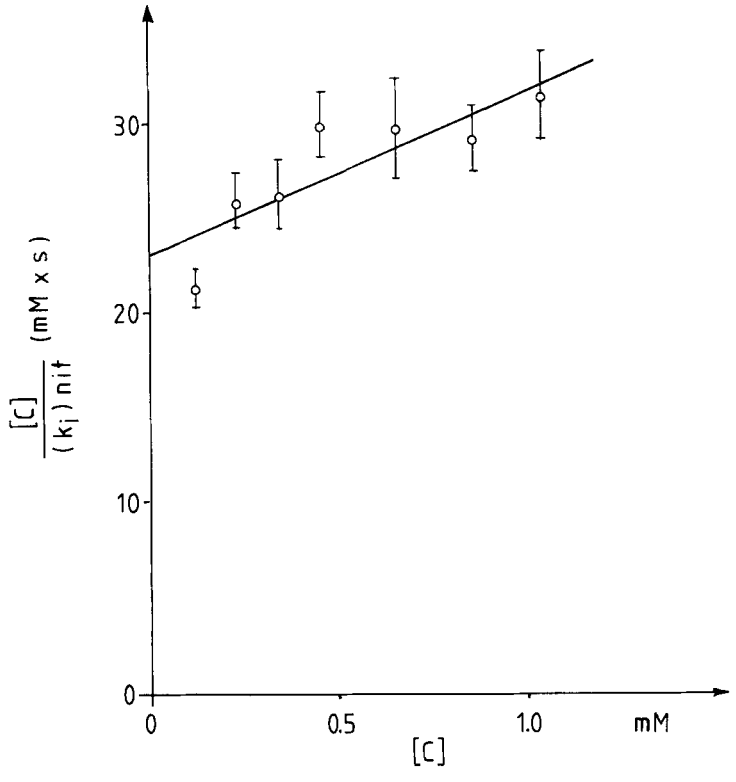


FIGURE 3 Rate of inactivation by cefoxitin: plot of $[C]/(k_i)_{nit}$ versus $[C]$. The values are ± S. D. (4 determinations). For conditions, see text.

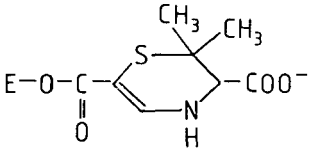
TABLE III
Hydrolysis of cefoxitin by various β-lactamases

Enzyme	K (mM)	k_2 (s^{-1})	k_2/K (M^{-1} s^{-1})	k_3 (s^{-1})
S. cacaoi	2.1	0.11	52	2.7×10^{-4}
RTEM	3.9	0.024	6	4×10^{-3}
P99	ND	ND	ND	$\geqslant 0.06$

Apparently, the *S. cacaoi* enzyme hydrolyzed cefoxitin according to the same pathway as the β-lactamases of *Enterobacter cloacae* P99 (J. M. Frère, unpublished) and *Escherichia coli* (RTEM plasmid)[12]. Table III compares the values of the kinetic parameters which are presently available.

Compared to cefoxitin, β-iodopenicillanate was a more efficient inactivator of the β-lactamase of *S. cacaoi*: k_2/K was 60-fold larger and the final product of inactivation could no longer be hydrolyzed. A chromophore absorbing near 325 nm was obtained, similar to that formed upon inactivation of β-lactamase I of Bacillus cereus by β-bromopencillanate[13,14]. As observed by the same authors[13,14], disappearance of enzymatic activity and appearance of the chromophore (probably **3**, where E is the enzyme) were concomitant events, indicating that the rearrangement of the acyl

enzyme, accompanied by the loss of the iodine atom, was not the rate-limiting step of the complete process.



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Contrarily to the *S. albus* G β-lactamase, the *S. cacaoi* enzyme did not turn over β-iodopencillanate. This difference did not, however, imply that the two enzymes belonged to different classes. In fact, some class A β-lactamases catalysed the hydrolysis of that inactivator while others did not (R. Pratt, personal communication: F. De Meester and J. M. Frère, unpublished results; Refs. 13 and 14).

In consequence, there does not seem to be any direct correlation between the chemical class to which a β-lactamase belongs and the presence or absence of turnover of a given suicide substrate. It will be interesting, in the future, to determine the structural factors which are responsible for branched pathway interactions. The experiments described in the present paper certainly did not demonstrate any kind of similarity between the two *Streptomyces* β-lactamases. The obtaining of primary structure data appears to be necessary to decide to which class a β-lactamase belongs. Work is now in progress in our laboratory which should supply this type of information concerning the two β-lactamases of *Streptomyces* discussed in the present paper. As noted in the introduction, all the data presently available indicate that both *Streptomyces* β-lactamases are probably serine enzymes. The results presented here are in complete agreement with that hypothesis.

ACKNOWLEDGEMENTS

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