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DD-Carboxypeptidase-Transpeptidase and Killing Site of β-Lactam Antibiotics in *Streptomyces* Strains R39, R61, and K11

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Additional evidence is given that in *Streptomyces* strains R39, R61, and K11 the same enzyme performs DD-carboxypeptidase and transpeptidase activities and that this enzyme is the killing site of β-lactam antibiotics. With strain R61, it was found that the exocellular enzyme has a sensitivity towards some antibiotics different from that of the membrane-bound enzyme. Under the growth conditions used in the present investigations, β-lactamase activity was not involved in susceptibility to β-lactam antibiotics.

The susceptibility of bacteria to penicillins and cephalosporins is a complex phenomenon. It depends upon (i) the sensitivity of the target itself, i.e., the membrane-bound transpeptidase which cross-links the peptides of the nascent peptidoglycans (14, 15), (ii) the β-lactamase activity of the bacteria (2), and (iii) the permeability of the bacterial envelope to the antibiotics and the nonspecific fixation of the antibiotic by cell-envelope components other than the transpeptidase (12). During growth, *Streptomyces* strains R39, R61, and K11 excrete β-lactamases and DD-carboxypeptidases-transpeptidases. The efficiency and specificity profile of the β-lactamases of strains K11 and R39 towards β-lactam antibiotics has been described (7). The DD-carboxypeptidases-transpeptidases excreted by strains R39, R61, and K11 were shown to function as hydrolytic enzymes (carboxypeptidases) or synthesizing enzymes (transpeptidases) depending upon the availability of nucleophilic groups (water or NH_2-R). These enzymes had a considerable specificity in their substrate requirements both for donor peptides and for acceptor amino groups, strongly suggesting that they were the membrane-bound transpeptidases which had undergone solubilization (8, 9, 11).

In the experiments described in this paper, we studied the in vivo susceptibility of *Streptomyces* strains to β-lactam antibiotics as related to their β-lactamase activity and the properties of their exocellular DD-carboxypeptidases-transpeptidases and membrane-bound transpeptidases.

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MATERIALS AND METHODS

Antibiotics. 6-Aminopenicillanic acid, benzylpenicillin, substituted benzylpenicillins, phenoxymethylpenicillin, and various cephalosporins were used (see Fig. 1 in the accompanying paper [7]).

Culture media. Peptone-oxoid medium and glycerol-casein medium have been described previously (8). Agar-CGY medium contained, per liter of final volume: agar, 20 g; Casamino Acids (Difco), 3 g; glycerol, 30 ml; and yeast extract (Difco), 3 g. Agar-KC medium contained, per liter of final volume: agar, 20 g; partially hydrolyzed keratin from hens' feathers, 0.25% (w/v); partially hydrolyzed casein, 0.25% (w/v); NaCl, 0.5 g; $CaCO_3$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2HPO_4, 1 g; and $FeSO_4 \cdot 7H_2O$, 0.1 g. Hydrolyzed keratin and casein were prepared as described previously (4). Agar-APG medium contained, per liter of final volume: agar, 20 g; asparagine, 0.5 g; peptone, 0.5 g; glucose, 10 g; and K_2HPO_4, 0.5 g.

Streptomyces strains. *Streptomyces* strains R39, R61, and K11 were used. R61 and K11 were maintained on slants of agar-APG, and R39 was maintained on slants of agar-KC. Viable counts were determined as follows. Conidia were collected from slants with the help of a 1:10,000

dilution of sodium dodecyl sulfate (6). After thorough shaking, the mycelial debris was removed from the suspensions by filtration on Whatman no. 2 paper. The conidia suspensions were diluted to contain 100 to 200 "colony-forming units" per ml. Viable counts were made by spreading 1 ml of the suspensions on agar-CGY plates. Incubation was at 30 C.

β-Lactamase activity of isolated Streptomyces colonies. The enzyme activity of isolated colonies was estimated by using the starch-iodine test of Foley and Perret (3), modified as follows. About 5 to 10 conidia (colony-forming units) were spread on GA-6 filters (porosity, 0.45 μm; diameter, 47 mm; Gelman Instrument Co.), deposited on agar-CGY plates. Incubation at 30 C was maintained until the colonies were 2 mm in diameter. The filters were then transferred to a freshly prepared starch-iodine indicator agar containing: agar, 1.8% (w/v); iodine, 0.0127% (w/v); KI, 0.0254% (w/v); soluble starch, 0.3% (w/v); sodium phosphate buffer (*p*H 7.4), 10 mM; and benzylpenicillin, 200 μg/ml. β-Lactamase activity provokes decolorization around the colonies. The diameters of decolorized areas were measured after increasing times at 30 C (up to 90 min). When penicillin was not present in the starch-iodine agar, decolorization around the colonies did not occur under the above conditions.

Exocellular DD-carboxypeptidases-transpeptidases. The purified, exocellular DD-carboxypeptidases - transpeptidases from *Streptomyces* strains R39, R61, and K11 have been described (8, 9). One enzyme unit catalyzes the hydrolysis of 1 nanoequivalent of D-alanyl-D-alanine linkage per hr at 37 C when the enzyme is exposed to $N\alpha^{a},N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine at concentrations 10 times the K_m values, in the absence of any acceptor amino group. The K_m values are 0.8 mM for the R39 enzyme and 12 mM for R61 and K11 enzymes.

Carboxypeptidase activity of exocellular enzymes and its inhibition by β-lactam antibiotics. N^{a},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (1.23 mM, final concentration) was incubated with the enzymes for 2 hr at 37 C, in 0.03 M K_2HPO_4, in the absence and in the presence of various concentrations of antibiotics. The amount of D-alanine liberated was estimated by the fluorodinitrobenzene technique (5). In the absence of antibiotic, the yield of hydrolysis was 90% of the theoretical value.

Transpeptidase activity of exocellular enzymes and its inhibition by β-lactam antibiotics. The conditions of the tests were as described above for the determination of carboxypeptidase activity except that ^{14}C-glycine was added to the reaction mixtures. The molar ratios of ^{14}C-glycine (i.e., the acceptor) to N^{a},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (i.e., the donor) were 10:1 for enzymes from R61 and K11 and 20:1 for enzyme from R39. The amount of N^{a},N^{ϵ}-diacetyl-L-lysyl-D-alanyl transferred from the tripeptide donor to ^{14}C-glycine (i.e., the yield of transpeptidation) was determined as described previously (11). The transpeptidation product, N^{a},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-^{14}C-glycine, was separated from the excess of ^{14}C-glycine by paper electrophoresis at *p*H 5.6, and the amount of radioactivity incorporated was measured with a Packard Tri-Carb liquid scintillation spectrometer. In the absence of antibiotic, the yield of transpeptidation was 40%, expressed as conversion of the donor tripeptide.

Membrane-bound transpeptidase from Streptomyces R61. Membranes were prepared essentially as described by Munoz et al. (10). *Streptomyces* R61 was grown (volume of inoculum, 5%) for 48 hr at 29 C, with vigorous shaking, in 1-liter flasks containing 500 ml of peptone-Oxoid medium. This medium was used rather than glycerol-casein medium because less carboxypeptidase-transpeptidase is excreted by the growing bacteria in peptone-Oxoid medium than in glycerol-casein medium. The mycelium (from 2.5 liters of culture) was harvested, washed four times with distilled water by decantation, resuspended in water, homogenized with a Potter Elvehjem tissue homogenizer, and centrifuged at 10,000 × *g* for 20 min. The pellet was washed twice by centrifugation (10,000 × *g*, 20 min) with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, and finally resuspended in 100 ml of the same buffer supplemented with 0.05 M $MgCl_2$ and 25 g of sucrose. After homogenization as above, 250 mg of lysozyme was added, and the suspension was kept at 4 C for 20 hr. The pellet obtained after centrifugation at 12,000 × *g* for 15 min was suspended in 100 ml of 0.05 M Tris-hydrochloride buffer, *p*H 7.5, containing 0.005 M $MgCl_2$ and 75 mg of deoxyribonuclease I (pancreatic). The suspension was kept at room temperature until complete lysis occurred (usually 16 hr). The lysate was centrifuged at 1,100 × *g* for 10 min; the pellet was resuspended in 100 ml of 0.05 M Tris-hydrochloride buffer (*p*H 7.5) plus 0.005 M $MgCl_2$ and centrifuged again at 1,100 × *g* for 10 min. The supernatant fluids of the two last centrifugations were combined and centrifuged at 37,000 × *g* for 30 min, yielding the membrane preparation. Membranes were suspended in a volume of 0.03 M K_2HPO_4 such that the suspension contained about 25 mg of protein per ml. The membrane suspensions could be stored at 4 C, in the presence of thymol, for 6 weeks without loss of transpeptidase activity. The same technique as above was applied to *Streptomyces* R39, but the membranes did not exhibit transpeptidase or carboxypeptidase activities.

Activity of the Streptomyces R61 membrane-bound transpeptidase and its inhibition by β-lactam antibiotics. N^{a},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (1.35 mM) and ^{14}C-glycyl-glycine (13.5 mM) were incubated for 16 hr at 37 C, with 500 μg (in protein) of R61 membrane, in a final volume of 35 μliters of 0.016 M K_2HPO_4 containing 0.3% (v/v) Triton X-100 (final con-

centrations). Incubations were carried out in the absence and in the presence of various concentrations of antibiotics. The transpeptidation product, N^{α},N^{ϵ} - diacetyl - L - lysyl - D - alanyl-^{14}C-glycyl-glycine, was separated from the excess of ^{14}C-glycyl-glycine by paper electrophoresis at *p*H 5.6 for 2 hr at 30 v/cm, and the amount of radioactivity incorporated was measured as described above. In the absence of antibiotic, the yield of transpeptidation (expressed as conversion of the donor tripeptide) was 30 to 40%, depending upon the membrane preparation. Transpeptidation reactions with acceptors other than glycyl-glycine were also carried out under the same conditions. When no radioactive acceptor was available, the radioactive tripeptide ^{14}C-diacetyl-L-lysyl-D-alanyl-D-alanine was used, and the radioactive transpeptidation product ^{14}C-diacetyl-L-lysyl-D-alanyl-acceptor was separated by electrophoresis at *p*H 6.45 for 4 hr at 60 v/cm and estimated (Perkins et al., Biochem. J., *in press*).

RESULTS

Effect of β-lactam antibiotics on growth of Streptomyces (LD_{50} values). As for many other bacteria, the susceptibility of *Streptomyces* to β-lactam antibiotics is critically dependent upon the size of the cell inoculum. To ensure maximal reproducibility in the quantitative estimates, we measured susceptibility by observing the effect of the presence of various concentrations of antibiotics on the ability of conidia to form "single-cell" colonies. Colonies were counted daily until maximal values were obtained (5 to 8 days, depending upon the strain, the antibiotic, and its concentration). In the absence of antibiotic, control plates contained about 100 to 200 colonies. The LD_{50} values (Table 1 to 3) were determined graphically as the concentrations of antibiotics allowing 50% cell survival. The data show that the antibiotic susceptibility of strains R61 and K11 was almost the same. These strains were less susceptible than strain R39, except to 6-aminopenicillanic acid and cephalosporin C. Great variations were observed depending upon the antibiotic.

Effect of β-lactam antibiotics on activity of the exocellular carboxypeptidases-transpeptidases of Streptomyces (ID_{50} values, soluble enzyme). The effect of the antibiotics on carboxypeptidase activity was expressed as the concentration which decreased by 50% the amount of D-alanine liberated from N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. Similarly, the effect of antibiotics on transpeptidase activity was expressed as the concentration which reduced by 50% the amount of transpeptidation products. The ID_{50} values are also reported in Tables 1–3. These data show that inhibition of the transpeptidase activity of the exocellular enzymes always occurred at those concentrations of antibiotics which inhibited the carboxypeptidase activity. When the whole series of antibiotics is taken into account, there appears to be no correlation at all between the in vivo LD_{50} values and the in vitro ID_{50} values for the exocellular enzymes. The in vivo susceptibility of the *Streptomyces* strains may be either similar to, or considerably higher or lower than, the in vitro sensitivity of the exocellular enzymes.

Membrane-bound carboxypeptidase-transpeptidase activity of Streptomyces R61. Since the catalytic properties of an enzyme

TABLE 1. *Effect of β-lactam antibiotics on Streptomyces strain R39*[a]

Antibiotic	LD_{50}	ID_{50}			ID_{50} (avg)/LD_{50}
		Carboxy-peptidase	Trans-peptidase	Avg	
6-Aminopenicillanic acid	5,100	500	650	575	0.113
Penicillin G	70	3.8	7.1	5.4	0.077
Penicillin V	140	3.5	4.6	4.0	0.029
Ampicillin	240	3.25	3.6	3.4	0.014
Carbenicillin	830	195	200	197	0.24
Oxacillin	710	12	33	22	0.031
Cloxacillin	530	29	46	37	0.070
Methicillin	730	42	53	47	0.064
Cephalosporin C	9,000	4.7	6.0	5.3	0.00059
Cephaloglycine	120	5.4	7.4	6.4	0.053
Cephalexin	15	26	17	21	1.4
Cephalothin	90	2.9	5.5	4.2	0.047

[a] Comparison between the lethal action (LD_{50} values) and the inhibition of the exocellular DD-carboxypeptidase-transpeptidase activity (ID_{50} values). Values for LD_{50} and ID_{50} represent $10^8 \times$ molar concentration.

TABLE 2. *Effect of β-lactam antibiotics on Streptomyces strains K11 and R61*[a]

Antibiotic	LD_{50}		ID_{50}				ID_{50}/LD_{50}[b]	
			Carboxypeptidase		Transpeptidase			
	K11	R61	K11	R61	K11	R61	K11	R61
6-Aminopenicillanic acid	1,160	1,850	135,000	130,000		130,000	116	70
Penicillin G	390	560	15	12		15	0.038	0.024
Penicillin V	390	510	110	190	135		0.31	0.37
Ampicillin	670	540	3,300	3,000		2,200	4.9	4.8
Carbenicillin	3,080	3,310	400	640		900	0.13	0.23
Oxacillin	3,300	4,710	2,100	1,900	3,400		0.83	0.40
Cloxacillin	2,630	5,040	3,500	7,000	4,700		1.6	1.4
Methicillin	3,180	4,410	15,000	14,000	17,500		5.1	3.2
Cephalosporin C	5,620	11,220	51	46		42	0.0091	0.0039
Cephaloglycine	1,630	2,860	5,000	6,600		7,600	3.1	2.5
Cephalexin	510	2,060	50,000	60,000		50,000	98	27
Cephalothin	590	1,400	62	80		80	0.105	0.057

[a] Comparison between the lethal action (LD_{50} values) and the inhibition of the exocellular DD-carboxypeptidase-transpeptidase activity (ID_{50} values). Values for LD_{50} and ID_{50} represent $10^8 \times$ molar concentration.

[b] When the data are available, the ID_{50} values used in this ratio are the average values between carboxypeptidase and transpeptidase. In the other cases, the carboxypeptidase values alone were used.

TABLE 3. *Effect of β-lactam antibiotics on Streptomyces strain R61*[a]

Antibiotics	LD_{50}	ID_{50} for membrane-bound transpeptidase	ID_{50}/LD_{50}
6-Aminopenicillanic acid	1,850	8,000	4.3
Penicillin G	500	215	0.38
Penicillin V	510	1,850	3.6
Ampicillin	540	800	1.5
Carbenicillin	3,310	3,400	1.0
Oxacillin	4,710	8,800	1.9
Cloxacillin	5,040	3,900	0.78
Methicillin	4,410	3,500	0.79
Cephalosporin C	11,220	51,000	4.5
Cephaloglycine	2,860	6,200	2.2
Cephalexin	2,060	6,000	2.9
Cephalothin	1,400	7,000	5.0

[a] Comparison between the lethal action (LD_{50} values) and the inhibition of the transpeptidase activity of the membrane-bound enzyme. Values for LD_{50} and ID_{50} represent $10^8 \times$ molar concentration.

may depend upon its localization, whether it occurs extracellularily or is membrane-bound, the carboxypeptidase-transpeptidase activities exhibited by the plasma membranes prepared from strain R61 (Materials and Methods) were tested under a variety of experimental conditions. Figure 1 shows the effect of increasing the molar ratio of acceptor to donor from 1:1 to 20:1 on the amount of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-glycyl-glycine (transpeptidation product) formed from N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (donor, at a constant concentration of 1.68 mM) and ^{14}C-glycyl-glycine (acceptor) by the R61 membrane-bound enzyme. Incubation was for 16 hr at 37 C with R61 membranes (500 μg of protein) in a final volume of 37 μliters of 0.017 M K_2HPO_4 containing 0.27% Triton X-100. At a molar ratio of acceptor to donor of 10:1, the yield of transpeptidation was almost maximal (35%, expressed as conversion of the tripeptide donor) and was proportional to the amount of membrane present in the reaction mixture. Triton X-100 (0.27%) was not necessary for the transpeptidation reaction to occur and did not solubilize the enzyme activity which remained sedimentable (40,000 × *g*, 30 min). However, the presence of the detergent in the reaction mixture facilitated the separation of the transpeptidation product by paper electrophoresis (Materials and Methods). Table 4 shows that the specificity profile of the membrane-bound transpeptidase for various acceptors (with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine as donor) resembles that of the exocellular carboxypeptidase-transpeptidase enzyme. Glycine, D-alanine, glycyl-glycine, glycyl-L-alanine, racemic diaminoadipic

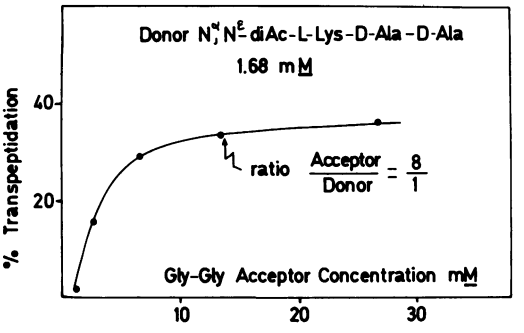

FIG. 1. *Effect of increasing ratios of acceptor to donor on transpeptidation by the membrane-bound enzyme from Streptomyces R61. The reaction mixtures contained, in a final volume of 37 μliters: membranes, 500 μg of protein; N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, 1.68 mM; glycyl-glycine, from 1.68 to 33.6 mM; Triton X-100, 0.27%; and K_2HPO_4, 0.017 M. Incubation was for 16 hr at 37 C. Transpeptidation is expressed as conversion of the tripeptide donor.*

acid, and 2-amino-D-galacturonic acid are good acceptors for both exocellular and membrane-bound enzymes. Glycyl-D-alanine is a poor acceptor, and L-alanine, L-alanyl-L-alanine, and D-alanyl-D-alanine are not acceptors at all. However, in spite of this similarity between the substrate requirements, the relative yields of transpeptidation with the various acceptors are not identical for the two enzyme preparations.

Under the same conditions as above, and even in the absence of acceptor, the R61 membrane preparation did not hydrolyze the tripeptide donor into D-alanine and N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine. This lack of carboxypeptidase activity is no doubt related to the fact that the purified exocellular DD-carboxypeptidases-transpeptidases from *Streptomyces* R61 and R39 can be forced to work preferentially as transpeptidase rather than as carboxypeptidase by properly adjusting the conditions of the in vitro assays (Ghuysen et al., Frère et al., *in preparation*).

Effect of β-lactam antibiotics on the transpeptidase activity of the membrane-bound enzyme from Streptomyces R61. In marked contrast to what was observed with the exocellular enzyme, there was a high correlation between the ID_{50} values of the membrane-bound enzyme and the in vivo LD_{50} values of strain R61 (Table 3). In comparing Tables 2 and 3, one should note that, in those instances when the correlation between the ID_{50} values of the exocellular enzyme and the LD_{50} values is good (in the range of 0.5 to 5.0), the correlation between the corresponding ID_{50} values for the membrane-bound enzyme and the LD_{50} values remains of the same order of magnitude. However, in the cases where the ID_{50} values for the soluble enzyme do not correlate with the LD_{50} values, the corresponding ID_{50} values for the membrane-bound enzyme do correlate well with the LD_{50} values.

β-Lactamase activity of Streptomyces strains R39, R61, and K11. The β-lactamase activity of *Streptomyces* colonies on benzylpenicillin was estimated under growth conditions similar to those used for the estimations of the in vivo LD_{50} values (Materials and Methods). The tests were performed on isolated colonies of identical size by the starch-iodine method. The diameters of the decolorized zones around the colonies were 0, 2.5, and 4 mm, respectively, for strains R61, R39, and K11 after 10 min at

TABLE 4. *Profiles of the membrane-bound enzyme and exocellular enzyme from Streptomyces strain R61 for acceptors in transpeptidation reactions with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine as donor*[a]

Acceptor	Membrane-bound enzyme		Exocellular enzyme	
	Acceptor to donor ratio	Yield of transpeptidation	Acceptor to donor ratio	Yield of transpeptidation
Glycine	10:1	18	1:1	14
D-Alanine	10:1	25	1:1	8
L-Alanine	10:1	0	10:1	0
Glycyl-glycine	10:1	26	1:1	18
	2:1	17		
Glycyl-L-alanine	10:1	11	1:1	25
Glycyl-D-alanine	10:1	2	1:1	4
D-Alanyl-D-alanine	10:1	0	1:1	0
L-Alanyl-L-alanine	10:1	0	1:1	0
2-Amino-β-galacturanic acid	2:1	12	5:1	18
Racemic diaminoadipic acid	2:1	9	5:1	54

[a] Yield of transpeptidation is expressed as percentage of conversion of the tripeptide donor. For the experiments carried out with the membrane-bound enzyme, the tripeptide donor was not radioactive and the acceptors were ^{14}C-labeled (glycine, D-alanine, L-alanine, glycyl-glycine) or, conversely, the tripeptide donor was ^{14}C-labeled on the acetyl groups and the acceptors were not radioactive. Conditions used with membrane-bound enzyme are given in the text; those for exocellular enzyme are given in reference 12.

30 C; after 90 min of incubation, the corresponding diameters were 5, 9, and 10 mm. Thus, strain K11 has a higher β-lactamase activity than strain R61, although these two strains are equally susceptible to the antibiotics. Similarly, strain R39 has a higher β-lactamase activity than strain R61, although strain R39 is more susceptible to the antibiotics than strain R61.

If β-lactamase activity is involved in the relative susceptibility of bacteria to β-lactam antibiotics, the susceptibility of a given strain to a series of antibiotics should bear an inverse relationship to the catalytic efficiency and specificity profile of the β-lactamase for the same antibiotics. Comparison of the LD_{50} values of the antibiotics for *Streptomyces* K11 and R39 with the profiles and efficiencies of the corresponding β-lactamases (see Table 2 in the accompanying paper [7]) clearly confirms that the susceptibility of these *Streptomyces* strains to β-lactam antibiotics is not related to their β-lactamase activities, at least under the growth conditions used for the tests.

DISCUSSION

A complete lack of correlation of the in vivo susceptibility of the *Streptomyces* strains to β-lactam antibiotics, the β-lactamase activity of the strains, and the efficiency and specificity profiles of their β-lactamases was observed. β-Lactamase activity thus appears to be irrelevant to antibiotic susceptibility, at least under the conditions used for the determination of the ability of conidia to form colonies in the presence of antibiotics. Although for many bacteria there is a high correlation between penicillinase production and penicillin resistance, there are also numerous examples in the literature which are not consistent with the idea that penicillinase is the prime factor involved in resistance (13).

Irrespective of the structure of the β-lactam antibiotics used, inhibition of the transpeptidase activity of the three exocellular enzymes from *Streptomyces* R39, R61, and K11 always occurred at those antibiotic concentrations that inhibited carboxypeptidase activity. This provides additional support for the idea previously proposed that in *Streptomyces* sp. the same enzyme performs both carboxypeptidase and transpeptidase activities.

The exocellular enzymes from *Streptomyces* R61 and K11 are indistinguishable from each other but differ greatly from the exocellular enzyme from *Streptomyces* R39 with respect to substrate requirements for peptide donor and acceptor, to K_m and V_{max} values in carboxypeptidase assays, and to sensitivity to β-lactam antibiotics. Parallel to this, the in vivo LD_{50} values of the β-lactam antibiotics for strains R61 and K11 are very similar, and these values differ greatly from those for strain R39. However, with the three *Streptomyces* strains studied, the lethal concentrations of some antibiotics are very different from those that inhibit the in vitro activity of the exocellular enzymes. The experiments carried out with the membrane-bound enzyme from *Streptomyces* R61 provide the explanation of these discrepancies. These experiments show that the sensitivity of the R61 exocellular enzyme towards some antibiotics may be very different from that of the corresponding membrane-bound enzyme. An increased resistance of the membrane-bound enzyme, compared to that of the exocellular enzyme, can be easily explained, since nonspecific binding sites for these antibiotics are likely to occur on the membrane. An increased sensitivity of the membrane-bound enzyme is more difficult to interpret. It suggests conformational changes of the enzyme as the result of its integration in the lipophilic environment of the membrane. Integration of the enzyme into the membrane, however, does not seem to modify its general specificity profile for peptide acceptors.

The present work suggests that in *Streptomyces* sp. carboxypeptidase and transpeptidase activities are performed by the same enzyme, and this enzyme, when integrated into the membrane, seems to be the killing site for β-lactam antibiotics. Contrary to what has been observed in *Streptomyces* sp., it has been proposed by Blumberg and Strominger (1) that in *Bacillus subtilis* the killing site, i.e., the transpeptidase, is distinct from the carboxypeptidase. Their conclusion was mainly based on the fact that 6-aminopenicillanic acid inactivated at least 95% of the carboxypeptidase activity (toluene-treated cells) at nonlethal concentrations (liquid cultures) and, conversely, that cephalothin was lethal at concentrations that did not inactivate the enzyme. One should note, however, that a similar conclusion would have been drawn in the case of *Streptomyces* R61 if, for example, one had limited the investigations to the simple comparison between the LD_{50} values for 6-aminopenicillanic acid and cephalosporin C and the corresponding ID_{50} values of the exocellular carboxypeptidase-transpeptidase. Indeed, 6-aminopenicillanic acid is 70 times more active on the soluble enzyme than in vivo and, conversely, cephalosporin C is 200 times less active on the soluble enzyme than in vivo.

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