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The Peptidoglycan Crosslinking Enzyme System in *Streptomyces* Strains R61, K15 and *rimosus*

Exocellular, Lysozyme-Releasable and Membrane-Bound Enzymes

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The DD-carboxypeptidase-transpeptidase enzyme system in *Streptomyces* strain K15 consists of: (1) a membrane-bound transpeptidase capable of performing low DD-carboxypeptidase activity; and (2) a set of DD-carboxypeptidases: (a) membrane-bound, (b) lysozyme-releasable and (c) exocellular, having low transpeptidase activities in aqueous media and at low acceptor concentrations. The DD-carboxypeptidases are related to each other and may belong to the same pathway leading to enzyme excretion. A similar enzyme system occurs in *Streptomyces* strain R61 except that the membrane-bound DD-carboxypeptidase activity is low when compared with the membrane-bound transpeptidase activity. In *Streptomyces rimosus* the enzyme system consists almost exclusively of the membrane-bound transpeptidase and the levels of membrane-bound, lysozyme-releasable and exocellular DD-carboxypeptidases are very low.

The peptide moiety of the wall peptidoglycan in *Streptomyces* sp. consists of L-Ala-D-αGln┌─(L1)-A2pm-(L1)-D-Ala peptide units crosslinked through a glycine residue that extends between an (L2) amino group of the diaminopimelic acid residue of one peptide unit and the carboxyl group of the D-alanine residue of another peptide unit [1,2]. From what is known of the wall synthesis in bacteria [3,4], peptide crosslinking in *Streptomyces* sp. must occur by a transpeptidation reaction through which the carboxyl group of the penultimate D-alanine residue of one hexapeptide unit

L-Ala-D-αGln┌─(L1)-A2pm-(L1)-D-Ala-D-Ala
Gly-(L2) ─┘

is transferred to the amino group of the glycine residue of a second peptide unit. N^{α}-(D-Ala)-Gly linkages are formed and equivalent amounts of D-alanine residues are released.

Streptomyces R61 excretes during growth an exocellular enzyme which performs three activities. (a) When exposed to the tripeptide Ac_2-L-Lys-D-Ala-D-Ala, it catalyses the release of the C-terminal D-Ala residue (Ac_2-L-Lys-D-Ala-D-Ala + H_2O → Ac_2-L-Lys-D-Ala + D-Ala; DD-carboxypeptidase action); (b) When exposed to the same tripeptide donor and the dipeptide Gly-Gly acceptor, it catalyses concomitantly both the hydrolysis of the donor and the transfer reaction Ac_2-L-Lys-D-Ala-D-Ala + Gly-Gly → Ac_2-L-Lys-D-Ala-Gly-Gly + D-Ala (transpeptidase action). In water, at neutral pH and at low acceptor concentration, the enzyme functions mainly as a DD-carboxypeptidase. Transfer is preferentially favored over hydrolysis by raising the pH value of the reaction mixture, by increasing the concentration of the acceptor and by decreasing the polarity of the medium. (c) It fragments penicillin into N^{α}-acylglycine and *N*-formyl-D-penicillamine. The reaction proceeds via the transitory formation of an inactive,

Abbreviations. Cetavlon, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; T/Hy, ratio of transpeptidase to carboxypeptidase activity as measured by the transfer/hydrolysis ratio with the substrate Ac_2-L-Lys-D-Ala-D-Ala.

stoichiometric enzyme · β-lactam complex of rather high stability (the half-life of the complex formed with benzylpenicillin is 80 min at 37 °C). The properties of this exocellular DD-carboxypeptidase-transpeptidase are described in two recent reviews [5, 6].

Streptomyces R61 also possesses a membrane-bound enzyme which catalyses the transfer reaction between Ac_2-L-Lys-D-Ala-D-Ala and Gly-Gly with high efficiency. In contrast to the exocellular enzyme, the ability of the membrane-bound enzyme to hydrolyse the tripeptide donor is very low. This transpeptidase has been solubilized by extracting the membranes or the washed mycelium with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide and has been partially purified by filtration on Sephadex G-100 [7–9].

Experiments were undertaken to establish the relationships which might exist between the exocellular and the membrane-bound enzymes. These studies were carried out on three different strains of *Streptomyces*, strains R61, K15 and *rimosus* respectively. The results obtained are reported in this paper and in the following ones.

MATERIALS AND METHODS

Enzymatic Assays

Unless otherwise specified, the standard substrate concentrations were: 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala (DD-carboxypeptidase assay) and 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala + 15 mM Gly-Gly (transpeptidase assay). The incubations were always carried out at 37 °C. The techniques used for the measurement of the enzyme activities were published previously; details can be found in [6].

DD-Carboxypeptidase Activity (Ac_2-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-Lys-D-Ala). This activity was estimated either by measuring enzymatically the amount of D-Ala liberated from the non-radioactive tripeptide Ac_2-L-Lys-D-Ala-D-Ala (D-amino acid oxidase technique) or by measuring the amount of [^{14}C]Ac_2-L-Lys-D-Ala formed from [^{14}C]-Ac_2-L-Lys-D-Ala-D-Ala (with the radioactive label in the acetyl groups). The radioactive dipeptide was separated from the residual radioactive tripeptide by paper electrophoresis at pH 6.5. When membranes were assayed, the release of free D-alanine was always estimated using the radioactive tripeptide.

Transpeptidase Activity (Ac_2-L-Lys-D-Ala-D-Ala + [^{14}C]Gly-Gly → D-Ala + Ac_2-L-Lys-D-Ala-[^{14}C]-Gly-Gly). This activity was estimated by measuring the amount of radioactive tetrapeptide Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly formed during the reaction. The product was separated from the excess of [^{14}C]Gly-Gly by paper electrophoresis at pH 5.6.

T/Hy Ratio Values. In the present paper, these values express the ratios of the rate of transformation of the tripeptide donor (1.5 mM) into Ac_2-L-Lys-D-Ala-Gly-Gly in the presence of 15 mM Gly-Gly (transpeptidase assay) to the rate of hydrolysis of the same tripeptide donor (1.5 mM) in the absence of acceptor Gly-Gly (DD-carboxypeptidase assay).

Buffers

The buffers were prepared and their ionic strength expressed according to Dawson *et al.* [10].

Streptomyces *Strains*

Streptomyces strain R61 has been used previously [5]. *Streptomyces* strain K15 also belongs to the local collection. *Streptomyces rimosus* was kindly provided by Dr D. A. Hopwood (John Innes Institute, Norwich NOR 70F, U.K.). The wall peptidoglycans of all three strains are identical and typical of those of *Streptomyces* sp. with a glycyl-LL-diaminopimelyl sequence in the bridging position.

Growth Media

The glycerol/casein and peptone Oxoid media of Leyh-Bouille *et al.* [11] were used.

Exocellular Enzymes

Streptomyces R61 excretes an exocellular DD-carboxypeptidase in the glycerol/casein medium. Maximal activity (5.4 nmol tripeptide hydrolysed min^{-1} ml culture $filtrate^{-1}$) occurs after 3–4 days of growth at 28 °C. The enzyme has been purified to protein homogeneity [5, 6].

Streptomyces strain K15 is also a good producer of exocellular enzyme. Under the same conditions as above, the maximal activity is 3 nmol tripeptide hydrolysed min^{-1} ml culture $filtrate^{-1}$. This enzyme has been partially purified with an 800-fold increased specific activity and an overall recovery of 45% through the following steps. Once the enzyme was adsorbed on amberlite (see step 1, below) all the ensuing operations were carried out at 0 °C. Step 1: the enzyme in the culture filtrate (0.4 nmol tripeptide hydrolysed min^{-1} mg $protein^{-1}$) was adsorbed on amberlite CG-50 H^+ at pH 6.0 (25 g resin/l), and then desorbed from the resin by 0.1 M K_2HPO_4 with the pH value maintained at 8.0 by careful addition of concentrated ammonia (specific activity of the extract: 2 nmol min^{-1} mg $protein^{-1}$). Step 2: the enzyme was precipitated from the solution obtained after step 1 by addition of 1.6 vol. of acetone (precooled at −20 °C) and the pellet dissolved in 0.5 M Tris-HCl buffer, pH 8.0, containing 0.3 M NaCl (specific activity: 17 nmol min^{-1} mg $protein^{-1}$). Step 3: the solution obtained after step 2 was filtered on a column of Dowex

AG 1X2 (100–200 mesh) previously equilibrated against the same Tris-NaCl buffer as above. A 600-ml column of resin was used for an enzyme solution containing 200 mg total protein. The enzyme was not adsorbed on the column and was recovered in the effluent with a substantially increased specific activity (330 nmol min^{-1} mg $protein^{-1}$).

Streptomyces strain *rimosus* is a very poor producer of exocellular enzyme in both peptone and glycerol/casein media. Maximal activity is about 0.03 nmol min^{-1} ml culture $filtrate^{-1}$. Attempts were not made to purify it. The culture filtrate was concentrated 10-fold by ultrafiltration on UM 10 Amicon membranes.

Lysozyme-Releasable Enzymes: Conditions for Enzyme Release

Streptomyces rimosus and K15 were grown (volume of inoculation: 2%) for 40 h at 27 °C with vigorous shaking in 1-l flasks containing 500 ml peptone Oxoid medium. The mycelia (from 2.5 l of the corresponding cultures) were harvested, washed with water and homogenised to disperse the mycelium. The mycelia were resuspended in 100 ml of 0.1 M Tris-HCl buffer pH 7.5 containing 25 g sucrose, 250 mg lysozyme and 50 mM $MgCl_2$. The suspensions were shaken gently at 4 °C for several hours until complete transformation into protoplasts. The protoplasts were collected by centrifugation at 27000 × *g* for 30 min. The supernatant from the centrifugation is termed the 'lysozyme-releasable enzyme preparation'. The same procedure was applied to *Streptomyces* R61 except that step 2 was carried out in the absence of added Mg^{2+} ions and by using a 2-fold decreased lysozyme concentration.

Partially Purified Lysozyme-Releasable Enzymes

Samples (50 ml) of the lysozyme-releasable enzyme preparations were dialysed for 12 h at 4 °C against 2 l 33 mM Tris-HCl buffer pH 7.5. This treatment was repeated four times, the dialysed fractions were concentrated to 5 ml by ultrafiltration on UM 10 Amicon membranes and then centrifuged at 100000 × *g* for 3 h at 4 °C. The pellets thus obtained had no DD-carboxypeptidase activity and were discarded. The DD-carboxypeptidase activities of the supernatant fractions were 1000, 44000 and 80000 pmol tripeptide hydrolysed min^{-1} l original $culture^{-1}$ in the cases of strains *rimosus*, K15 and R61, respectively. The supernatant fractions were separately filtered at 4 °C on 800-ml columns of Sephadex G-100 previously equilibrated against 33 mM Tris-HCl buffer, pH 7.5. The DD-carboxypeptidase activity was eluted at a K_D value of 0.3, well separated from the bulk of materials absorbing at 280 nm (K_D value: 0–0.2). The active fractions were pooled and concentrated to 5 ml by ultrafiltration. The DD-carboxypeptidase activities of these fractions, when compared with those of the corresponding fractions before Sephadex filtration, were of 80% for the *rimosus* enzyme, 90% for the K15 enzyme and 240% for the R61 enzyme. Table 1 gives the final recoveries. The specific activities of the final preparations (in 3 mM Tris-HCl buffer pH 7.5) were 35 pmol tripeptide hydrolysed min^{-1} mg $protein^{-1}$ for the *rimosus* enzyme, 4000 for the K15 enzyme and 5000 for the R61 enzyme.

Cytoplasmic Membranes

The *Streptomyces* were transformed into protoplasts as described above (see: Lysozyme-Releasable Enzymes: Conditions for Enzyme Release) either in the presence (standard procedure) or in the absence of added Mg^{2+} ions (modified procedure). After centrifugation, the pellets were suspended in 100 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and 7.5 mg deoxyribonuclease (pancreatic) and the suspensions maintained at room temperature until complete lysis occurred. The membranes were then isolated from the lysates by differential centrifugation as described previously [7]. The membrane preparations were resuspended in 14 mM phosphate buffer, pH 7.5, at a final protein concentration of 25 mg/ml as determined by the technique of Lowry. The yields of membrane proteins/l original cultures were about 50, 60 and 80 mg for strains K15, *rimosus* and R61 respectively. All the experiments described below were carried out either with fresh membrane preparations or with membrane suspensions stored at 4 °C for less than 1 week.

Cetavlon (N-cetyl-N,N,N-trimethylammonium Bromide)-Releasable Enzymes

The mycelia (from 15 l original culture) were washed, homogenised and resuspended in 1 l of a 2% cetavlon solution made in 33 mM Tris-HCl buffer, pH 7.5 (strains R61 and *rimosus*) or in 30 mM potassium phosphate buffer pH 7.5 (strain K15) and the suspensions were maintained at 37 °C for 45 min. After removal of the disrupted cells, the extracts were maintained at 0 °C overnight (which caused precipitation of a major part of the detergent) and the supernatant fractions were concentrated to 50 ml by ultrafiltration (for more details, see [8]).

Anti-R61 Exocellular Enzyme Antisera

Rabbit antisera against the purified exocellular enzyme from *Streptomyces* R61 were prepared and used as described by Nguyen-Distèche *et al.* [12].

Slab Gel Electrophoresis and Detection of Penicillin-Binding Proteins

Proteins were separated by electrophoresis in polyacrylamide slab gels containing sodium dodecylsulphate essentially as described in [13]. Gels were stained with Coomassie brilliant blue [14] and prepared for fluorography using the techniques developed by Bonner and Laskey [15] and Laskey and Mills [16].

Preparation of Samples for Detection of Penicillin-Binding Proteins

Samples containing cetavlon or samples greater than 50 µl in volume were precipitated with 4 vol. of acetone at −10 °C after treatment with [^{14}C]benzylpenicillin (40−100 µM; 52 Ci/mol) for 20 min at 37 °C followed by non-radioactive benzylpenicillin for 30 s. The precipitate was collected by centrifugation, dried *in vacuo* and redissolved in 10 mM Tris-HCl, pH 7.2, containing 10% glycerol, 1% sodium dodecylsulphate, 1% 2-mercaptoethanol and 0.002% bromophenol blue by boiling for 1 min. Samples lacking cetavlon and less than 50 µl in volume were treated with [^{14}C]benzylpenicillin and non-radioactive penicillin as above; this treatment was followed by the addition of 0.2 vol. of dissolving buffer containing the same reagents as above but at 5 times the concentration, and boiling for 1 min.

RESULTS

With the three strains selected, the enzyme activities were measured: (a) in the culture filtrates (exocellular enzymes); (b) in the supernatants obtained after transformation of the mycelia into protoplasts by lysozyme (lysozyme-releasable enzymes); (c) in the cytoplasmic membrane preparations (membrane-bound enzymes); and (d) in the extracts obtained by treatment of the mycelia with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (cetavlon-releasable enzymes). The results are summarized in Table 1. All the enzymes studied catalysed both hydrolysis and transfer reactions. However, for the sake of simplicity, enzymes are designated as DD-carboxypeptidases when the rates of the reactions catalysed are such that the T/Hy ratio is < 1, and as transpeptidases when the reaction rates are such that the T/Hy ratio is > 1.

Table 1. *Exocellular, lysozyme-releasable and membrane-bound DD-carboxypeptidases-transpeptidases in* Streptomyces *sp.*
Cbpase: DD-carboxypeptidase activity. Tpase: transpeptidase activity. All the determinations were carried out under standard substrate concentrations (Materials and Methods); (a) standard procedure (Materials and Methods); (b) modified procedure (Materials and Methods); (c) depending upon the strains, the exocellular enzymes were purified to different stages of purity. The R61 enzyme was the only one which was purified to protein homogeneity [6]. The K15 enzyme was partially purified (see text). The *rimosus* enzyme was a crude concentrated culture filtrate (see text)

Enzymes	Strains	Procedure used	Specific activity			Total activity	
			Cbpase	Tpase	T/Hy	Cbpase	Tpase
			pmol min^{-1} mg protein^{-1}			pmol min^{-1} l original culture^{-1}	
Exocellular culture filtrates	*rimosus*	see text	c	c	0.61	3.1×10^4	1.89×10^4
	K15		c	c	0.36	3×10^6	1.08×10^6
	R61		c	c	0.10	5.4×10^6	5.4×10^5
Lysozyme-releasable after dialysis and Sephadex filtration	*rimosus*	a	35	12	0.34	1000	340
	K15	a	4000	1400	0.35	40000	13000
	R61	b	5000	1400	0.28	190000	53000
Membrane-bound (isolated membranes)	*rimosus*	a	6	370	60	340	21300
	K15	a	2	7	3.5	110	370
	K15	b	4	30	7.5		
	R61	a	2	37	19	150	2960
	R61	b	4	140	35	320	11200
Membrane-bound (isolated membranes + 1.5% cetavlon)	*rimosus*	a	8	440	55	490	24800
	K15	a	4	22	5.5	240	1100
	K15	b	4	30	7.5		
	R61	a	2	93	46	135	7400
	R61	b	4	200	50	320	16000
Mycelia extracts (cetavlon)							
Fraction K_D 0.45	*rimosus*	see text	8000	100000	12.5	8000	100000
Fraction K_D 0.35	K15		10600	1640	0.15	8300	1300
Fraction K_D 0.45	K15		1400	32000	23	680	15700
Fraction K_D 0.45	R61		3000	42000	14	1300	18200

EXOCELLULAR
AND LYSOZYME-RELEASABLE ENZYMES

Streptomyces strains R61 and K15 are good producers of exocellular DD-carboxypeptidase and contain large amounts of lysozyme-releasable DD-carboxypeptidase (Table 1). All these enzymes catalysed transfer reactions with low efficiency (Table 1). The specific DD-carboxypeptidase and transpeptidase activities of the exocellular R61 enzyme (T/Hy = 0.1) have been described [5,6]. Those of the partially purified exocellular K15 enzyme were 500 and 180 nmol min^{-1} mg $protein^{-1}$, respectively (in Tris-HCl buffer pH 9, $I = 0.03$ M; T/Hy = 0.36). The R61 and K15 lysozyme-releasable enzymes exhibited approximately the same 0.3 T/Hy ratio value (in Tris-HCl buffer pH 7.5, $I = 0.01$ M).

The pH and ionic strength effects on the DD-carboxypeptidase activity of the R61 enzyme were described previously [5,6]. Maximal activity occurs at pH 7 and at low (0.01 M) ionic strength. In DD-carboxypeptidase assays the exocellular K15 enzyme had a broad pH optimum between 8 and 10. In Tris-HCl buffer pH 9, the ionic strength (up to 0.15 M) had little effect, although maximal activity occurred at about $I = 0.03$ M. Under these conditions the K_m value for the tripeptide donor was 1.4 mM with a V value of 1 µmol min^{-1} mg $protein^{-1}$ (Table 2). The R61 and K15 lysozyme-releasable enzymes were very sensitive to variations of the ionic strength. In Tris-HCl buffers pH 7.5, optimal activity occurred at I lower than 0.002 M. In 0.5 M ionic strength buffer, the activity was inhibited by 90%. In 0.01 M ionic strength buffer, the K_m values for the tripeptide were 8–9 mM and the V values were about 40 nmol min^{-1} mg $protein^{-1}$ (Table 2).

With the lysozyme-releasable enzymes, the rate of hydrolysis was maximal at pH 6–7 (in phosphate buffer, $I = 0.02$ M) whereas the rate of transpeptidation was maximal at pH 9 or more (in Tris-HCl buffer or L-alanine-NaOH buffer, $I = 0.02$ M) (Fig. 1 A, B). Hence, as previously observed with the exocellular enzyme from strain R61 (see the introduction), the channelling of the total activity of the lysozyme-releasable enzymes into either of these pathways also depended upon the environmental conditions.

One property of the R61 exocellular enzyme is its ability to hydrolyse Ac-L-Lys-D-Ala-D-Ala into D-Ala
Gly—┘
and Ac-L-Lys-D-Ala and to utilize it as donor and
Gly—┘
acceptor in a transfer reaction leading to the formation of peptide dimer. Transfer is favoured over hydrolysis by performing the reaction in a medium of low polarity [17]. The exocellular K15 enzyme and both R61 and K15 lysozyme-releasable enzymes exhibited the same property.

When incubated with [^{14}C]benzylpenicillin and subjected to dodecylsulphate slab gel electrophoresis,

Table 2. *Kinetic parameters for the hydrolysis of Ac$_2$-L-Lys-D-Ala-D-Ala in the absence of acceptor (DD-carboxypeptidase activity)*
All the incubations were carried out at 37 °C

Enzymes	Strains	K_m	V	Remarks
		mM	nmol min^{-1} mg $protein^{-1}$	
Exocellular	*rimosus*	n.d.	n.d.	concentrated culture filtrate (see text)
	K15[a]	1.4	1000	partially purified (see text)
	R61[b]	10	80000	purified to protein homogeneity [17]
Lysozyme-releasable	*rimosus*	n.d.	n.d.	
	K15[c]	9	40	partially purified (see text)
	R61[d]	8	40	
Cetavlon-solubilised transpeptidase (K_D: 0.45)	*rimosus*[e]	7	30	
	K15[f]	3	5	partially purified (see text)
	R61[g]	3	10	
Cetavlon-solubilised DD-carboxypeptidase (K_D: 0.35)	K15[h]	25	130	partially purified (see text)

[a] 0.6 µg protein in 60 µl $I = 0.04$ M Tris-HCl buffer pH 9.
[b] See [6].
[c] 17 µg protein in 30 µl $I = 0.05$ M phosphate buffer pH 7.5.
[d] 9 µg protein in 40 µl $I = 0.003$ M Tris-HCl buffer pH 7.5.
[e] 7 µg protein in 30 µl $I = 0.024$ M Tris-HCl buffer pH 7.5.
[f] 15 µg protein in 35 µl $I = 0.046$ M phosphate buffer pH 7.5.
[g] 13 µg protein in 35 µl $I = 0.006$ M Tris-HCl buffer pH 7.5.
[h] 17 µg protein in 30 µl $I = 0.05$ M phosphate buffer pH 7.5.

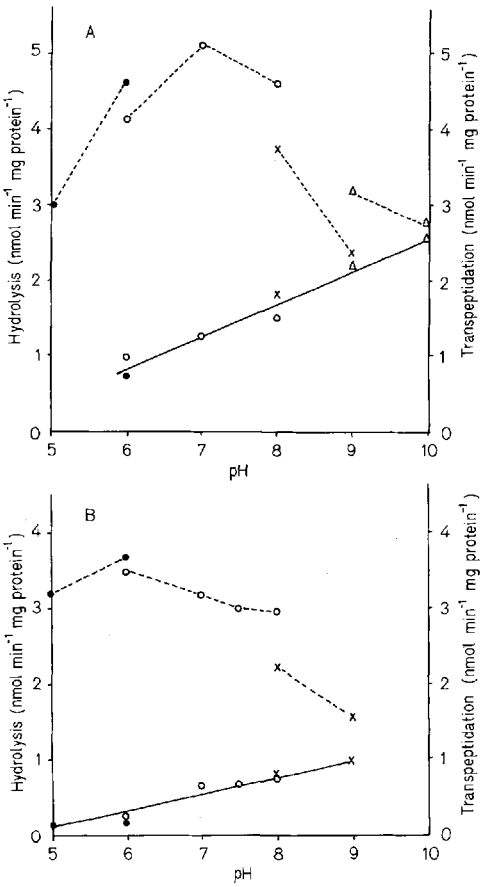

Fig. 1. *Effect of pH on the hydrolysis and transfer activities of the lysozyme-releasable* DD-*carboxypeptidases-transpeptidases from* Streptomyces *K15 (A) and* Streptomyces *R61 (B).* Enzyme activities were assays under standard substrate concentrations (Materials and Methods). DD-Carboxypeptidase activity (----); transpeptidase activity (——). All the buffers used were: $I = 0.02$ M. Cacodylate buffer for pH 5 and 6 (●); phosphate buffer for pH 6–8 (○); Tris-HCl buffer for pH 8 and 9 (×); L-alanine-NaOH for pH 9 and 10 (△) [10]

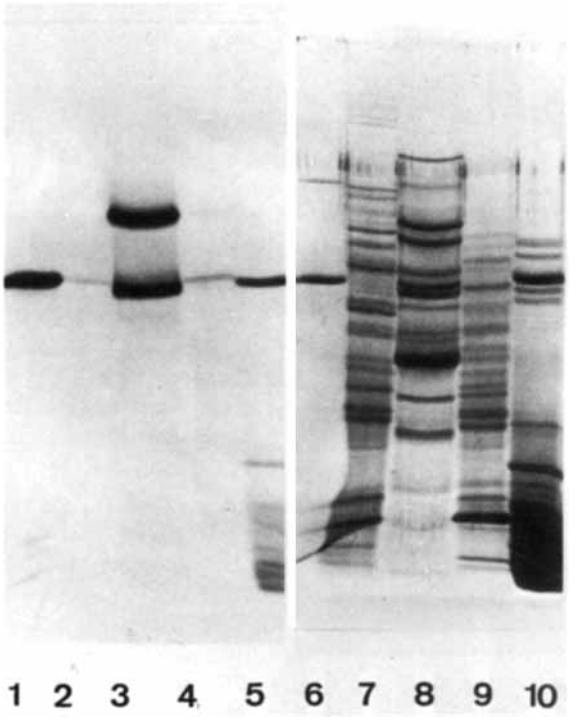

Fig. 2. *Dodecylsulphate slab gel electrophoresis and fluorography of fractions containing* DD-*carboxypeptidase activity from* Streptomyces *strains R61 and K15, after treatment with [^{14}C]benzylpenicillin.* Fractions (20–100 µl) were treated with [^{14}C]benzylpenicillin (40–80 µM) for 10 min at 37 °C. Unlabelled benzylpenicillin (5 µl; 10 mM) was added and the samples prepared for dodecylsulphate electrophoresis [18], with (samples 7–10) or without (sample 6) prior precipitation with 4 vol. of acetone. Proteins were separated by slab gel electrophoresis (10% acrylamide) and the protein components stained with Coomassie brilliant blue (tracks 6–10). The gel was prepared for fluorography (see Materials and Methods) and developed for 2 weeks at −70 °C (tracks 1–5). The samples were not run in adjacent tracks of the gel so the photographs of the stained gel and fluorogram were cut up and re-aligned. Tracks 1 and 6: R61 exocellular enzyme. Tracks 2 and 7: R61 lysozyme-releasable enzyme. Tracks 3 and 8: K15 exocellular enzyme. Tracks 4 and 9: K15 lysozyme-releasable enzyme. Tracks 5 and 10: K15 cetavlon-releasable extract, filtered on G100 Sephadex, fraction K_D 0.35 (carboxypeptidase activity)

each of the exocellular and lysozyme-releasable enzymes from strains R61 and K15 gave rise to a radioactive band migrating at exactly the same position (the R61 exocellular enzyme has a molecular weight of 38000) (Fig. 2). A second benzylpenicillin-binding protein was found only in the K15 exocellular enzyme preparation (M_r approximately 62000) (Fig. 2). The activities of the exocellular enzyme from strain R61 and of the lysozyme-releasable enzymes from strains R61 and K15 were inhibited by 75–80% by the antiserum prepared against the R61 exocellular enzyme [12]. Obviously all these enzymes must be closely related proteins. The activity of the exocellular enzyme preparation from strain K15 was only inhibited by 30%, suggesting that only the penicillin-binding protein of M_r 38000 was a DD-carboxypeptidase immunologically related to the former ones.

In contrast to strains R61 and K15, strain *rimosus* contains a very small amount of lysozyme-releasable DD-carboxypeptidase and it only excretes a very small amount of exocellular DD-carboxypeptidase. The specific DD-carboxypeptidase and transpeptidase activities of the concentrated culture filtrate of strain *rimosus* were 22 and 13 pmol min^{-1} mg $protein^{-1}$ respectively (T/Hy = 0.61). With the lysozyme-releasable enzyme, the T/Hy ratio was 0.34 (in Tris-HCl buffer pH 7.5, $I = 0.01$ M) (Table 1).

MEMBRANE-BOUND ENZYMES

Specific Activities of Isolated Membranes

All the measurements were carried out in 14 mM potassium phosphate buffer pH 7.5. From the data

of Table 1 the following observations were made. (a) In the absence of added cetavlon, the transpeptidase activities of the membranes were high with strain *rimosus* (370 pmol transpeptidation product min^{-1} mg $protein^{-1}$), moderate with strain R61 (37 or 140 pmol min^{-1} mg^{-1} depending upon the method used for the preparation of the membranes) and low with strain K15 (7 or 30 pmol min^{-1} mg^{-1}, also depending upon the method of preparation). In all cases, the specific DD-carboxypeptidase activities were low: from 2 to 6 pmol tripeptide hydrolysed min^{-1} mg^{-1}. (b) Addition of cetavlon to the reaction mixtures (1.5%, final concentrations) caused increased specific transpeptidase activities. The phenomenon was especially noticeable with the membranes from strains K15 and R61. In all cases cetavlon had very little effect on the specific DD-carboxypeptidase activities and the T/Hy ratio values remained high (≈ 50) with the membranes from both strains *rimosus* and R61 and low (≈ 5) with the membranes from strain K15; (c) the data of Table 1 probably do not express the full activities of the isolated plasma membranes. Indeed, the procedure used for their isolation may be damaging for the bound enzymes. Thus, for example, when the membranes of *Streptomyces* strains R61 and K15 were prepared in the absence of Mg^{2+} ions during digestion of the mycelia with lysozyme (see Materials and Methods), they had a specific transpeptidase activity which was 2–4-fold higher than that of the membranes prepared under standard conditions (Table 1). Moreover, there are indications that the isolated membranes contain endogenous substrates that may react with their own bound enzymes. Thus for example, when incubated with the membranes of *Streptomyces* strain R61, the tripeptide [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala was in part hydrolysed into D-Ala and [^{14}C]Ac_2-L-Lys-D-Ala (DD-carboxypeptidase activity) and was in part converted into a radioactive product exhibiting at pH 6.5 an electrophoretic mobility between that of the original substrate and that of the hydrolysed dipeptide. This compound did not occur when the incubation was carried out in the presence of benzylpenicillin and, hence, it is probably a product formed by transpeptidation between [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala and a membrane-endogenous acceptor. Its electrophoretic mobility suggested that glycine might be the endogenous acceptor involved.

Composition of the Membrane-Bound Enzyme System

The low levels of DD-carboxypeptidase activity found in the isolated membranes from the three strains examined may be due to the ability of the transpeptidases to react with water with a low efficiency and/or to the occurrence of distinct DD-carboxypeptidases. Different techniques were used in order to make a choice between the two alternatives. (a) Because of their low T/Hy ratio values, the membranes from strain K15 appeared to be especially suitable for fractionation purposes. Membranes (300 mg protein) were extracted with 1% cetavlon for 45 min at 37 °C in 14 mM phosphate pH 7.5 and after elimination of part of the detergent at 0 °C, the supernatant was concentrated to 2 ml by ultrafiltration on UM 10 Amicon membranes. The extract (containing 66 mg protein) was then filtered on a 120-ml column of Sephadex G-100 in 30 mM phosphate pH 7.5 containing 0.05% cetavlon. The filtration profile thus obtained showed a partial dissociation between DD-carboxypeptidase activity and transpeptidase activity (Fig. 3) demonstrating the presence of two distinct enzymes in the membrane/detergent extract. (b) The anti-exocellular R61 enzyme antiserum had no detectable effect on the transpeptidase activity of the membranes of strain K15 but caused a significant decrease of their DD-carboxypeptidase activity. Neither the transpeptidase nor the DD-carboxypeptidase activities of the membranes of strain R61 and *rimosus* was affected by the antiserum [12].

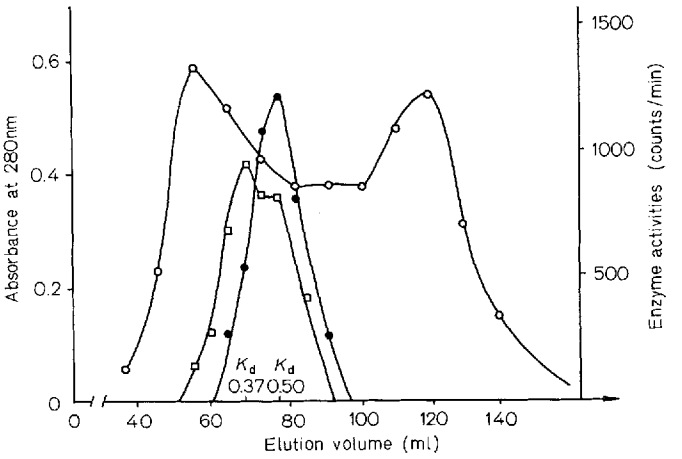

Fig. 3. *Filtration on Sephadex G-100 of a cetavlon extract of membranes of* Streptomyces *strains K15.* Samples (30 µl) of each fraction were incubated either with 4 mM [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala, DD-carboxypeptidase assay (□——□) or with 4 mM Ac_2-L-Lys-D-Ala-D-Ala + 15 mM [^{14}C]Gly-Gly, transpeptidase assay (●——●) for 16 h at 37 °C. The amounts of tripeptide hydrolysed and that of tripeptide transpeptidated are expressed in counts/min. Absorbance at 280 nm (○——○)

From the foregoing it can be concluded that strain K15 contains both a membrane-bound transpeptidase which apparently is not related to the lysozyme-releasable and exocellular enzymes and, in addition to it, a membrane-bound DD-carboxypeptidase which is related to these former enzymes. Obviously, this DD-carboxypeptidase is responsible for the low T/Hy ratio value exhibited by the K15 membranes. The membranes from strains R61 and *rimosus* also contain a transpeptidase similar to that present in strain K15 but the technique failed to reveal the occurrence

of a distinct DD-carboxypeptidase. The membranes isolated from the three *Streptomyces* strains were treated with [^{14}C]benzylpenicillin and analysed by dodecylsulphate slab gel electrophoresis. The results were complex. They will be illustrated and discussed elsewhere (P. E. Reynolds, J. Dusart, M. Leyh-Bouille, and J. M. Ghuysen, unpublished results).

CETAVLON-RELEASABLE ENZYMES

In order to characterize further the cell-bound enzyme systems, the washed mycelia of the three strains studied were directly extracted with cetavlon and the extracts were concentrated (to 50 ml from 15 l of original culture) by ultrafiltration as described in Materials and Methods. Small samples were used for measuring the DD-carboxypeptidase and transpeptidase activities; then the concentrated extracts were divided into 3 samples and each of them was filtered separately at 4 °C on columns of Sephadex G-100 ($V_0 + V_i = 800$ ml) equilibrated against either 33 mM Tris-HCl buffer containing 0.05% cetavlon (strains *rimosus* and R61) or 30 mM phosphate buffer pH 7.5 containing 0.05% cetavlon (strain K15).

Specific Activities of the Mycelia Extracts

From the data of Table 1, the following observations were made.

a) Depending upon the strains, the yields of solubilized transpeptidase activity present in the mycelial extracts (in pmol min^{-1} l original culture^{-1}) were 2–10-fold higher and the specific transpeptidase activities (in pmol min^{-1} mg protein^{-1}) were 5–20-fold higher than those obtained via the isolation of the plasma membranes followed by treatment with cetavlon.

b) With strain *rimosus* (which had very little lysozyme-releasable enzyme), the increased yield of DD-carboxypeptidase activity paralleled that of transpeptidase activity with the result that the mycelial extract had the same high T/Hy ratio value as the isolated membranes (50–60), strongly suggesting that the enzyme solubilized from the mycelium essentially arose from the membranes (and that the isolation of the membranes caused substantial loss of the enzyme activity associated with them).

c) With strains R61 and K15, extraction of the mycelia with cetavlon caused a preferential increase of the DD-carboxypeptidase activity and, consequently, the mycelia extracts had lower T/Hy ratio values than the isolated membranes. These ratio values were 5 in the case of strain R61 (instead of 50) and 0.8 in the case of strain K15 (instead of 5).

d) Treatment of the R61 and K15 lysozyme-releasable enzymes with cetavlon (0.05%, final volume, at 4 °C for 18 h or 2%, final volume, at 37 °C for 45 min) did not cause any loss of activity. Consequently, if one assumed that the lysozyme-releasable enzymes were loosely associated with the mycelial envelope, the cetavlon extracts were expected to have a level of DD-carboxypeptidase activity at least equal to that of the corresponding lysozyme-releasable preparations. Despite the observed increases, the yields of solubilized DD-carboxypeptidase activity in the mycelial extracts from strains K15 and R61 were considerably lower. Hence the lysozyme-releasable enzymes, or at least a major part of them, must escape solubilization by the detergent, suggesting that they are firmly bound to some lysozyme-sensitive structure of the cell envelope, probably to the bacterial wall. Following this interpretation, it seems likely that the DD-carboxypeptidase activity found in the mycelial extracts originates essentially from the plasma membranes.

Fractionation of the Mycelial Extracts

a) With the three strains examined, filtration of the mycelial extract on Sephadex G-100 yielded an enzyme which was eluted at K_D 0.45 and exhibited a high T/Hy ratio value. The active fractions (from the three gel filtrations for each of the extracts) were pooled, concentrated by ultrafiltration and the resulting enzyme solutions were submitted to two additional Sephadex filtrations. Irrespective of the strains, the final preparations thus obtained had similar T/Hy ratio values (13–23; see Table 1) and a 50–70-fold increased specific activity. The yields were 50–60% for strains *rimosus* and R61 and 140% for strain K15. On the basis of the T/Hy ratio values (Table 1), the solubilized and partially purified membrane-transpeptidases from strains R61 and *rimosus* appeared to have a higher capability to react with water (*i.e.* to perform DD-carboxypeptidase activity) than the isolated membranes.

b) The above technique failed to reveal the presence in the extract from strain *rimosus* of any enzyme other than the transpeptidase eluted at K_D 0.45.

c) The first Sephadex filtration of the mycelial extract from strain K15 showed the presence of a second enzyme, which eluted at K_D 0.35 and had a low T/Hy ratio (Fig. 4). The active fractions were pooled, concentrated and submitted to two additional Sephadex filtrations. The enzyme preparation thus obtained had a T/Hy ratio value of 0.15 and a 40-fold increased specific DD-carboxypeptidase activity (Table 1). The yield was about 60%. Dodecylsulphate slab gel electrophoresis and fluorography of the enzyme preparation previously treated with [^{14}C]-benzylpenicillin revealed one single radioactive band. This compound had exactly the same migration as the exocellular and lysozyme-releasable enzymes.

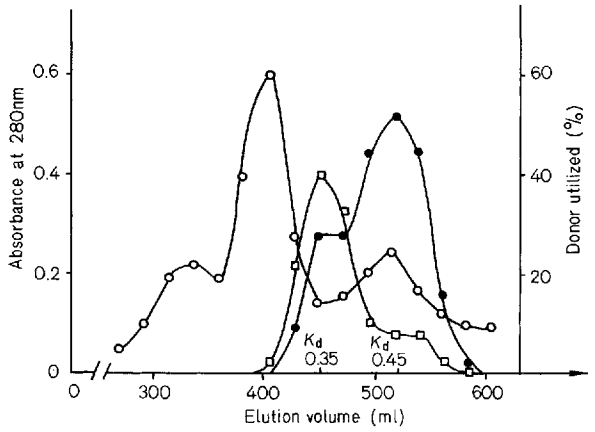

Fig. 4. *Filtration on Sephadex G-100 of the cetavlon mycelial extract from* Streptomyces *K15. Separation of the* DD-*carboxypeptidase and transpeptidase.* Samples (50 µl) of each fraction were incubated with substrate under standard concentration for 10 h at 37 °C, DD-carboxypeptidase activity (□——□); transpeptidase activity (●——●). Absorbance at 280 nm (O—— O)

Table 3. *Antibiotic concentrations required to inhibit by 50% the enzyme activities of the main* DD-*carboxypeptidase* ($K_D = 0.35$) *and the main transpeptidase* ($K_D = 0.45$) *isolated from the cetavlon mycelium extract of* Streptomyces *strain K15*

Samples of each enzyme preparation [*i.e.* (a) 15 µg protein for the inhibition of either the DD-carboxypeptidase activity of the main DD-carboxypeptidase enzyme preparation or the transpeptidase activity of the main transpeptidase enzyme preparation; and (b) 60 µg protein for the inhibition of either the transpeptidase activity of the main DD-carboxypeptidase enzyme preparation or the DD-carboxypeptidase activity of the main transpeptidase enzyme preparation] 7 mM Ac_2-L-Lys-D-Ala-D-Ala alone (DD-carboxypeptidase activity) or 5 mM Ac_2-L-Lys-D-Ala-D-Ala + 7 mM [^{14}C]-Gly-Gly (transpeptidase activity) and increasing antibiotic concentrations were incubated together in 35 µl (final volume) of 20 mM phosphate pH 7.5, at 37 °C for 30 min (a) or 30–60 min (b)

Antibiotic	Main DD-carboxypeptidase ($K_D = 0.35$) inhibition		Main transpeptidase ($K_D = 0.45$) inhibition	
	DD-carboxypeptidase activity	transpeptidase activity	DD-carboxypeptidase activity	transpeptidase activity
	µM			
Benzylpenicillin	0.4	0.6	50	30
Carbenicillin	3	6	1000	300

Finally, the possibility that the low transpeptidase activity of the main DD-carboxypeptidase (eluted at a K_D value of 0.35) and the low DD-carboxypeptidase activity of the main transpeptidase (eluted at a K_D value of 0.45) might be due to cross-contamination, was ruled out by the fact that the transpeptidase and DD-carboxypeptidase activities of the main DD-carboxypeptidase were much more sensitive to benzylpenicillin and carbenicillin than the DD-carboxypeptidase and transpeptidase activities of the main transpeptidase (Table 3).

d) With the mycelial extract obtained from strain R61, analysis of the fractions obtained after the first Sephadex filtration revealed the presence of an enzyme eluted at a low 0.15 K_D value and exhibiting a low T/Hy ratio value. The yield, however, was low (about 10%) and this enzyme was lost in the course of the subsequent gel filtration.

Properties of the Cetavlon-Extracted and Partially Purified Enzyme Preparations

The cetavlon-extracted and partially purified enzymes had a broad pH optimum between 6 (cacodylate buffer) and 8 (phosphate or Tris-HCl buffers). The ionic strength (between 0.01 and 0.1 M) had no effect on the activities of the transpeptidase and the DD-carboxypeptidase from strain K15. The transpeptidases from both strains R61 and *rimosus* had maximal activities at $I = 0.01$ M or less. From Lineweaver-Burk plots $1/v$ *vs* $1/[Ac_2$-L-Lys-D-Ala-D-Ala] (DD-carboxypeptidase assay), the K_m and V values shown in Table 2 were obtained.

DISCUSSION

Streptomyces R61 and K15, but not *Streptomyces rimosus*, excrete during growth large amounts of DD-carboxypeptidase capable of performing low transpeptidase activity (in aqueous media and at low acceptor concentrations). Parallel to this, *Streptomyces* R61 and K15, but not *Streptomyces rimosus*, possess substantial amounts of lysozyme-releasable DD-carboxypeptidases capable of performing transpeptidase activity with low efficiency. The exact cellular location of the lysozyme-releasable enzymes is not known. However, they appear to be firmly bound to some lysozyme-sensitive structure of the cell envelope, perhaps the wall peptidoglycan. The enzymatic transformation of the mycelia into protoplasts in a sucrose medium is the procedure of choice for their release. Exocellular and lysozyme-releasable enzymes are very similar proteins: they exhibit the same molecular weight and they are immunologically related to each other. At present, *Micrococcus lysodeikticus* is the only bacterium known which also contains a DD-carboxypeptidase that can be released into the protoplasting medium [19].

Streptomyces rimosus contains very low levels of both lysozyme-releasable and exocellular enzymes, suggesting that the less lysozyme-releasable enzyme present, the lower is the amount of exocellular enzyme excreted in the culture medium.

The isolated membranes of the three *Streptomyces* strains contain a transpeptidase capable of performing DD-carboxypeptidase activity with low efficiency. The transpeptidase activity is not affected by the anti-exocellular R61 enzyme antiserum. The membrane-bound transpeptidases can be extracted directly from the mycelia with cetavlon and substantially purified by Sephadex filtration.

In addition to the membrane-bound transpeptidase, *Streptomyces* K15 possesses a membrane-bound DD-carboxypeptidase (capable of performing low transpeptidase activity), which is immunologically related to the lysozyme-releasable and exocellular enzymes. This enzyme can also be extracted directly from the mycelium with cetavlon and substantially purified by Sephadex filtration. Its K_D value is lower than that of the transpeptidase. As shown by dodecylsulphate slab gel electrophoresis of the complex formed with [^{14}C]benzylpenicillin, it has the same molecular weight as the lysozyme-releasable and exocellular enzymes. *Streptomyces* R61 may also possess some membrane-bound DD-carboxypeptidase but the activity was found to be labile and the corresponding enzyme could not be isolated. *Streptomyces rimosus* does not possess any detectable membrane-bound DD-carboxypeptidase.

It seems highly probable that in *Streptomyces* K15, the membrane-bound, lysozyme-releasable and exocellular DD-carboxypeptidase enzymes are members of a single pathway leading to enzyme excretion. Their possible relationship with the membrane-bound transpeptidase remains unknown.

The present study has been carried out with cells collected after 72 h of growth for the exocellular enzymes and after 40 h of growth for both lysozyme-releasable and membrane-bound enzymes. A time course experiment might be revealing with regard to the possible relationship between these three types of enzymes of the peptide crosslinking enzyme system in *Streptomyces* sp. One may postulate that basically a similar situation occurs in all *Streptomyces* but that, depending upon the strain and/or the stage of the mycelial development, large variations in the amounts of enzymes present may occur with the result that some of them may be beynd the limits of detection. The membrane-bound transpeptidase, however, is always detectable.

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