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Membrane-Bound DD-Carboxypeptidase and LD-Transpeptidase of *Streptococcus faecalis* ATCC 9790

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Isolated membranes of *Streptococcus faecalis* ATCC 9790 exhibit DD-carboxypeptidase activity (standard reaction: Ac_2-L-Lys-D-Ala-D-Ala → D-alanine + Ac_2-L-Lys-D-Ala) and LD-transpeptidase activity (standard reaction: Ac_2-L-Lys-D-Ala + acceptor → D-alanine + Ac_2-L-Lys-acceptor). The DD-carboxypeptidase activity has a considerable specificity for peptides with a C-terminal L-R_3-D-Ala-D-Ala sequence where R_3 is an amino acid residue and a long side-chain at the L-R_3 position. A corresponding DD-transpeptidation reaction yielding the product Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala from the system Ac_2-L-Lys-D-Ala-D-Ala + D-[^{14}C]alanine was not detected. The LD-transpeptidase activity has a considerable specificity for peptide donors that have an N^{α}-substituted, C-terminal L-R_3-D-Ala sequence with a free ω-amino group at the end of a long side-chain at the L-R_3 position, and a considerable specificity for amino group acceptors that are located on a D-carbon in α-position to a free carboxyl group. In the absence of acceptor, hydrolysis of the dipeptide Ac_2-L-Lys-D-Ala (LD-carboxypeptidase activity) was not observed. Both DD-carboxypeptidase and LD-transpeptidase activities are inhibited by β-lactam antibiotics, but their relative sensitivity differs according to the particular antibiotic used.

Three enzyme activities, transpeptidase, DD-carboxypeptidase and LD-carboxypeptidase are involved in the synthesis and the control of the crosslinking between the peptide units during the biosynthesis of the bacterial wall peptidoglycans. (a) The transpeptidase activity catalyses the transfer of the penultimate C-terminal D-alanine residue of a peptide donor ending in a L-R_3-D-Ala-D-Ala sequence, to the ω-amino group at the L-R_3 position of a peptide acceptor of the same composition [1,2]. N^{ω}-(D-Ala)-L-R_3 interpeptide bonds are formed (at least in peptidoglycans of chemotypes I, II and III [3]) and equivalent amounts of D-alanine are released from the peptide donor. This transpeptidation reaction makes the peptidoglycan network insoluble. (b) The DD-carboxypeptidase activity (also called carboxypeptidase I) catalyses the hydrolysis of the C-terminal D-alanyl-D-alanine peptide bond without concomitant transfer reaction [4]. This activity probably limits the number of peptide donors available for transpeptidation. The relationship between transpeptidase and DD-carboxypeptidase activities is still a matter of speculation. Thus far, no membrane-bound transpeptidase has ever been isolated, purified and characterized. Strains of *Streptomyces*, however, excrete during growth single polypeptide enzymes which catalyse concomitant hydrolysis (DD-carboxypeptidase activity) and transfer (transpeptidase activity) reactions and the proportion of enzyme activity that can be channelled into either pathway depends upon the experimental conditions [5–7]. It is thought that these enzymes might be the exoforms of the membrane-bound transpeptidases. DD-Carboxypeptidases which seem to be unable to catalyse transfer reactions and which therefore function as simple hydrolases also exist [8–10]. They may be "uncoupled" transpeptidases lacking the binding site for peptide acceptors. (c) The LD-carboxypeptidase activity (also called carboxypeptidase II) catalyses the hydrolysis of the C-terminal L-R_3-D-alanine peptide bond exposed by prior action of a DD-carboxypeptidase [8]. Based on the known primary structures of the wall peptidoglycans [3,11], there was no indication until recently (*vide infra*) that LD-carboxypeptidases might function as LD-transpeptidases and catalyse transfer reactions *in vivo*. The possible physiological function of this activity is therefore obscure.

The present paper describes investigations carried out on the membrane-bound, peptide crosslinking

Abbreviations. The nomenclature of the amino acids is according to the rules of the IUPAC-IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem. 27*, 201–207 (1972).

system in *Streptococcus faecalis* ATCC 9790 (*S. faecium*). In this organism, a major portion of the peptide moiety of the wall peptidoglycan is composed of N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine tetrapeptides that are crosslinked through single D-isoasparaginyl residues extending from the C-terminal D-alanine of one tetrapeptide to the N^{ϵ}-L-lysine of another [12]. The bonds made by transpeptidation are thus N^{α}-(D-alanyl)D-isoasparaginyl linkages. Evidence for the presence of a heretofore unknown LD-transpeptidase activity in the isolated membranes of this organism will be presented.

MATERIALS AND METHODS

Growth Conditions

Growth medium contained per l: 10 g yeast extract Difco; 10 g Bactopeptone Difco; 0.442 g KH_2PO_4; 0.305 g K_2HPO_4; 25.65 g Na_2HPO_4; 16.45 g NaH_2PO_4; 20 g glucose. *S. faecalis* was grown at 37 °C without shaking in 1-l flasks containing 500 ml medium.

Plasma Membranes

Unless otherwise stated, all the operations were carried out at 4 °C. Cells of *S. faecalis* (6 l of culture near the end of the exponential growth phase; absorbance at 550 nm = 1.0) were collected by centrifugation, washed once with 500 ml 40 mM ammonium acetate buffer pH 7 and suspended in 150 ml of the same buffer supplemented with 0.5 M sucrose, 1 mM magnesium acetate and 133 μg/ml (final concentration) of hen's egg-white lysozyme. The suspension was maintained at 37 °C for about 1 h. During the incubation, 20-μl samples were diluted with 500 μl 1 mM sodium phosphate buffer pH 7 and the absorbance at 550 nm was measured. Maximum osmotic fragility was considered to be attained when the absorbance of the diluted suspension was 5—10% of its original value. The protoplasts were collected by centrifugation at 40000 × *g* for 1 h and suspended in 30 ml 1 mM phosphate buffer pH 7. To the suspension 30 ml water, 120 μl 1 M magnesium acetate and a few crystals of DNAse and RNAse were added and the suspension was homogenized and maintained at room temperature for 10—15 min. The lysate (checked for the presence of intact cells or protoplasts by phase-contrast microscopy) was centrifuged at 40000 × *g* for 1 h and the pellet was suspended in 60 ml 1 mM phosphate buffer pH 7 supplemented with 2 mM magnesium acetate. The suspension was first centrifuged at 3000 × *g* for 10 min to remove debris and intact cells and then at 40000 × *g* for 1 h: the procedure yielded about 180 mg membrane protein (as estimated by the Lowry technique [13]). Membranes were suspended in water (about 20 mg protein/ml) and stored in 0.5-ml aliquots at −20 °C. No loss of activity was observed after months of storage.

Amino Acids

The non-radioactive amino acids were purchased from Merck, Calbiochem, Fluka AG and General Biochemical Inc. Monoacetyl-LL-diaminopimelic acid was prepared as described in [14]. D-[^{14}C]Alanine (48.5 mCi/mmol) and [^{14}C]glycine (113 mCi/mmol) were purchased from Radiochemical Center (Amersham, U.K.).

Peptides

All peptides, except those listed below were used in previous studies [7, 9, 14—17]. Gly-Gly was purchased from E. Merck. D-Ala-D-Ala, L-Ala-D-Ala and D-Ala-L-Ala were purchased from Cyclo Chemical Corp. D-Ala-Gly, Gly-D-Ala, L-Lys-D-Ala, αAc-L-Lys-D-Ala, Ac_2-L-Lys-D-Ala, Ac_2-L-Lys-Gly, Ac_2-L-Lys-L-Ala, Ac_2-L-Lys-D-Leu, Ac-Gly-D-Ala, Ac-D-Ala-D-Ala, Ac-L-Ala-D-Ala, Ac_2-L-A_2bu-D-Ala and Ac_2-L-Orn-D-Ala were prepared as described in [18] and the dipeptide Ac_2-L-Lys-D-Ala radioactively labelled with ^{14}C in the acetyl groups (specific activity: 8.36 mCi/mmol), as described in [14].

The disaccharide pentapeptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine was prepared by removing the C-terminal D-alanine residue from the corresponding D-alanyl-D-alanine-ending disacharide hexapeptide [15] by treatment with the R39 DD-carboxypeptidase-transpeptidase [7] in 0.05 M Tris-HCl buffer pH 7.5, under conditions where the enzyme functions as a hydrolase. A mixture of N^{α}-(lactyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(β- and α-D-aspartyl)-L-lysyl-D-alanine, in which the β-isomer greatly predominated, was prepared by treating the above disaccharide pentapeptide with 0.05 N NaOH at 37 °C under conditions known to catalyse the β-elimination of the lactylpeptide together with the deamidation of the isoasparaginyl residue and the interconversion of the peptide into a mixture of N^{ϵ}-(β- and α-aspartyl)-lysyl peptides [12]. The two lactylpeptides were not separated from each other but were purified by paper electrophoresis at pH 6.5 (at this pH, both are anionic and equally charged).

Estimation of Free Amino Acids

This was made by using the fluorodinitrobenzene technique [19] without prior elimination of the membranes from the reaction mixtures. Free D-alanine was also estimated enzymatically. Membrane-containing reaction mixtures (20—40 μl) were diluted with water to 100 μl final volumes, centrifuged at 20000 × *g* for 20 min and the D-alanine present in 50-μl supernatant samples was estimated with D-amino acid oxidase [19] as described in [20].

Table 1. *Inhibition of growth of* S. faecalis *(minimal inhibitory concentration or MIC values) and of membrane-bound* DD*-carboxypeptidase and* LD*-transpeptidase (ID_{50} values) by β-lactam antibiotics (in μM)*
For determination of minimal inhibitory concentration and ID_{50} values, see text

Group	Antibiotics	MIC values	DD-Carboxypeptidase		LD-Transpeptidase	
			ID_{50}	ID_{50}/MIC	ID_{50}	ID_{50}/MIC
		μM			μM	
I	Penicillin V	1	1	1	140	140
	Benzylpenicillin	2	1.2	0.6	190	95
	Ampicillin	10	6	0.6	2400	240
	Carbenicillin	25	40	1.6	1200	48
II	Methicillin	250	1070	4.3	280	1.1
	6-Aminopenicillinic acid	1000	800	0.8	3400	3.4
III	Cephalothin	12.5	190	15	46	3.7
	Oxacillin	25	740	30	180	7
	Cloxacillin	50	420	8.4	40	0.8
	Cephalexin	250	12500	50	970	4
IV	Cephaloglycin	225	850	3.8	15	0.067
	Cephalosporin C	2000	3000	1.5	230	0.11

Estimation of Radioactive Compounds

To each membrane-containing reaction mixture (40 μl) 10 μl of a 20% (w/v) Triton X-100 solution was added and the entire volume was spotted on a strip of Whatman No. 3 MM paper. Radiocative compounds were separated from each other by electrophoresis at pH 6.5 (collidine—acetic acid—water, 9.1:2.65:1000, v/v/v) or at pH 2 (formic acid 0.5 N) for 2 to 4 h at 60 V/cm, using a Gilson high voltage (10000 V) electrophorator model DW. The radioactive compounds were located on the strips using a Packard radiochromatogram scanner and the radioactivity was estimated as described in [7].

Antibiotics

D-Cycloserine was purchased from Calbiochem. The various β-lactam antibiotics were those previously used [21].

Minimal Inhibitory Concentrations

Minimal inhibitory concentrations values were determined by inoculating tubes containing 6 ml broth (see Growth Conditions in Materials and Methods) and various concentrations of each antibiotic with 0.1 ml of a 1/1000 dilution of a late log phase culture of *S. faecalis*. The minimal antibiotic concentrations which prevented growth were recorded after 18 h of incubation at 37 °C (stationary cultures). The value for each antibiotic is given in Table 1.

RESULTS

The Membrane-Bound DD*-Carboxypeptidase Activity*

Standard Reaction Catalysed. When exposed to the isolated membranes, the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was hydrolysed into D-alanine and the dipeptide Ac_2-L-Lys-D-Ala.

Optimal Conditions for Activity. The tripeptide Ac_2-L-Lys-D-Ala-D-Ala (1.5 mM, final concentration) was incubated with 125 μg membrane proteins (final volumes, 40 μl) at 37 °C for 30 min under various conditions, after which time free D-alanine was estimated. In buffers of 0.01 ionic strength, the rate of the reaction was maximal at pH 6 in sodium cacodylate buffer or at pH 7 in sodium barbital buffer (Fig. 1). At pH 6, the rate of the reaction was maximal in 50 mM cacodylate buffer (Fig. 2). Under these latter conditions, EDTA (up to 10 mM), $MgCl_2$, $MnCl_2$ and $CaCl_2$ (5 mM) had no effect; $ZnSO_4$ and $FeSO_4$ (5 mM) decreased the activity by 35%; Triton X-100 at concentrations higher than 0.1% (w/v) enhanced it (Fig. 3).

DD-Carboxypeptidase Unit. One unit (U) catalysed the degradation of 1 μmol Ac_2-L-Lys-D-Ala-D-Ala into D-alanine and Ac_2-L-Lys-D-Ala per min at 37 °C when 38 mM tripeptide (*i.e.* 10 × the K_m value, *vide infra*) was incubated with the membranes in 50 mM sodium cacodylate buffer pH 6. Specific activity of the isolated membranes was about 16 DD-carboxypeptidase mU per mg protein.

Specificity. The substrate requirements of the membrane-bound DD-carboxypeptidase were studied on peptides of the general type X-L-R_3-R_2-R_1 (OH) where R_3, R_2 and R_1 are amino-acid residues and X an N^{α}-substituent of the R_3 residue, in 20 μl (final volumes) of 50 mM sodium cacodylate buffer pH 6 (Table 2). The rate of hydrolysis (liberation of the R_1 residue) was measured at a fixed, 3-mM concentration of peptide. For each peptide, the amount of membrane and the incubation time at 37 °C were such that less than 25% of the tripeptide was hydrolysed (Table 2).

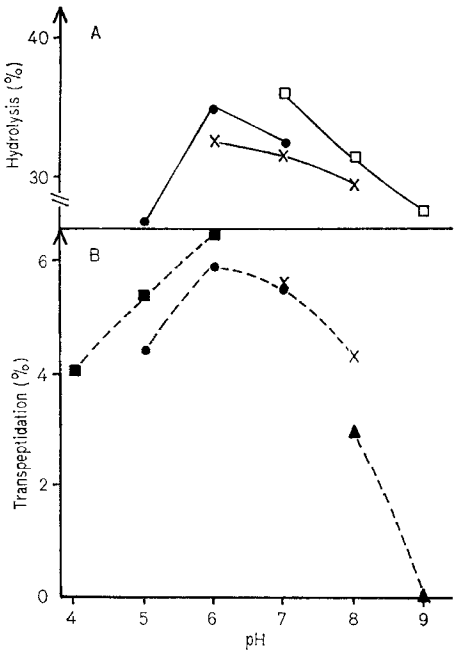

Fig. 1. *Effects of pH on (A) DD-carboxypeptidase and (B) LD-transpeptidase activities.* For conditions, see text: the buffers (0.01 ionic strength) were: (●——●) cacodylate; (×——×) phosphate; (□——□) barbital; (■——■) acetate; (▲----▲) Tris. For DD-carboxypeptidase, results were expressed as percentage of Ac_2-L-Lys-D-Ala-D-Ala hydrolysed. For LD-transpeptidase, results were expressed as percentage of Ac_2-L-Lys-D-Ala converted into Ac_2-L-Lys-D-[^{14}C]Ala

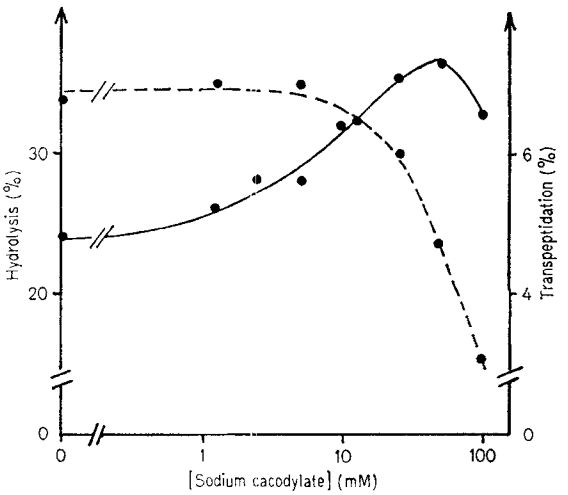

Fig. 2. *Effects of concentration of sodium cacodylate buffer pH 6 on (●——●) DD-carboxypeptidase and (●----●) LD-transpeptidase activities.* For conditions, see text. Results were expressed as in Fig. 1

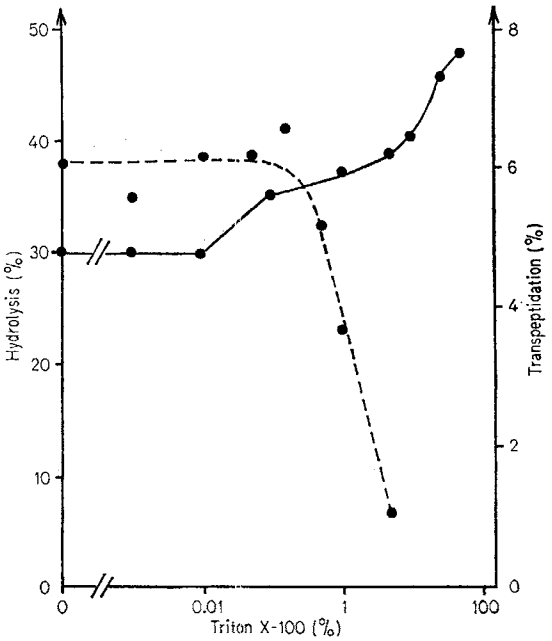

Fig. 3. *Effects of Triton X-100 at pH 6 on (●——●) DD-carboxypeptidase and (●----●) LD-transpeptidase activities.* For conditions, see text. Results were expressed as in Fig. 1

The *S. faecalis* membrane-bound DD-carboxypeptidase showed a considerable specificity for peptides with a C-terminal L-R_3-D-alanyl-D-sequence (compare peptides no. 1—7) and a long side-chain at the L-R_3 position (compare peptides no. 8—11). Introduction of a charged amino group at the end of the L-R_3 side-chain slightly increased the rate of reaction (compare peptides no. 1 and 12). The nature of these N^{ω}-terminal groups as well as the nature of the N^{α}-substituent of the L-R_3-D-Ala-D-Ala sequence (the X residue or groups) seemed to exert little influence (compare peptides no. 13, 15, 16, 17). The diacetylated pentapeptide no. 14, however, was a poor substrate. Interestingly, the disaccharide hexapeptide no. 17, in which the peptide moiety was that of the wall peptidoglycan precursor of *S. faecalis* (see Introduction), was an excellent substrate, whereas the dimer no. 18 was hydrolysed more slowly.

K_m and V Values. The K_m (in mM) and V (in $\mu mol \times mg$ membrane protein$^{-1} \times min^{-1}$) were determined for a few peptides (in the absence of Triton X-100) on the basis of initial velocity measurements. Typical Michaelis-Menten kinetics were observed. K_m values were 3.8, 7.5 and 1.45 mM for peptides 1, 14 and 17 (Table 2), respectively. V values were 0.020 $\mu mol \times mg^{-1} \times min^{-1}$ for these three peptides.

Effects of β-Lactam Antibiotics (ID_{50} values). Ac_2-L-Lys-D-Ala-D-Ala (3 mM, final concentration) and membranes (250 µg protein) were incubated in 20 µl (final volumes) of 50 mM sodium cacodylate buffer pH 6 for 45 min at 37 °C, in the absence and in the presence of various concentrations of antibiotics. In the absence of antibiotic, 90% of the tripeptide was hydrolysde. The concentrations of

Table 2. *Specificity of the* S. faecalis *DD-carboxypeptidase*
Release of the R_1 residue from peptides X-L-R_3-R_2-R_1(OH). For conditions, see text. Peptide concentrations were 3 mM. Origin of the peptides: No. 8, see Materials and Methods; No. 14, see [16]; No. 17 and 18, see [15]; all the other peptides, see [9]. Disaccharide: β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl

No.	Peptides	Activity
		nmol × mg protein^{-1} × min^{-1}
1	Ac_2-L-Lys-D-Ala-D-Ala	7.60
2	Ac_2-L-Lys-D-Ala-Gly	1.10
3	Ac_2-L-Lys-D-Ala-D-Leu	0.07
4	Ac_2-L-Lys-D-Ala-L-Ala	0
5	Ac_2-L-Lys-Gly-D-Ala	0.09
6	Ac_2-L-Lys-D-Leu-D-Ala	0
7	Ac_2-L-Lys-L-Ala-D-Ala	0
8	D-Ala-D-Ala	0
9	Ac-L-Ala-D-Ala-D-Ala	0.40
10	Ac_2-L-A_2bu-D-Ala-D-Ala	1.65
11	Ac_2-L-Orn-D-Ala-D-Ala	4.50
12	N^{α}-Ac-L-Lys-D-Ala-D-Ala	8.85
13	D-Glu ┐— L-Lys-D-Ala-D-Ala └	9.42
14	Ac-L-Ala-D-Glu ┐— L-Lys-D-Ala-D-Ala └ | Ac	1.30
15	L-Ala-D-αGln ┐— L-Lys-D-Ala-D-Ala └ └— $(Gly)_5$	14.10
16	Disaccharide-L-Ala-D-αGln ┐— L-Lys-D-Ala-D-Ala └ └— $(Gly)_5$	9.40
17	Disaccharide-L-Ala-D-αGln ┐— L-Lys-D-Ala-D-Ala └ | D-αAsn	11
	Disaccharide-L-Ala-D-αGln ┐— L-Lys-D-Ala-D-Ala └ |	
18	Disaccharide-L-Ala-D-αGln ┐— L-Lys-D-Ala-D-αAsn └ | D-αAsn	3.20

antibiotics which inhibited the enzymic activity by 50% (ID_{50} values) were listed in Table 1 and compared with the minimal inhibitory concentration values. The ID_{50} values ranged from 1—2 μM (benzylpenicillin and penicillin V) to 12.5 mM (Cephalexin).

Assays of Transfer Reaction. Attempts to catalyse transfer reactions with the *S. faecalis* membranes were undertaken under the following conditions. The tripeptide Ac_2-L-Lys-D-Ala-D-Ala (1.5 mM) was incubated with 500 μg membrane protein at 37 °C in 40 μl 50 mM sodium cacodylate buffer pH 6, in the absence and in the presence of various concentrations of D-[^{14}C]alanine (up to 30 mM). In the absence of D-[^{14}C]alanine, hydrolysis of the tripeptide was complete in about 45 min. In the presence of D-[^{14}C]alanine, concomitant hydrolysis and transfer reactions occurred. However, the product of the transpeptidation reaction was not the expected Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala, but was the dipeptide Ac_2-L-Lys-D-[^{14}C]Ala. Using a molar ratio of tripeptide to D-[^{14}C]alanine of 1 to 5, 6% of tripeptide was converted into radioactive dipeptide after 18 h of incubation. The formation of this radioactive dipeptide resulted from a sequence of two reactions. First, the tripeptide was hydrolysed into D-alanine and the dipeptide Ac_2-L-Lys-D-Ala by the action of the DD-carboxypeptidase. Second, the Ac_2-L-Lys residue was transferred from this latter dipeptide to D-[^{14}C]alanine by an LD-transpeptidase.

The Membrane-Bound LD-Transpeptidase Activity

Standard Reaction Catalysed. Incubation of Ac_2-L-Lys-D-Ala and D-[^{14}C]alanine with the membranes resulted in the formation of the radioactive dipeptide Ac_2-L-Lys-D-[^{14}C]Ala. In the absence of D-[^{14}C]-alanine, the dipeptide was not hydrolysed.

Optimal Conditions for Activity. Dipeptide Ac_2-L-Lys-D-Ala (1.5 mM, final concentration) and D-[^{14}D]alanine (specific activity: 1 mCi/mmol; 5 mM,

Table 3. *Specificity of the* S. faecalis *LD-transpeptidase for peptide donors*
Transpeptidation between non-radioactive peptide donors and 5 mM D-[^{14}C]alanine acceptor is measured as a percentage of D-[^{14}C]alanine incorporated. For conditions of incubation and electrophoresis, see text. The transpeptidation products with peptides 1 to 3 and 5 to 9 were anionic at pH 6.5. The products with peptides 10, 14 and 15 were cationic at pH 2. Origin of peptides: No. 1—13 and 15, see Materials and Methods; No. 14, see [17]

No.	Peptide donors	Transpeptidation for peptide donor/D-[^{14}C]alanine molar ratios of:			Mobility of transpeptidation product relative to	
		1 to 1	5 to 1	10 to 1	Ac_2-L-Lys-D-Ala at pH 6.5	D-Alanine at pH 2
		%				
1	Ac_2-L-Lys-D-Ala	14	42	53	1	—
2	Ac_2-L-Lys-Gly	1	1.8	2.3	1	—
3	Ac_2-L-Lys-L-Ala	0.4	3.0	4.4	1	—
4	Ac_2-L-Lys-D-Leu	0	0	0	—	—
5	Ac_2-L-Orn-D-Ala	3.3	—	—	1	—
6	Ac_2-L-A_2bu-D-Ala	1.3	2.2	—	1.1	—
7	Ac-L-Ala-D-Ala	3	14	26	1.4	—
8	Ac-D-Ala-D-Ala	1.3	4.4	10	1.4	—
9	Ac-Gly-D-Ala	—	1.0	1.6	1.49	—
10	αAc-L-Lys-D-Ala	53	82	—	—	0.81
11	L-Lys-D-Ala	0	0	0	—	—
12	L-Ala-D-Ala	0	0	0	—	—
13	D-Ala-D-Ala	0	0	0	—	—
14	Disaccharide-L-Ala-D-αGln ┌—L-Lys-D-Ala	38	64	—	—	0.4
15	Disaccharide-L-Ala-D-αGln ┌—L-Lys-D-Ala D-αAsn	32.5	—	—	—	0.4

final concentration) were incubated with 500 μg membrane proteins in 40 μl (final volumes) for 18 h at 37 °C under various conditions. The transpeptidation product Ac_2-L-Lys-D-[^{14}C]Ala and the excess of D-[^{14}C]alanine were separated by paper electrophoresis and the radioactive dipeptide was estimated (Materials and Methods). In buffers of 0.01 ionic strength, the rate of the reaction was maximal at pH 6 in sodium cacodylate (Fig. 1). At pH 6, the rate of the reaction decreased at concentrations of sodium cacodylate higher than 10 mM (Fig. 2). In 10 mM sodium cacodylate buffer pH 6, the addition of 1 mM EDTA resulted in a 30% increased activity; the addition of Triton X-100 at concentrations higher than 0.2% (w/v) strongly inhibited the activity (Fig. 3). $CaCl_2$, $MgCl_2$ and $FeSO_4$ (up to 10 mM) had no effect on the reaction while 0.02 mM $CuSO_4$, 0.2 mM $MnCl_2$ and 0.4 mM $ZnSO_4$ decreased the activity by 50%.

Specificity for Peptide Donors. The substrate requirements of the membrane-bound LD-transpeptidase for peptide donors were studied on peptides of the general type X-L-R_3-R_2(OH) by using D-[^{14}C]alanine as acceptor. The general reaction catalysed was X-L-R_3-R_2(OH) + D-[^{14}C]alanine → R_2 + X-L-R_3-D-[^{14}C]Ala. A fixed concentration of D-[^{14}C]alanine (5 mM, final concentration) and various concentration of each peptide donor (5, 25 and 50 mM) were incubated with 1 mg membrane protein for 18 h at 37 °C in 40 μl (final volume) of 10 mM sodium cacodylate buffer pH 6 supplemented with 1 mM EDTA. The transpeptidation products X-L-R_3-D-[^{14}C]Ala were estimated and the yields of the reactions were expressed as percentage of D-[^{14}C]alanine incorporated into products (Table 3). The *S. faecalis* LD-transpeptidase showed a considerable specificity for peptide donors with a C-terminal L-R_3-D-Ala sequence and a long side-chain at the L-R_3 position. Replacement of the C-terminal D-alanine residue in the standard dipeptide Ac_2-L-Lys-D-Ala (peptide no. 1 in Table 3) by glycine, L-alanine or D-leucine (peptides no. 2, 3 and 4, respectively) resulted in a much decreased effectiveness or in a complete lack of donor function. A similar effect was observed when the length of the side-chain of the L-R_3 residue was decreased (compare peptide no. 1 with peptides no. 5, 6 and 7). Replacement of the L-alanine residue in the dipeptide Ac-L-Ala-D-Ala by D-alanine or glycine also resulted in a decreased donor function (peptides no. 7—9). The introduction of a charged amino group at the end of the L-R_3 side-chain (peptides no. 10, 14, 15) markedly increased the rate of the reaction. The nature of the N^{α}-substituent of the L-R_3-D-Ala peptide seemed to exert little influence (peptides no. 10, 14, 15). However, N^{α}-unsubstituted dipeptides such as L-Lys-D-Ala, L-Ala-D-Ala or D-Ala-D-Ala

Table 4. *Specificity of the* S. faecalis *LD-transpeptidase for acceptors: transpeptidation between amino acid and peptide acceptors and 3 mM Ac_2-L-Lys-D-Ala donor*
For conditions of incubation and electrophoresis, see text. All transpeptidation products were anionic except with peptides no.7 and 13. Origin of the peptides: No. 1 to 18 and 25, see Materials and Methods; No. 19, 20, see [14]; No. 21 to 24, see [7]

No.	Acceptors	Transpeptidation at acceptor/Ac_2-L-Lys-D-Ala molar ratios of:			Mobility of transpeptidation product relative to Ac_2-L-Lys-D-Ala
		1 to 1	5 to 1	10 to 1	
		%			
1	D-Alanine	6.6	7.6	8.3	1.00
2	D-Alanine (+ 30 mM D-cycloserine)	8.8	12	22	1.00
3	L-Alanine	3.5			1.00
4	L-Alanine (+ 30 mM D-cycloserine)	0			1.00
5	D-Aspartic acid	0.75	0.9	2.2	1.65
6	L-Aspartic acid	0	0	0	
7	D-Lysine	8.8	10.7	10.8	−0.15
8	L-Lysine	0	0	0	
9	*meso*-Diaminopimelic acid	4.7	7.6	8	0.81
10	LL-Diaminopimelic acid	0	0		
11	Ac-LL-A_2pm	0	0		
12	Glycine	2.3	5	7	1.00
13	Glycinamide	0.5	1.7	3.2	−0.12
14	D-Cycloserine	0	0.7	1.9	0.73
15	D-Ala-L-Ala			0	
16	D-Ala-Gly			0	
17	Gly-D-Ala			0	
18	Gly-Gly			0	
19	Ac-L-Lys(Gly)			0	
20	Ac-LL-A_2pm(Gly)			0	
21	L-Ala-D-Glu(L-A_2pm(D))	1.0	2.0		1.13
22	L-Ala-D-αGln(L-A_2pm(D))-D-Ala	2.2	2.8		0.55
23	Disaccharide-L-Ala-D-Glu(L-A_2pm(D))-D-Ala	0.8	2.0		1.15
24	L-Ala-D-Glu(L-A_2pm(D)(amide))	0	0		
25	Lactyl-L-Ala-D-αGln(L-Lys-D-Ala)(D-Asp)	0	0.5		1.15

(peptides no. 11—13) were not utilized at all by the enzyme. Among the best substrates was the major disaccharide peptide unit (peptide no. 15) found in the wall peptidoglycan of *S. faecalis* ATCC 9790.

Specificity for Acceptors. The substrate requirements of the *S. faecalis* membrane-bound LD-transpeptidase for acceptors were studied by using Ac_2-L-Lys-D-Ala as the peptide donor and various amino acids, di- and oligopeptides as acceptors. A fixed concentration of Ac_2-L-Lys-D-Ala donor (3 mM) and various concentrations of each acceptor (3, 15 and 30 mM) were incubated with 500 μg membrane protein for 18 h at 37 °C in 40 μl (final volume) of 1 mM EDTA in 10 mM sodium cacodylate buffer pH 6. Radioactive [^{14}C]Ac_2-L-Lys-D-Ala (specific activity: 0.91 mCi/mmol) and non radioactive acceptor were used except when D-alanine, glycine and L-alanine were tested as acceptors in which cases non-radioactive Ac_2-L-Lys-D-Ala and the ^{14}C-labeled amino acid were used. The transpeptidation products

([^{14}C]Ac_2-L-Lys-acceptor, Ac_2-L-Lys-D-[^{14}C]Ala or Ac_2-L-Lys-[^{13}C]Gly) were isolated by paper electrophoresis and estimated. The yields were expressed as percentage of Ac_2-L-Lys-D-Ala converted into transpeptidation products (Table 4).

D-Amino acids and glycine were utilized as acceptors (acceptors no. 1, 2, 5, 7, 9, 12). D-Alanine and D-lysine were especially good substrates. D-Cycloserine (acceptor no. 14) was also an acceptor. It has been argued that because of its zwitterion properties, D-cycloserine has charges similar to those of an α-amino acid [14]. The addition of 30 mM D-cycloserine to the systems Ac_2-L-Lys-D-Ala + D-[^{14}C]-alanine (no. 2) or Ac_2-Lys-D-Ala + L-[^{14}C]alanine (no. 4) resulted in an increased rate of transfer with D-alanine and in the complete absence of reaction with L-alanine. These results suggested the presence of an alanine racemase in the isolated membranes and demonstrated that L-alanine was, like the other L-amino acids tested (no. 6, 8, 10, 11), not a substrate. Since L-amino acids including LL-diaminopimelic acid were not acceptors, the transpeptidation reaction observed with *meso*-diaminopimelic acid (no. 9) probably involved the amino group located on the D-carbon.

The presence of an amide group on glycine (no. 13) decreased its effectiveness as an acceptor (compare with no. 12). The dipeptides no. 15—20 where the amino group potentially available for transfer reaction was not in α-position to a free carboxyl group, were not utilized as acceptors. Particularly the dipeptides D-Ala-Gly (no. 16) and Gly-D-Ala (no. 17) remained unutilized even when tested in the presence of 0.1 mM benzylpenicillin, a concentration below that which inhibited the LD-transpeptidase activity (*vide infra*) but sufficient to completely inhibit the DD-carboxypeptidase activity (Table 1). Inhibition of DD-carboxypeptidase with benzylpenicillin would prevent the hydrolysis of the tripeptides [^{14}C]Ac_2-L-Lys-D-Ala-Gly and [^{14}C]Ac_2-L-Lys-Gly-D-Ala if these peptides were formed by a transfer reaction.

The tripeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid (no. 21) functioned as an acceptor. Acceptor activity was seen even when the glutamic acid residue was amidated and a C-terminal D-alanine was present (peptide no. 22); and when and N-terminal disaccharide was added (peptide no. 23). However acceptor function was abolished by the amidation of the carboxyl group of *meso*-diaminopimelic acid in α-position to the amino group acceptor (peptide no. 24). Finally the lactylpentapeptide no. 25 functioned as a poor acceptor. It should be noted however that this peptide no. 25 as well as peptides no. 22 and 23, contained L-D-alanine sequences suitable for donor function.

Apparent K_m *Values.* Both double-reciprocal plots $1/v$ *vs* $1/[Ac_2$-L-Lys-D-Ala] at a fixed, 5 mM, concentration of D-[^{14}C]alanine and $1/v$ *vs* $1/[$D-[^{14}C]Ala) at a fixed, 3 mM, concentration of Ac_2-L-Lys-D-Ala gave rise to straight lines from which apparent K_m values of 40 mM for Ac_2-L-Lys-D-Ala and of 140 mM for D-[^{14}C]alanine could be calculated. The incubations were carried out for 18 h at 37 °C with 500 μg membrane proteins in 10 mM sodium cacodylate buffer pH 6, 1 mM EDTA and 15 mM D-cycloserine (final volume: 40 μl).

Effects of β-Lactam Antibiotics (ID_{50} Values). Ac_2-L-Lys-D-Ala (5 mM, final concentration) and D-[^{14}C]alanine (5 mM) were incubated with 500 μg membrane protein for 18 h at 37 °C in 40 μl (final volume) of 10 mM sodium cacodylate buffer pH 6 and 1 mM EDTA, in the absence and in the presence of various concentrations of the antibiotics. In the absence of antibiotic, 6.5% of the non radioactive dipeptide was converted into Ac_2-L-Lys-D-[^{14}C]Ala. The concentrations of antibiotics which inhibited the enzyme activity by 50% (ID_{50}) values were listed in Table 1. The ID_{50} values ranged from 15 μM (cephaloglycin) to 3.4 mM (6-aminopenicillinic acid). The same ID_{50} values for benzylpenicillin, oxacillin and cephaloglycin as those given in Table 1 were obtained when the time of incubation was decreased from 18 h to 3 h indicating that the observed ID_{50} values did not depend upon the time of incubation.

DISCUSSION

The *S. faecalis* membranes contained a DD-carboxypeptidase activity which catalysed the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala into D-alanine and Ac_2-L-Lys-D-Ala. The specific activity of the enzyme was about 16 mU per mg membrane protein. This value was close to the 6 DD-carboxypeptidase mU per mg protein found for membranes of *E. coli* K12 [using UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl (L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine as substrate] [22—24]. Specific activities of the purified exocellular DD-carboxypeptidase from *Streptomyces* were 86 units (R61 enzyme [25]) and 15 units (R39 enzyme, unpublished results) per mg protein (using Ac_2-L-Lys-D-Ala-D-Ala as substrate). The *S. faecalis* DD-carboxypeptidase had a considerable specificity for peptides with a C-terminal L-R_3-D-Ala-D-Ala sequence and a long side-chain at the L-R_3 position. The occurrence of charged amino groups at the end of this side-chain exerted relatively little influence. Such a specificity profile was very similar to those of the DD-carboxypeptidases from *Streptomyces* strains R61 and R39 [16,26]. Unlike these latter enzymes, however, the *S. faecalis* membranes failed to catalyse transfer reactions at least with the system Ac_2-L-Lys-D-Ala-D-Ala + D-[^{14}C]alanine and under the incubation conditions used. In view of the nature of the peptide crosslinking in the peptidoglycan of *S. faecalis* ATCC 9790, the apparent absence of a DD-transpeptidase activity in the membrane prep-

arations seems remarkable. However, other substrates and conditions of incubation must be examined before the absence of such an activity could be stated.

Exposure of Ac_2-L-Lys-D-Ala, *i.e.* the end-product of DD-carboxypeptidase activity, to *S. faecalis* membranes did not result in the liberation of D-alanine. Hence the membranes appeared to be devoid of LD-carboxypeptidase activity. However, when the same Ac_2-L-Lys-D-Ala dipeptide and various suitable amino acids or peptide were simultaneously exposed to the membranes, transfer reactions occurred according to the reaction Ac_2-L-Lys-D-Ala + acceptor → D-alanine + Ac_2-L-Lys-acceptor. Hence the *S. faecalis* membranes exhibited the transpeptidase activity that one would expect for an LD-carboxypeptidase reacting with an amino nucleophile (instead of H_2O as it occurs in simple hydrolysis). The *S. faecalis* LD-transpeptidase showed a considerable specificity for peptide donors presenting a N^{α}-substituted, C-terminal L-R_3-D-Ala sequence with a free ω-amino group located at the end of a long side-chain at the L-R_3 position, and for amino acid and peptide acceptors with an amino group located on a D-carbon in α-position to a free carboxyl group.

If functional *in vivo*, the *S. faecalis* LD-transpeptidase could catalyse the transfer of the penultimate C-terminal L-lysine of a pentapeptide donor N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}(β-D-aspartyl)-L-lysyl-D-alanine (*i.e.* the end-product of DD-carboxypeptidase activity on a hexapeptide precursor) to the amino group of the D-aspartic residue of another pentapeptide acceptor. Note that amidation of the α-carboxyl group of D-aspartic acid would abolish the acceptor function but not the donor function. The resulting interpeptide bond would be a C-terminal L-Lys-D-Asp linkage. Previous studies [12] showed the presence of L-Lys-D-Ala-D-αAsn interpeptide linkages as a major component of the wall peptidoglycan of exponential phase cells (see Introduction). The occurrence of L-Lys-D-Asp linkages as minor peptidoglycan component might have escaped detection or the synthesis of this latter type of interpeptide bond might prevail under different culture conditions, as those which lead to wall thickening [27]. Recent investigations support the idea that atypical types of peptide crosslinking which might result from LD-transpeptidase activities, occur in peptidoglycans of some bacteria. LL-Diaminopimelylglycyl-LL-diaminopimelyl was proposed as the structure of a minor type of crosslinking in *Streptomyces albus* G and *Clostridium perfringens* type A (see Fig. 10 in [28]). Similarly, *meso*-diaminopimelyl-*meso*-diaminopimelic acid is the proposed structure for a minor type of crosslinking in the peptidoglycan of *Mycobacterium smegmatis* (J. Wietzerbin *et al.*, unpublished results).

Depending upon the antibiotic (Table 1), inhibition of growth occurred either at (a) concentrations which were about the same as those which inhibited the DD-carboxypeptidase but much lower than the concentrations required to inhibit the LD-transpeptidase (group I); (b) concentrations which were about the same as those which inhibited both the DD-carboxypeptidase and the LD-transpeptidase (group II); (c) concentrations which inhibited the LD-transpeptidase but were considerably lower than those required to inhibit the DD-carboxypeptidase (group III). These results would suggest that both enzymes might be killing targets of the β-lactam antibiotics and that inhibition of either of them would result in inhibition of cell growth. This conclusion, however, was not supported by the observation that inhibition of the LD-transpeptidase by cephaloglycin and cephalosporin C (group IV) occurred at lower concentrations than those required to inhibit growth and DD-carboxypeptidase. Whatever the possible physiological function of the LD-transpeptidase, its inhibition by β-lactam antibiotics *in vitro* is not consistent with the hypothesis [1] that the penicillin molecule would act as an analogue of a C-terminal D-alanyl-D-alanine sequence.

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