Streptomyces DD Carboxypeptidases-Transpeptidases and Mechanism of Action of Penicillin

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Summary : A major part of penicillin resistance in *Streptomyces* sp results from a mechanism much more fundamental than penicillinase production. This basic mechanism would reside in structural and/or conformational features of the "DD carboxypeptidase-transpeptidase" system, which do not affect its catalytic properties but which independently alter its capability of binding the penicillin molecule.

Primary Structures of the Peptidoglycans. The four Chemotypes.

The tensile strength of bacteria is imparted by a peptidoglycan polymer that forms a continuous network, 20 to 100 Å thick, and which is located at the inner boundary of the wall part of the cell envelope, in close proximity to the plasma membrane [1-13]. As its name suggests, this network is composed of glycan strands that are interconnected through peptide chains. Fig. 1 shows the structure of the peptidoglycan in walls of *Staphylococcus aureus* strain Copenhagen.

The peptidoglycans exhibit a remarkable consistency of structure throughout the bacterial world, even if at first sight, this unity is hidden by a wide variation in structural details. The glycan moiety consists of linear strands of β-1,4 linked N-acetyl-D-glucosamine pyranoside residues in which each alternate sugar is ether-linked at C_3 to a lactyl group which has the D configuration (this 3-O-D-lactyl-N-acetyl-D-glucosamine is currently called N-acetylmuramic acid). Galactosamine instead of glucosamine and galactomuramic acid instead of glucomuramic acid have never been encountered, suggesting that a chitin-like conformation and linear arrangement of the glycan strands are essential for the wall function of the polymer [14]. Depending upon the bacteria, variations that do not alter the basic conformation of the backbone may occur. For example, C_6 of muramic acid may be O-acetylated or substituted by a phosphodiester group [4]. Muramic acid can occur as N-glycolylmuramic acid [15] (instead of the usual N-acetylmuramic acid) or in the form of a lactam derivative [16].

The D-lactic groups of the glycan, or at least some of them, are substituted by tetrapeptide units L-alanyl (or L-seryl, or glycyl)-γ-D-glutamyl-L-R_3-D-alanine. The lin-

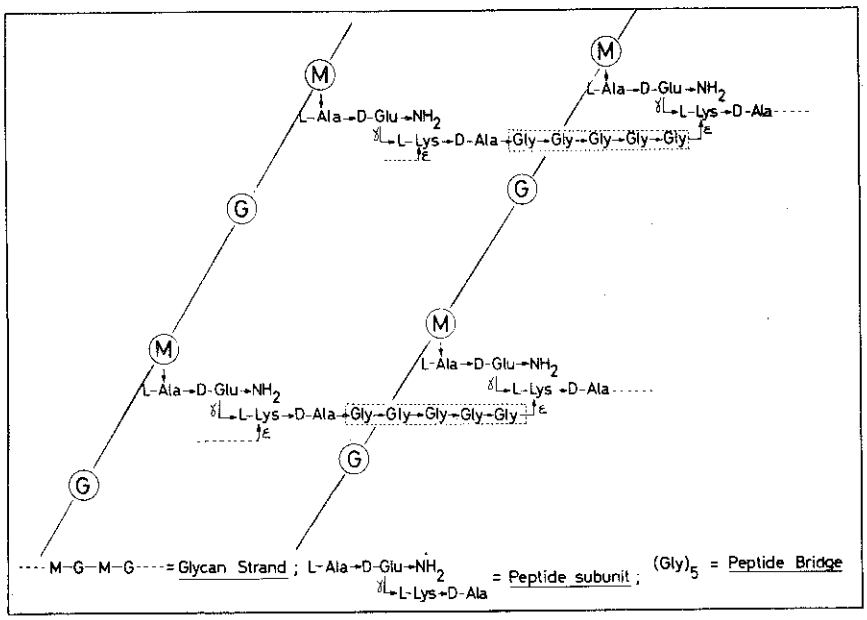

Fig. 1 : The peptidoglycan in *S. aureus* Copenhagen. The glycan chains consist of β-1,4 linked N-acetylglucosamine (G) and N-acetylmuramic acid (M). The tetrapeptide units have the sequence L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine. The peptide bridges are pentaglycine sequences. The arrows indicate the CO → NH direction of linkage. α-Peptide bonds are represented by horizontal arrows.

kages are α except the glutamyl bond which is γ . According to the bacterial species, the L-R_3 residue may be either a neutral amino acid such as L-alanine or L-homoserine, or a diamino acid such as L-diaminobutyric acid, L-ornithine, L-lysine, LL-diaminopimelic acid or *meso*-diaminopimelic acid. In the latter case, both the amino group linked to D-glutamic acid and the carboxyl group linked to D-alanine are located on the same L-carbon of *meso*-diaminopimelic acid [17]. Thus, there appears to be a consistent DLDLD sequence for the backbone of all lactyl-tetrapeptides, with the exception that glycine can occur at the amino terminus of the tetrapeptides. Depending upon the bacteria, the α-carboxyl group of glutamic acid can be either free or amidated or substituted by a C-terminal glycine residue. Similarly, the carboxyl group of diaminopimelic acid residue not engaged in peptide bond may be either free or amidated.

The peptide units are, in turn, crosslinked through "specialized" bridges [4]. In chemotypes I, II and III, the bridges extend from the C-terminal D-alanine of one peptide to the ω-amino group of the L-R_3 diamino acid of another peptide. The bridging may consist of direct N^{ω}-(D-alanyl-R_3) peptide bonds (chemotype I). Bridging may be mediated *via* a single additional amino acid or *via* an intervening short peptide (chemotype

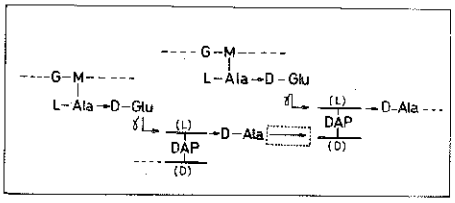

Fig. 2 : Peptidoglycan in *Escherichia coli* (chemotype I). The crosslinking between two tetrapeptide units. The dotted area contains the interpeptide bridge. G = N-acetylglucosamine . M = N-acetylmuramic acid .

II). It may also be composed of one or several peptides each having the same amino acid sequence as the peptide unit (chemotype III). Finally, in chemotype IV and in contrast to the foregoing, bridges extend from the C-terminal D-alanine residue of one peptide unit to the α-carboxyl group of D-glutamic acid of another peptide unit. This latter type of bridging extends between two carboxyl groups and, therefore, necessarily involves either a diamino acid residue or a diamino acid-containing peptide. Strikingly, the crosslinking between two peptide units always involves the C-terminal D-alanine of one of them, regardless of the chemotype. The primary structures of some peptidoglycans are illustrated in Fig. 2, 3 and 4 . One should note that the interpeptide bonds in the case of *Escherichia coli* (a D-alanyl-(D)-*meso*-diaminopimelic acid linkage; Fig. 2) and in the case of *Corynebacterium poinsettiae* (a N^{α}-(D-alanyl)-D-ornithine linkage; Fig. 4) are simultaneously in both an internal and C-terminal position. Consequently, the lytic "endopeptidases" that specifically hydrolyze these bonds are in fact DD carboxypeptidases. The structural features of the wall peptidoglycans are criteria of taxonomic importance and have been used in studies dealing with the classification of *Corynebacteria*, *Micrococcaceae* and other microorganisms [18-19].

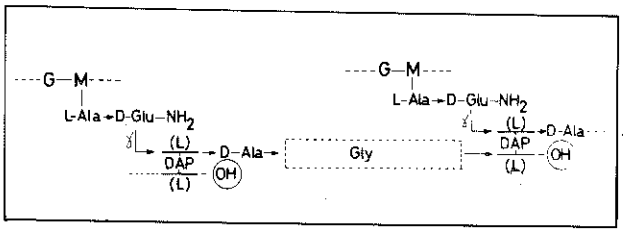

Fig. 3 : Peptidoglycan in *Streptomyces* strains *albus* G and R61 (chemotype II). The crosslinking between two tetrapeptide units. The dotted area contains the interpeptide bridge. G and M : see Fig. 2 . The peptidoglycan of *Staphylococcus aureus* (Fig. 1) is another example of chemotype II .

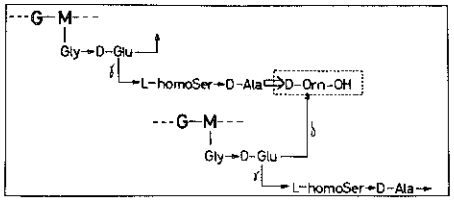

Fig. 4 : Peptidoglycan in *Corynebacterium poinsettiae* (chemotype IV). The crosslinking between two tetrapeptide units. The dotted area contains the interpeptide bridge. G and M : see Fig. 2 .

Biosynthesis of the Nascent Wall Peptidoglycan. A survey.

Many important steps of the biosynthesis of the wall peptidoglycans are carried out on the plasma membrane from the two following activated nucleotide precursors : Uridine-5'-pyrophosphoryl(UDP)-N-acetylglucosamine and UDP-N-acetylmuramyl-L-alanyl (or L-seryl, or glycyl)-γ-D-glutamyl-L-R_3-D-alanyl-D-alanine. Note that in this latter precursor, the peptide ends in a C-terminal D-alanyl-D-alanine sequence. The biosynthetic machinery is complex (for details, see [7]) and involves a polyisoprenoid alcohol phosphate carrier which is clearly relevant to problems of orientation and transport and a series of synthetic and hydrolytic enzymes which catalyze a coordinated sequence of reactions. Synthetases bring about the formation of β-1,4-N-acetylglucosaminyl-N-acetylmuramyl peptide units. They catalyze, if necessary, appropriate modifications of the peptides. Such modifications include the substitution of the L-R_3 residue or the α-carboxyl group of D-glutamic acid by those residues which will function as "specialized" bridges in the completed wall peptidoglycan. Finally, synthetases insure the transport of the completed units through the membranes to the extracellular sites of incorporation. Autolysins (hydrolytic enzymes) may be responsible for the creation of the appropriate receptor sites needed for the insertion of the newly synthesized units in the expanding wall. The overall reactions are reasonably well understood at the molecular level. Our concepts, however, of the mode of growth and replication of the wall at the cellular level (for details, see [12]), of the intimate functioning of the membrane and of the exact topology of the wall-synthesizing enzymes, are still largely obscure.

Peptide Crosslinking in the Nascent Peptidoglycan.

The insertion of newly synthesized (but as yet uncrosslinked) disaccharide peptide units into the wall peptidoglycan, whatsoever its precise mechanism, must be followed by the closure of the bridges between the peptide units if the process is to yield an insoluble network. This last reaction is thought to occur by transpeptidation [4, 20,

21]. The penultimate C-terminal D-alanine residue of a donor peptide is transferred to the amino group of an acceptor peptide of the same composition. Interpeptide bonds are formed and equivalent amounts of D-alanine residues are released from the donor peptides. Fig. 5 illustrates such reactions.

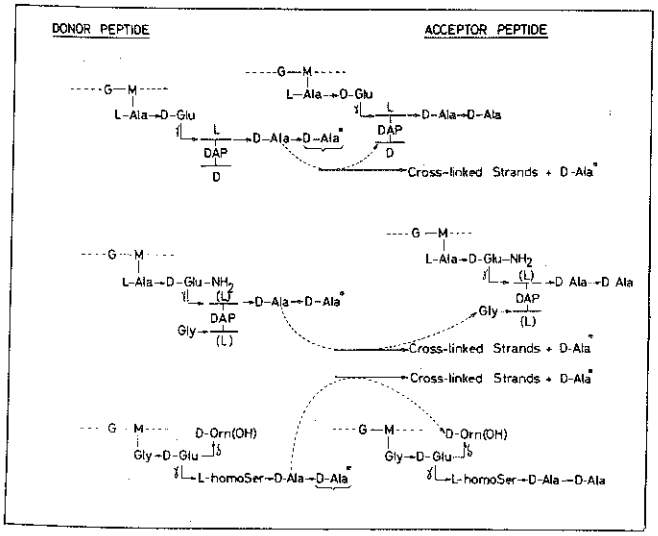

Fig. 5 : The bridge closure reaction in *Escherichia coli* (upper part; compare with Fig. 2), in *Streptomyces albus* G and R61 (middle part; compare with Fig. 3) and in *Corynebacterium poinsettiae* (lower part; compare with Fig. 4). The reactions in *Streptomyces* and in *C. poinsettiae* are hypothetical. They are deduced from the primary structures of the completed wall peptidoglycans.

In *Escherichia coli*, the acceptor group is the amine located on the D-carbon of *meso*-diaminopimelic acid at the R_3 position. In *Streptomyces* sp, the acceptor group is a N-terminal glycyl-diaminopimelic acid sequence at the same R_3 position. In *Corynebacterium poinsettiae*, the acceptor group is the α amino group of the D-ornithine residue which substitutes, through its δ amino group, the α-carboxyl group of D-glutamic acid.

Particulate preparations obtained from *E. coli* and *Salmonella* strains were able to perform the transpeptidation reaction [22]. Efforts made in several laboratories to extend these studies to other microorganisms have not yet achieved this desirable goal. That transpeptidation is probably ubiquitous, however, rests upon several pieces of evidence. (1) A transpeptidation reaction effectively explains why a single D-alanine residue is involved in crosslinking the peptide units in all bacterial peptidoglycans whereas the peptides in nucleotide precursors always end in a C-terminal D-alanyl-D-alanine sequence. (2) Transpeptidation does not involve large changes

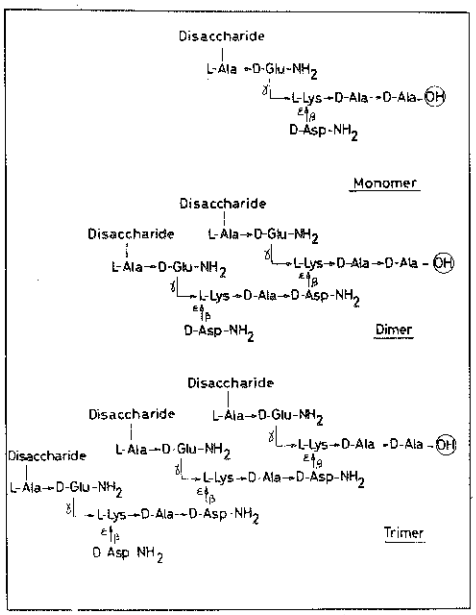

Fig. 6 : The peptide moiety in walls of *Lactobacillus acidophilus* strain 63 AM Gasser, after degradation of the glycan into disaccharide units. 10 % of the peptide units occur as monomers, 37 % as dimers and 30 % as trimers. The C-termini of the peptides have retained the D-alanyl-D-alanine sequence of the nucleotide precursor. In most bacteria, the C-terminal D-alanine residue or the C-terminal D-alanyl-D-alanine sequence is absent.

in free energy and therefore, is likely to bring about peptide bond synthesis at extracytoplasmic sites where ATP is not available. (3) For the same reason, transpeptidation probably never reaches completion, and thus the efficiency of the reaction is likely to vary according to the bacteria. This again explains, at least in part, the large variations in the extent of peptide crosslinking of the wall peptidoglycans which are observed among the bacteria. Estimation of the extent of peptide crosslinking indicates that the reaction is very efficient in *S. aureus* (80 %) [4] but poorly efficient in *L. acidophilus* strain 63 AM Gasser (30 %) [23]. With these two bacteria, the residual non-crosslinked C-termini of the wall peptide moieties have retained the D-alanyl-D-alanine sequence found in the nucleotide precursors (Fig. 6), thus demonstrating that peptide hydrolases are not involved in the control of the size of the peptide moieties. In most bacteria, however, the residual non-crosslinked C-termini of the wall peptides do not retain the D-alanyl-D-alanine sequences, thereby indicating the active presence of D-alanyl-D-alanine carboxypeptidases and their involvement in the control of the extent of peptide crosslinking.

No bacterial transpeptidase has yet been isolated from membranes, purified and characterized. From recent reports in the literature, more than one synthesizing system

(and perhaps more than one transpeptidase) might occur in a single cell. These systems would be responsible for cell elongation and septum formation, respectively [24, 12].

Transpeptidase as the Target of the Penicillin Molecule.

Penicillin, when added at sublethal dose levels to growing *Staphylococcus aureus* was shown to reduce the extent of peptide crosslinking. Walls of *S. aureus* isolated from cells grown in the presence of small amounts of penicillin were found to contain higher amounts of uncrosslinked peptide units ending in C-terminal D-alanyl-D-alanine sequence, than walls isolated from cells grown in the absence of penicillin [20, 21, 25]. Subsequently, penicillin was shown to abolish the transpeptidase activity of the particulate preparation obtained from *E. coli* [7,22]. Surprisingly, however, and for reasons that are not clearly understood, a reduction of the extent of peptide crosslinking in *E. coli* could not be observed in the *in vivo* biosynthesis of the peptidoglycan after brief exposure to penicillin [24]. Similarly, it was reported [26] that the peptidoglycan from the normal rod-shaped Gram-negative *Proteus mirabilis* and that from its penicillin-induced unstable L-form differed very little, if at all, in their extent of peptide crosslinking. Regardless of these discrepancies, however, it is generally accepted that an early and important step in penicillin action is the reduction or the abolition of the efficiency of the membrane-bound transpeptidase.

Despite a number of hypotheses, little is known about the exact molecular basis of penicillin action. Essentially, three hypotheses have been proposed. (1) One postulates a combination between penicillin and the peptide which undergoes transpeptidation, presumably on the amino acceptor site, thus preventing the substrate from attack by the transpeptidase [27]. (2) According to a second hypothesis, penicillin is a structural analogue of the nascent peptidoglycan and, consequently, directly acts upon the membrane-bound transpeptidase. Penicillin might resemble either N-acetylmuramic acid [28], or the L-alanyl-γ-D-glutamyl part of the peptide unit [21], or its C-terminal acyl-D-alanyl-D-alanine sequence [20, 7]. Models show that one edge of the penicillin molecule, i.e. the 6-aminopenicillanic acid part of it, has a conformation that may resemble one of the conformations of the C-terminal D-alanyl-D-alanine backbone. In fact, however, the D-alanyl-D-alanine peptide bond is about 25 % longer than the corresponding bond in the β-lactam ring of penicillin and the angle around the D-alanyl-D-alanine bond is considerably smaller than the angle around the corresponding bond in the β-lactam [29]. Moreover, the 6-methyl derivative of penicillin which is a better analogue of D-alanyl-D-alanine than penicillin since it has a methyl group in the position where penicillin has a hydrogen atom, is completely inactive as an antibiotic [30]. (3) The third hypothesis rests upon the inactivation of the transpeptidase by acylation through the highly reactive CO-N bond of the penicillin lactam ring, resulting in the formation of an inactive penicilloyl-enzyme complex [7, 22]. At the present time, it

is advisable to hold reservations on the possible involvement of penicilloylation in the lethal action of penicillin. Indeed, it has been shown [27] that the irreversible fixation of the great majority of the penicillin molecules on living bacteria such as *S. aureus* takes place on sites which are not concerned with peptidoglycan biosynthesis and which, therefore, are irrelevant to the killing by penicillin. It has been reported (J.L. STROMINGER : report given at the Second Harden Conference. Wye College. England. September 1970) that at least three different proteins, separable by electrofocusing , are involved in the binding of penicillin by cells of *Bacillus subtilis*, but it is not known whether or not these proteins or some of them are the killing targets of penicillin. Finally, penicillin, when considered as an acylation agent, has no specificity. It is responsible, for example, for the formation of penicilloyl-allergen through acylation of serum protein.

The Hypothesis of the "Transpeptidase-DD Carboxypeptidase" System.

In an early work [31] dealing with the enzymatic degradation of the walls of *S. aureus*, it was observed that the enzyme complex excreted by *Streptomyces albus* G contained an enzyme which was able to hydrolyze the D-alanyl-D-alanine sequence at the C-termini of the peptide moiety of the peptidoglycan. In more recent works [17, 32, 33], it was recognized that the purified "KM endopeptidase" excreted by the same *Streptomyces* strain *albus* G, exhibited its lytic action upon certain walls through the hydrolysis of DD linkages that were in position α to a free carboxyl group. Finally [34, 35] it became clear that all these activities of the *Streptomyces albus* G culture filtrates could be assigned to a single, extracellular DD carboxypeptidase. Concomitantly, DD carboxypeptidases were also detected in and partially purified from *E. coli* [36], *B. subtilis* [37, 38] and the blue-green alga *Anabaena variabilis* [39]. So far, however, *Streptomyces* sp offer the unique and obvious advantage of possessing extracellular DD carboxypeptidases which can be isolated and purified.

From recent studies, the soluble DD carboxypeptidases from *Streptomyces* sp appear to be the *exo*forms of the membrane-bound transpeptidases. The difference in effective function of the protein would be that, after elimination of the C-terminal D-alanine residue (Fig. 5), the peptide-enzyme complex would react either with water (leading to simple hydrolysis; carboxypeptidase activity) or with an acceptor amino group (leading to peptide bond synthesis; transpeptidase activity).

DD Carboxypeptidases, Penicillinases and Penicillin Action *in vivo*

Penicillin resistance of *Streptomyces*, as for many other bacteria, is critically dependent upon the size of the inoculum. In order to ensure better quantitative esti-

mates, it was measured as the ability of conidia to form single-cell colonies on plates in the presence of different concentrations of penicillin G . Strains of *Streptomyces* were selected, which were either very sensitive to penicillin (LD 50 : 0.25 to 2 µg/ml) or highly resistant to it (LD 50 : 13 to 22 µg/ml) (Table I).

Table I : Inhibition of growth of *Streptomyces* sp by penicillin G (in µg/ml) in relation to penicillin inhibition of the DD carboxypeptidases (in µg/ml) and to penicillinase production (in units per ml of culture filtrates for exocellular enzymes).

Strains	Growth Inhibition		Extracellular Penicillinase in units/ml (b)	Inhibition of DD carboxypeptidases (c)
	Single cell colonies (LD 50) (a)	Heavy Inoculum (10^6 spores/ml)		
R39	0.25	100	4.4	5×10^{-3}
K11	1.5	50	3.1	5×10^{-2}
R61	2	50	< 0.02	5×10^{-2}
albus G	13	500	2.9	1×10^{4}
coelicolor A3(2)N2	22	750	0	1×10^{5}

(a) Expressed in amounts (µg/ml) of penicillin G required to reduce by 50 % the number of colonies. (b) units : amount of enzyme which catalyzes the degradation of 1 µmole of penicillin G per hour at 30° at pH 7 . (c) in µg/ml of penicillin required to produce 50 % inhibition. Peptide N° 1 (Table II) was used for enzymes from strains R39, K11, R61 and *albus* G. Peptide N° 12 (Table II) was used for enzyme from strain *coelicolor*. In all cases, the substrate concentrations were 0.45 mM.

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 [40]. In accord with this latter view, penicillin resistance of *Streptomyces* is not related to the ability of the strains to produce penicillinase (Table I). By contrast, penicillin resistance of the strains is reflected in penicillin resistance of the relevant, isolated DD carboxypeptidases (Table I). Inhibition of the DD carboxypeptidases was estimated as the dose levels of penicillin G required to inhibit by 50 % the enzyme activity. The substrate concentration were 0.45 mM, i.e. concentrations which, depending upon the enzyme, were equivalent to or far below the Km values (*vide infra*). Hence, the comparison of the penicillin resistance exhibited by the enzymes is only a rough approximation.

Specificity Profile of *Streptomyces* DD Carboxypeptidases.

The penicillin-sensitive DD carboxypeptidases from strains R39, K11 and R61 and the penicillin-resistant DD carboxypeptidase from strain *albus* G were isolated and their substrate requirements were studied on peptides with the general structure $X \rightarrow L\text{-}R_3 \rightarrow R_2 \rightarrow R_1(OH)$ [35, 41, 42]. Table II and III present the most significant results. K11 enzyme was very similar if not identical to R61 enzyme; hence, the data obtained with the K11 enzyme are not presented.

(1) The nature of the α-substituent of the $L\text{-}R_3$ residue (i.e. the X group) does not produce any striking effect on the enzyme efficiency (not shown in the tables).

(2) There appears to be a considerable specificity for an amino acid residue with a D configuration at the C-terminal position (the R_1 residue; Table II, peptides N° 1-5) and for a D-alanine residue at the penultimate C-terminal position (the R_2 residue; Table II, peptides N° 1 and 6-8). The presence of a D-alanine residue at the C-terminal position best fits the specificity profile of the enzymes. The replacement of D-alanine at this R_1 position by other C-terminal D amino acids such as D-leucine, D-lysine or N^{ω}-substituted D-diamino acids (Table II, peptide N° 13) is often accompanied by a decrease in enzyme efficiency; complete lack of activity may occur.

(3) Increasing the length of the side-chain of the $L\text{-}R_3$ residue without introduction of charged groups (for example, replacement of the acetyl-L-alanyl residue of the peptide acetyl-L-alanyl-D-alanyl-D-alanine, by N^{α},N^{γ}-bisacetyl-L-diaminobutyric acid, by N^{α},N^{δ}-bisacetyl-L-ornithine and by $N^{\alpha},N^{\varepsilon}$-bisacetyl-L-lysine) is always paralleled by a remarkable increase in enzyme efficiency (Table III and Table II, peptides N° 1 and 9).

(4) The influence exerted by the introduction of charged groups at the end of the side-chain of the $L\text{-}R_3$ residue was also examined (Table II; peptides N° 1 and 10-12). Presence of a free ε-amino group of L-lysine, transformation of this ε-amino group to an α-amino group by introduction of a carboxyl group in an α position (i.e. the replacement of lysine by diaminopimelic acid), and substitution of the ε-amino group of L-lysine by a pentaglycine sequence, differently influence the efficiency of the enzymes. In all cases, however, the presence of a N^{ε}-(pentaglycyl)-L-lysine group is compatible with a high enzyme efficiency.

From the foregoing, it thus appears that both the donor site (i.e. the C-terminal D-alanyl-D-alanine sequence) and the acceptor site (i.e. the $L\text{-}R_3$ group) involved in transpeptidation are recognized by the DD carboxypeptidases. Such a specificity profile is consistent with the idea that DD carboxypeptidases are the *exo*forms of the transpeptidases.

Table II : Substrate requirements of *Streptomyces* DD carboxypeptidases (Release of the C-terminal residue).

N°	Substrates[a]	R61			R39			*albus* G		
		Km	Vmax	Efficiency	Km	Vmax	Efficiency	Km	Vmax	Efficiency
1	Acetyl → L-Lys(Acetyl, ε) → D-Ala → D-Ala	12	890	72	0.8	330	410	0.33	100	300
2	D-Ala → D-Lys	13	90	7				0.80	85	106
3	D-Ala → D-Leu	10	50	5	0.7	230	320	0.33	33	100
4	D-Ala → Gly	36	200	6	2.5	100	40	2.50	60	24
5	D-Ala → L-Ala	virtually no hydrolysis			no hydrolysis			no hydrolysis		
6	Gly → D-Ala	15.5	1.7	0.1	no hydrolysis			15.0	107	7
7	D-Leu → D-Ala	10	10	1	no hydrolysis			no hydrolysis		
8	L-Ala → D-Ala	virtually no hydrolysis			no hydrolysis			no hydrolysis		
9	Acetyl → L-DAB(Acetyl, γ) → D-Ala → D-Ala				2.5	400	160			
10	Acetyl → L-Lys(H) → D-Ala → D-Ala	15	4	0.3	0.2	600	3,000	6.0	20	3
11	R_1 → (L)-*meso*-DAP-(L) → D-Ala → D-Ala	11	8	0.3	0.25	400	1,600	0.4	10	25
12	R_2 → L-Lys(ε-(Gly)$_5$) → D-Ala → D-Ala	14	800	57	0.30	420	1,400	0.28	9	32
13	*C. poinsettiae* bisdisaccharide peptide dimer	weak hydrolysis			no hydrolysis			1.50	78	52

(a) : R_1 = UDP-MurNAC-L-Ala-γ-D-Glu ; R_2 = N^{α}-[Disacch-L-Ala-γ-D-Glu(NH_2)] ; peptide N° 13 : see Fig. 4 .

Km values are expressed in mM ; Vmax values in µmoles, per mg of enzyme, per hour ; Efficiency in Vmax/Km .

Table III : Influence of the length of the side chain of the L-R_3 residue on DD carboxypeptidase activity (Release of the C-terminal residue).

Substrate [a]	Specific activity of enzyme from [b]		
	R61	R39	*albus* G
Acetyl → L-Ala → D-Ala → D-Ala	650	200	300
R_1 → L-homoSer → D-Ala → D-Ala	1,400	6,000	2,000
N^{α},N^{γ}-bisacetyl → L-DAB → D-Ala → D-Ala	4,000	14,000	22,000
N^{α},N^{δ}-bisacetyl → L-Orn → D-Ala → D-Ala	21,000	75,000	22,000
N^{α},N^{ε}-bisacetyl → L-Lys → D-Ala → D-Ala	47,000	90,000	40,000

(a) : R_1 = UDP-MurNAC-Gly-γ-D-Glu ;

(b) : expressed in nequiv of D-Ala → D-Ala linkage hydrolyzed per mg of enzyme, per hour, at 37°C (peptide concentration : 0.45 mM).

Very little is known about the specificity profile of DD carboxypeptidases other than those of *Streptomyces*. The DD carboxypeptidase of *E. coli*, however, is known to interact readily with UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine [36]. It has no action on the same peptide in which diaminopimelic acid has been replaced by L-lysine. Similarly, N^{α},N^{ε}-bisacetyl-L-lysyl-D-alanyl-D-alanine is not a substrate (E. BRICAS, personal communication).

Lytic DD Carboxypeptidases.

Peptides ending in a C-terminal D diamino acid of which the ω amino group is, in turn, substituted by long peptide chains, are substrates of the DD carboxypeptidases. For example, the *albus* G, R61, K11 and R39 *Streptomyces* enzymes are able to hydrolyze the peptide dimers of *E. coli* (Fig. 2) and *C. poinsettiae* (Fig. 4) into monomers by splitting the C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkage in the former, and the C-terminal D-alanyl-D-ornithine linkage in the latter (Table II, peptide N° 13). Similarly, the *E. coli* DD carboxypeptidase hydrolyzes the *E. coli* peptide dimer into monomers [43]. DD carboxypeptidases may thus exert endopeptidase activities. Among the DD carboxypeptidases isolated both from *Streptomyces* sp and from *E. coli*, that of *Streptomyces albus* G is unique in that it is the only one which lyzes walls (or cells) in which the interpeptide bonds are C-terminal D-alanyl-D linkages (for a list of sensitive walls, see Chart I in [34]). Moreover, the *Streptomyces albus* G enzyme is a cationic protein (even at pH 8) in marked contrast with all the other DD carboxypeptidases which are anionic. A likely conclusion is that the anionic properties of most of the DD carboxypeptidases prevent them from combining with the bacterial walls and, consequently, exerting a lytic activity even if they are able to exert endopeptidase activities on isolated peptide oligomers.

Enzyme Kinetics of DD Carboxypeptidases.

With the *Streptomyces* DD carboxypeptidases so far studied (i.e. the R39, K11, R61 and *albus* G enzymes), reaction is of zero order in the presence of saturating substrate concentrations and typical Michaelis-Menten kinetics are observed over a wide range of substrate concentrations. From the data of Table II, there appear to be different classes of enzymes [35, 41, 42]. The Vmax values reported in Table II depend upon the purity of the enzyme preparations. Hence, quantitative comparison between the *Streptomyces* enzymes with respect to these parameters is valid only if each of the preparations consists of one single protein. Polyacrylamide gel electrophoresis suggests that the enzyme preparations are at least highly purified.

(1) The R61 enzyme exhibits high Km values of 10 to 15 mM [41]. Peptides which are good substrates (Table II, peptides N° 1 and 12) have Km values as high as the poor

substrates but the good substrates have much higher Vmax values. Depending upon the substrates, Vmax values range from about 2 to 900 µmoles/mg protein/hour. By analogy with the mechanism of action of penicillinases [44], the properties of the R61 enzyme suggest a model involving conformational responses towards substrates (and possibly towards inhibitors : *vide infra*), i.e. some correlation between the structure of the peptide, its effect on the conformation of the enzyme and the catalytic activity. The R61 enzyme would have a binding surface which is not very specific, being able to bind even molecules that are only remotely analogous to the substrate. However, the alignment of the catalytic groups induced on binding would be incorrect or unfavourable for poor substrates, thus decreasing very much the Vmax or even preventing enzyme action. Such a model implies flexibility of the protein molecule. The enzyme is not activated by Mg^{++} and it is not inhibited by sodium ethylenediamine tetraacetate.

(2) The K11 enzyme exhibits properties very similar if not identical to those of the R61 enzyme [42].

(3) The R39 enzyme markedly differs from the foregoing ones [42]. The Km values are much lower and they vary from 0.2 m<u>M</u> for good substrates (Table II, peptides N° 10-12) up to 2.5 m<u>M</u> for poor substrates (Table II, peptides N° 4 and 9). The Vmax values are relatively little affected by the substrates and range from 100 to 600 µmoles/mg protein/hour. These observations show that, in this case, the enzyme efficiency reflects itself in Km rather than in Vmax. The R39 enzyme thus seems to have a better fit and a more specific binding surface than the R61 and K11 enzymes. A likely consequence is that peptides N° 5, 6, 7, 8 and 13 (Table II) which are poor substrates for the R61 enzyme but are still hydrolyzed to a measurable or at least detectable extent, completely escape attack by the R39 enzyme, probably because of lack of binding. The R39 enzyme is slightly activated by Mg^{++} . It is not inhibited by sodium ethylenediamine tetraacetate.

(4) The *albus* G enzyme [35] resembles the R39 enzyme. It exhibits Km values from 0.3 to 15 m<u>M</u> and Vmax values from 10 to 100 µmoles/mg protein/hour (Table II). Differences in enzyme efficiency, according to the substrates, thus also reflect variations in Km rather than in Vmax. The activity of the *albus* G enzyme is markedly increased by Mg^{++} and it is completely inhibited by sodium ethylenediamine tetraacetate.

<u>Inhibition of DD Carboxypeptidases by Penicillin</u>.

For the penicillin-sensitive DD carboxypeptidase from *E. coli*, Dixon plots of enzyme inhibition obtained from studies carried out in the presence of penicillin G gave straight lines meeting in points from which a Ki value of 1.6×10^{-8} <u>M</u> was calculated [36]. The assumed structural analogy between penicillin and the conformation of acyl-D-alanyl-D-alanine which had been evoked to explain the inactivation of the

E. coli membrane-bound transpeptidase by penicillin [7, 20], was then extended to explain the penicillin sensitivity of the *E. coli* DD carboxypeptidase [36]. In view of these apparent competitive kinetics, it was proposed that penicillin would resemble the substrate sufficiently to be bound in its stead on the catalytically active site of the enzyme.

Study of the inhibition of the carboxypeptidase activity of the *Streptomyces* enzymes does not support the above structural analogy hypothesis for the following reasons :

(1) Kinetically, the inhibition of the penicillin-sensitive DD carboxypeptidase from *Streptomyces* R39 is noncompetitive [42]. This suggests that penicillin combines with the enzyme at a site that is not identical with the substrate binding site. Moreover, increasing the penicillin concentration causes disproportionate decreases in the catalytic rate of hydrolysis, suggesting a conformational response of the enzyme towards the inhibitor.

(2) Insofar as can be judged from present analyses, penicillin competitively inhibits the two penicillin-sensitive DD carboxypeptidases from *Streptomyces* R61 and K11 [41, 42]. The Ki values were 6×10^{-8} M with K11 enzyme and 7.5×10^{-8} M with R61 enzyme. Competitive type kinetics, however, do not always or necessarily reflect the capacity of the inhibitor to compete with the substrate for a catalytically active site on the enzyme [45]. Altered penicillins that are competitive inhibitors of the hydrolysis of benzylpenicillin by some penicillinases act on the catalytic level by modifying the conformational responses of the enzymes [44].

(3) The conformation of the acceptor site in transpeptidation, i.e. the L-R_3 residue with its long side-chain, is recognized by the DD carboxypeptidases and constructed models do not show any analogy between penicillin and this acceptor site.

(4) Penicillin acts on the DD carboxypeptidases quite independently of the substrate specificity of the binding surfaces of the enzymes, at least as revealed by the Km values. Penicillin is a powerful inhibitor of the R61 and K11 enzymes which both exhibit high Km values and of the R39 enzyme which has low Km values (Tables I and II). Moreover, penicillin has virtually no action on *albus* G enzyme [46] which, like the R39 enzyme, also has low Km values (Tables I and II). Such behavior is reminiscent of those allosteric enzymes which can be made insensitive to effectors without affecting the catalytic activity.

The foregoing suggests a "conformational response" model for penicillin action on DD carboxypeptidases. Such a model has the advantage of stressing the independence of binding and hydrolysis and allows the possibility that molecules such as penicillin which are not obvious analogues, could be bound by some DD carboxypeptidases more strongly than the natural substrates. In the penicillin resistance of DD carboxypeptidases, a mechanism of "desensitization", independent of the catalytic activity, would be involved, suggesting the occurrence of specific penicillin-sensitive sites in the penicil-

lin-sensitive DD carboxypeptidases. The understanding of the reason why such sites would exist rests upon a better understanding of the role which is played by penicillinases in bacteria and of the relationship which may exist between penicillinases and DD carboxypeptidases.

In contrast to the *Streptomyces* and *E. coli* enzymes which were obtained in a water-soluble form, the DD carboxypeptidase from *B. subtilis* was obtained in the form of a particulate preparation [37, 38]. To all appearances, this latter enzyme was inactivated by penicillin through the formation of an inactive penicilloyl-enzyme complex. Further treatment of the penicillin-inactivated enzyme with hydroxylamine or ethylmercaptan reversed the binding. Penicilloyl-hydroxamate or ethylthio-penicilloate were formed and, concomitantly, the carboxypeptidase activity was restored [38]. However, the very large amount of penicillin bound by the preparation and other factors previously discussed with regard to the presumed acylation of the *E. coli* membrane-bound transpeptidase, make it uncertain that inhibition of the particulate *B. subtilis* DD carboxypeptidase is actually mediated by penicilloylation of the active site. Moreover, the inhibition of the purified *Streptomyces* R61 and R39 carboxypeptidases by penicillin was found to be reversible and to occur in the absence of any detectable acylation of the protein by the antibiotic [42 and unpublished results].

Transpeptidase Activity of *Streptomyces* DD Carboxypeptidases [47].

In the presence of a suitable carboxyl donor, such as N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine, and a proper amino acid acceptor, the purified enzymes from *Streptomyces* R61, R39 [47] and K11 [unpublished results] catalyze transpeptidation with concomitant release of the terminal D-alanine of the donor peptide. With either [^{14}C] D-alanine, [^{14}C] glycine or [^{3}H] *meso*-diaminopimelic acid as acceptor, these enzymes catalyze the formation of either diacetyl-L-lysyl-D-alanyl-[^{14}C] D-alanine, diacetyl-L-lysyl-D-alanyl-[^{14}C] glycine or diacetyl-L-lysyl-D-alanyl-D-[^{3}H] *meso*-diaminopimelic acid. In addition, the R61 enzyme is also able to transfer diacetyl-L-lysyl-D-alanine from the tripeptide donor to a glycyl-glycine dipeptide acceptor with the formation of diacetyl-L-lysyl-D-alanyl-glycyl-glycine tetrapeptide. Inhibition of both the carboxypeptidase and transpeptidase activity of the purified *Streptomyces* enzymes occur at the same concentrations of penicillin, providing additional evidence that at least in these *Streptomyces* sp, it is one and the same enzyme which is responsible for both activities. This, together with the ability to catalyze in the absence of an exogenous imput of energy, the synthesis of a C-terminal D-alanyl-D-*meso*-diaminopimelic acid linkage (i.e. the interpeptide bond in many peptidoglycans of chemotype I) and a D-alanyl-glycine bond in an endo-position (i.e. the interpeptide bond in several peptidoglycans of chemotype II) reinforce the idea that these DD carboxypeptidases-transpeptidases could easily be the *exo*forms of the enzymes that effect the closure of the bridges between the peptide units of the nascent peptidoglycans *in vivo*.

At present, the specificity profile for the transpeptidase activity of the *Streptomyces* enzymes is largely unknown. It is already apparent, however, that these enzymes have some specificity in their requirements for acceptors. Whereas D-alanine and glycine are good acceptors, L-alanine is not an acceptor at all and glycyl-glycine is an acceptor only for the R61 enzyme. Moreover, the *albus* G enzyme does not catalyze transpeptidation reactions with the donor-acceptor systems used so far. It is possible that in this instance, the natural *Streptomyces* substrate is required either for donor activity or acceptor activity or both of them, in order to obtain transpeptidation.

With the selected strains of *Streptomyces*, the dose level of penicillin which reduces by 50 % the number of viable single-cell colonies, ranges from 0.25 to 22 µg/ml. Penicillin inhibition of the corresponding soluble DD carboxypeptidases ranges from 0.005 µg/ml to 100,000 µg/ml, i.e. a 10^2 fold increase in the *in vivo* resistance is roughly paralleled by and amplified in a 10^7 fold increase in the *in vitro* resistance. This, of course, poses the problem of the functioning of the enzyme when it is integrated and acting as the transpeptidase, into the membrane. Evidently, the conditions which reside in the enzyme microenvironment in the membrane are far from being comparable to the situation of an aqueous solution where soluble enzyme, substrate and inhibitor are freely mixed. One may assume that a preferential enrichment of the penicillin molecules takes place on the membrane at the site of action or on some restricted areas around it. One may also assume that integration into the membrane imparts to the enzyme a modified tertiary structure (as is observed for example in the cell-bound γ-type penicillinase of *B. cereus* [44]) which is a preferential conformation enhancing responses to penicillin.

On the Diversity of the DD Carboxypeptidases-Transpeptidases.

The donor site in transpeptidation is most likely identical in all bacteria, i.e. a C-terminal D-alanyl-D-alanine sequence. By contrast and owing to the primary structures of the wall peptidoglycans, the structure, the location and the conformation of the acceptor site markedly differ according to the bacterial species. The occurrence of DD carboxypeptidases-transpeptidases of various specificities can thus be anticipated.

Studies of the *Streptomyces* enzymes show that, in addition, the DD carboxypeptidases-transpeptidases from strains within one bacterial genus or even species can exhibit variations affecting a number of parameters.

(1) Most of them are anionic at pH 8 but at least one is cationic at that pH .

(2) They exert endopeptidase activity on peptide oligomers in which the interpeptide bonds are mediated through C-terminal D-alanyl-D linkages. This "endopeptidase" acti-

vity, however, does not necessarily confer upon the enzymes the ability to lyse walls (or cells) containing those interpeptide bonds. Lytic activity seems to require the enzyme to be cationic.

(3) Although, in all cases, the length of the side-chain at the R_3 position is critical for activity, the presence of an α-amino group at this position is not required by all the enzymes, when acting as carboxypeptidase.

(4) The Km values for the good substrates may be high or low and the efficiency of the enzymes, acting as carboxypeptidases, may reflect itself either in the Vmax or the Km value.

(5) The enzymes, acting as transpeptidases, show differencies in their requirements for acceptors.

(6) Penicillin is sometimes but not always, a powerful inhibitor of the enzyme activity and probably acts on sites different from the catalytically active sites.

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