FEBS Symposium, Volume 20, 1970, pp. 59-69

Biochemistry of the Bacterial Wall Peptidoglycan in Relation to the Membrane

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The wall peptidoglycan and the inner plasma membrane are distinct but functionally interdependent organelles. Essentially, the peptidoglycan is a supporting structure which protects the membrane against deleterious influences. In particular, it allows the bacteria to live under environmental conditions which are usually hypotonic. Conversely, the membrane is actively involved in the peptidoglycan synthesis and, probably, in the functioning of several autolytic enzymes (that are possible factors of wall synthesis and regulation), as well as in the enzymatic inactivation of several antibiotics such as benzylpenicillin (which is known to inhibit the peptidoglycan synthesis).

During bacterial growth and division, the peptidoglycan layer undergoes many reactions, i.e. the creation of receptor sites by hydrolysis and the insertion of newly synthesized building blocks. In spite of the fact that the peptidoglycan is one of the most dynamic structures of the whole cell, its mechanical strength is never impaired, at least under well-balanced growth conditions. The biochemical expression of this remarkable property resides in the structure of the peptidoglycan, i.e. a network of glycan strands interlinked through peptide chains.

Essentially, the glycan chains are composed of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid pyranoside residues, i.e. a chitin-like structure, except that every other sugar is substituted by a 3-*O*-D-lactyl group and the average chain length is small (20 to 140 hexosamine residues, according to the bacterial species). The peptide chains are composed of tetrapeptide subunits which substitute through their N-termini the glycan D-lactic acid groups and of peptide bridges which cross-link the tetrapeptide subunits of adjacent glycan chains. The peptide moiety is a small size component with an average size of 1.5 to 10 cross-linked peptide subunits. Many terminal groups thus occur in both the glycan and the peptide parts of the peptidoglycan net, as it is isolated, which probably reflect the dynamics of the bacterial growth.

With the possible exceptions that the residue at the amino terminus is not

always L-alanine but sometimes L-serine or glycine, and that D-glutamic acid may be replaced by a derivative of it, such as 3-hydroxyglutamic acid, the tetrapeptide subunits have the general sequence:

$$\text{L-Ala-}\gamma\text{-D-Glu-}\underset{\displaystyle X}{\underset{|}{\text{CH}}}\text{-D-Ala (Fig. 1).}$$

Amide ammonia, glycine and D-serine may substitute the α-COOH group of D-glutamic acid. Amide ammonia may also substitute the free COOH group of diaminopimelic acid.

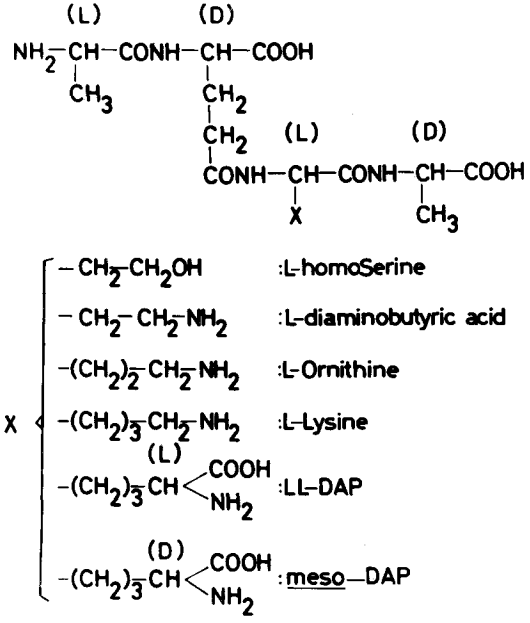


Figure 1. General structure of the tetrapeptide subunits. The L configuration of homoserine in *C. poinsettiae* has been proved by Dr. H. R. Perkins (personal communication).

Four types of cross-linking between the tetrapeptide subunits are known (Ghuysen, 1969). They represent all the possible basal variations unless unexpected mechanisms in the peptidoglycan synthesis were still to be discovered. The cross-linking always involves the C-terminal D-alanine of one peptide subunit and either the free amino group of the diamino acid (types I, II, III) or the α-carboxyl group of D-glutamic acid (type IV) of another peptide subunit. A type IV variant, which has not yet been encountered, might involve the carboxyl

group of diamino-pimelic acid instead of the α-carboxyl group of D-glutamic acid.

Type I (*Escherichia coli*; probably all the Gram negative bacteria) (Fig. 2) is characterized by a direct bond involving the amino group located on the D-carbon of *meso*-diaminopimelic acid, that is to say a D-alanyl-(D)-*meso*-diaminopimelic acid linkage (van Heijenoort *et al.*, 1969). Type II (some *Micrococcus* sp., *Staphylococcus, Gaffkya, Streptococcus, Lactobacillus, Leuconostoc, Streptomyces* sp., *Welchia perfringens*) (Fig. 3) consists in a single

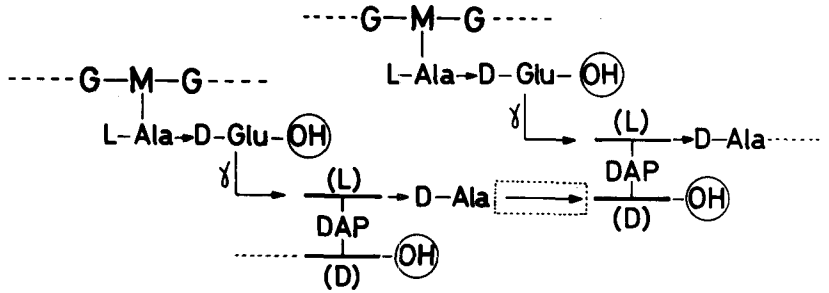


Figure 2. Peptidoglycan of type I. G = *N*-acetylglucosamine; M = *N*-acetylmuramic acid.

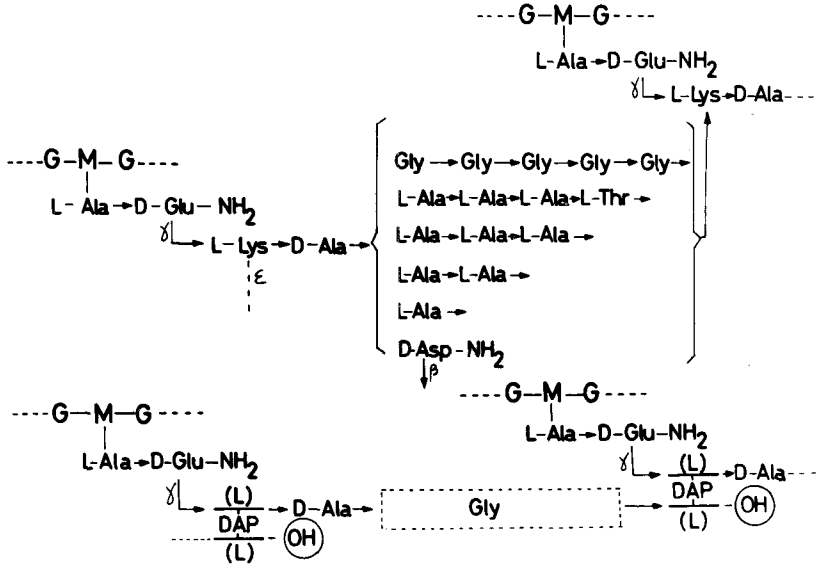


Figure 3. Peptidoglycan of type II. Upper part: see *Bact. Rev.* 1968, 32, 425. A pentaglycine bridge is typical of *Staphylococcus aureus.* Lower part: peptidoglycan of a *Streptomyces* sp. (R. Bonaly and J. M. Ghuysen, unpublished results) and of *Welchii perfringens* (Leyh-Bouille *et al.*, 1970a).

additional amino acid (glycine, L-amino acid, D-isoasparagine) or in a peptide chain (of glycine and/or L-amino acid residues) containing up to five residues, extending between the C-terminal D-alanine and the free amino group of the diamino acid (i.e. L-ornithine, L-lysine or LL-diaminopimelic acid). The α-COOH of the D-glutamic acid is often, if not always, amidated. Type III (*Micrococcus lysodeikticus* and related *Micrococci*) (Fig. 4) is similar to type II except that the peptide cross-linking consists in one or several peptides (interlinked through D-alanyl-L-alanine linkages) each having the same amino acid sequence as the peptide subunit (Campbell *et al.*, 1969), and that the α-COOH of D-glutamic acid is substituted by a glycine residue. The diamino acid is L-lysine. Type IV (Fig. 5) consists in a diamino acid residue such as D-lysine or D-ornithine, extending between the α-COOH of D-glutamic acid and the C-terminal D-alanine to which they are linked through their amino groups. The peptide subunits

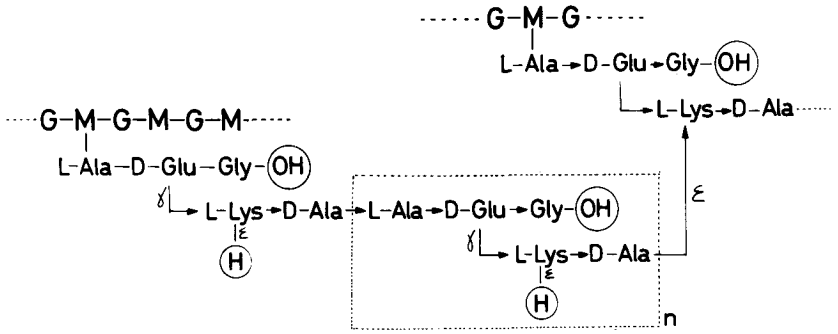


Figure 4. Peptidoglycan of type III.

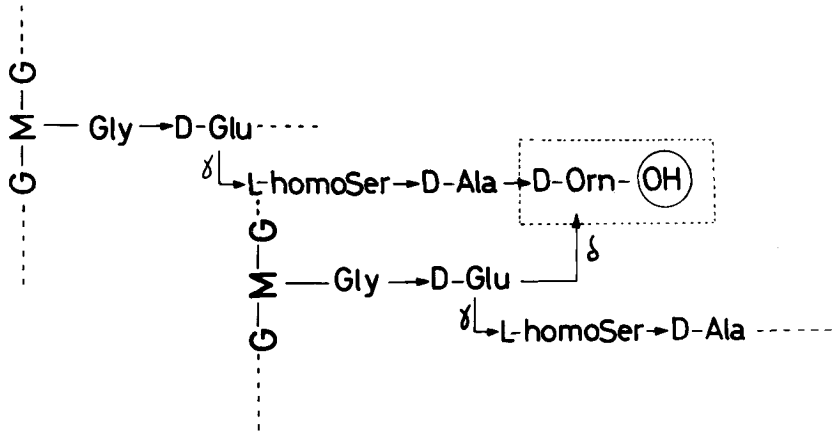


Figure 5. Peptidoglycan of type IV (from Perkins, H. R., 1965).

present rather unusual sequences: N^{α}-(L-Ser-γ-D-Glu)-L-Orn-D-Ala (*Butyribacterium rettgeri*) (Guinand *et al.*, 1969) and Gly-γ-D-Glu-homoSer-D-Ala (plant pathogenic *Corynebacteria*).

Electron microscopy studies strongly suggest that the peptidoglycan sheet in the Gram negative bacteria is a 20 Å-thick network (peptidoglycans of type I). The peptidoglycan layer in the Gram positive *Bacillaceae* is about 100 Å-thick although it is composed of the same *meso*-diaminopimelic acid peptide subunits as those of *E. coli* (van Heijenoort *et al.*, 1969). In one case, that of *B. megaterium* KM, not only *meso* but also DD-diaminopimelic acid residues are present. One may hypothesize that the DD-residues serve to interconnect several superimposed type I, *E. coli*-like, peptidoglycan monolayers. Similarly, in *Micrococcus varians*, the *meso*-diaminopimelic acid-containing peptide subunits appear to be cross-linked through polyglutamic acid bridges (with their amino termini linked to COOH groups of D-alanine) so that the *M. varians* peptidoglycan would actually belong to type II (M. Leyh-Bouille and J. M. Ghuysen, unpublished results).

Several particulate enzymes and a C_{55} polyisoprenoid alcohol monophosphate, which are believed to be parts of the membrane or, at least, to be located on it, are involved in a complex, multiple stage reaction (Fig. 6) which, according to Strominger and his colleagues (Strominger, 1969), deals with: (i) the assembly of the two precursors uridine-5′-pyrophosphoryl-*N*-acetylglucosamine (UDP-GlcNAc) and uridine-5′-pyrophosphoryl-*N*-acetylmuramylpentapeptide:

(L)
(UDP-MurNAc-R_1-γ-D-Glu-CH-D-Ala-D-Ala)
|
X

into β-1,4-GlcNAc-MurNAc-pentapeptide units. In this process, first MurNAc (pentapeptide)-monophosphate is transferred from the nucleotide precursor to the P-C_{55} lipid resulting in the liberation of UMP and the attachment of MurNAc-pentapeptide to the lipid by means of a pyrophosphate bridge. Next, GlcNAc is transglycosylated from UDP-GlcNAc with liberation of UDP and formation of disaccharide (pentapeptide)-P-P-C_{55} lipid. (ii) Possible modifications of the pentapeptide units, such as the amidation of carboxyl groups, the substitution of the α-COOH of D-glutamic acid by an additional amino acid, or the incorporation of those amino acid residues which in the completed peptidoglycan will play the role of "specialized" peptide bridges (types II, III and IV). Usually, the type II bridges incorporation is aminoacyl-tRNA-dependent, though ribosomes seem not to be involved in the process. The mechanisms involved in the incorporation of type III and IV bridges are

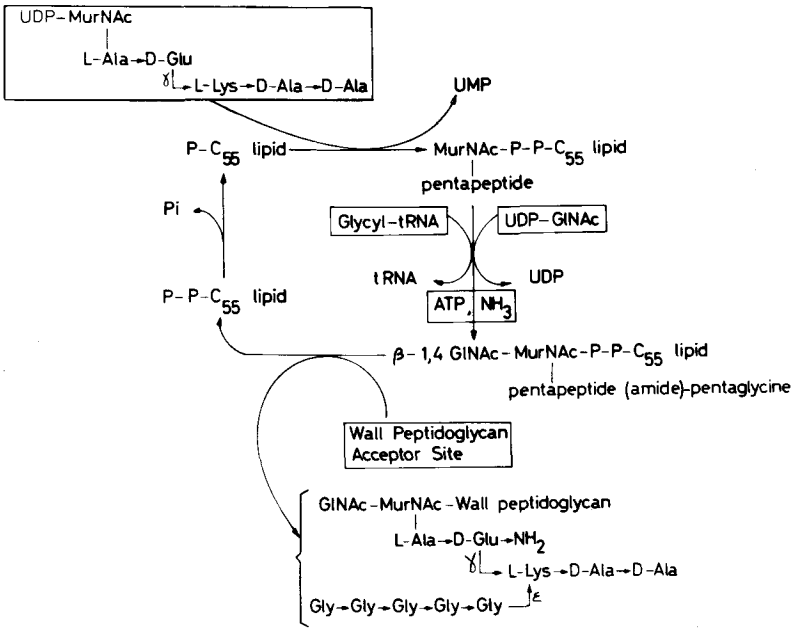


Figure 6. The lipd cycle in *Staphylococcus aureus* with formation of a nascent uncross-linked peptidoglycan (from Strominger, 1969). In *Escherichia coli,* the same cycle occurs except that D-glutamic acid is not amidated and no additional amino acids are incorporated in the pentapeptide moiety.

unknown. (iii) The transfer to the growing wall peptidoglycan of the newly synthesized disaccharide peptide units and their insertion into wall receptor sites. During this transfer, C_{55} lipid pyrophosphate is liberated and dephosphorylated so that the P-C_{55} carrier can begin a new cycle. The wall receptor sites are probably non-reducing *N*-acetylglucosamine termini in the glycan chains. Endo-*N*-acetylmuramidase autolysines may be involved in the creation of such receptors. (iv) The closure of the bridges between the peptide subunits so that the nascent peptidoglycan is being transformed into a rigid two- or three-dimensional network (Fig. 7). This last reaction would be a transpeptidation in which the bond energy of the terminal D-alanyl-D-alanine of one peptide subunit is utilized to transfer the carboxyl group of the penultimate D-alanine residue to the amino acceptor of a second peptide subunit. Concomitantly, the terminal D-alanine is released. The end product is always an insoluble network of glycan chains substituted by cross-linked tetrapeptides and the C-terminal D-alanine residue of these tetrapeptides are always involved in the cross-linking.

None of the membrane-bound enzymes involved in the lipid cycle and bridge

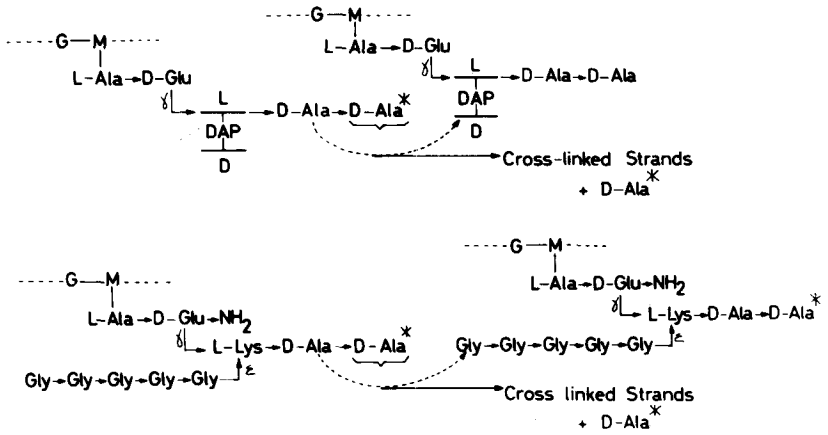
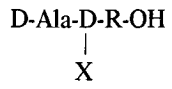


Figure 7. The bridge closure reaction in *Escherichia coli* (upper part, compare with Fig. 2) and in *Staphylococcus aureus* (lower part, compare with Fig. 3) (Strominger, 1969). A direct demonstration of the transpeptidation reaction has only been provided in the case of *Escherichia coli* (Izaki *et al.*, 1968).

closure reactions of the peptidoglycan synthesis pathway has been isolated and purified to the stage of enzymatic homogeneity. Recently, however, a D-alanine carboxypeptidase which releases the terminal D-alanine residue from L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala-D-Ala was detected in a particulate fraction of *E. coli* (Izaki *et al.*, 1968). It was partially purified in the form of a soluble enzyme (Izaki and Strominger, 1968) and was said to be involved in the regulation of the size of the peptide moiety of the *E. coli* peptidoglycan which is known to be very poorly cross-linked. Indeed, the removal at some stage of the peptidoglycan synthesis of the terminal D-alanine residues from some pentapeptides, consequently decreases the number of peptides susceptible to undergo the bridge closure reaction. A similar soluble D-alanine carboxypeptidase was isolated from a *Streptomyces* culture filtrate. In contrast to *E. coli*, the *Streptomyces* peptidoglycan belongs to type II, a single glycine residue being involved in the cross-linking between L-Ala-γ-D-Glu (amide)-(L_1)-LL′-DAP-(L_1)-D-Ala tetrapeptides (Fig. 3). Preliminary results* reveal that the release by the *Streptomyces* carboxypeptidase of the terminal D-alanine residue from pentapeptides L-Ala-γ-D-Glu-R_3-D-Ala-D-Ala is essentially controlled by the structure of the residue or chemical group at the R_3 position (Fig. 8). N^{α}-(L-Ala-γ-D-Glu)-L-Lys-D-Ala-D-Ala is hydrolyzed at a very slow rate. The replacement of L-lysine by N^{ϵ}-(D-isoasparaginyl)-L-lysine, homoserine, *meso*-diaminopimelic acid and N^{ϵ}-

* This research was initiated in collaboration with the Institut de Biochimie, Laboratoire des Peptides, Orsay, France (Dr. E. Bricas) (van Heijenoort *et al.*, 1969). A full report will be presented elsewhere (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970b).

(pentaglycyl)-L-lysine is paralleled by a 6, 15, 20 and 80-fold increase, respectively, of the rate of hydrolysis. Furthermore, the *Streptomyces* enzyme hydrolyses terminal:



linkages (Fig. 9) irrespective of the complexity of the X substituent, so that, in several cases, the carboxypeptidase acts, seemingly, as an endopeptidase. Again,

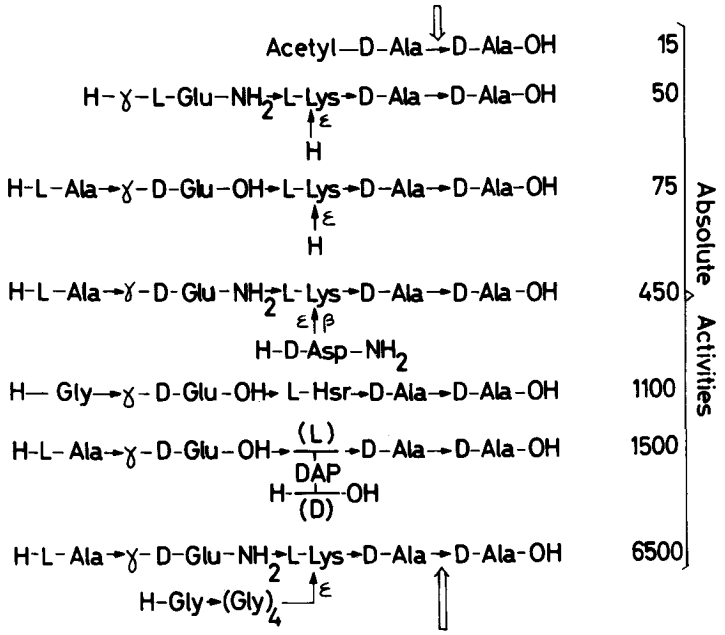


Figure 8. Substrate specificity of *Streptomyces* D-alanine carboxypeptidase. Action on D-alanyl-D-alanine linkages. Results (absolute activity) are expressed in nanoequivalents of hydrolyzed linkages/h/mg of protein. A typical assay mixture contains 15 nanoequiv. of D-Ala-D-Ala linkage, 1 to 10 μg of protein, in a total volume of 30 μl of 0.01 M Veronal buffer, pH 9. N^{α}-(UDP-MurNAc-L-Ala-γ-D-Glu)-L-Lys-D-Ala-D-Ala and UDP-MurNAc-Gly-γ-D-Glu-homoSer-D-Ala-D-Ala were gifts from Dr. H. R. Perkins. UDP-MurNAc-L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala-D-Ala was a gift from Dr. A. J. Garrett. These nucleotides were used to prepare the corresponding pentapeptides. Nucleotides and free pentapeptides have very similar enzymatic sensitivities. Origin of the peptides, from top to bottom: 1, synthetic; 2, synthetic; 3, *S. aureus*, nucleotide; 4, *L. acidophilus*, walls; 5, *C. poinsettiae*, nucleotide; 6, *B. subtilis* W23, nucleotide; 7, *S. aureus*, wall from cells treated with sublethal doses of penicillin.

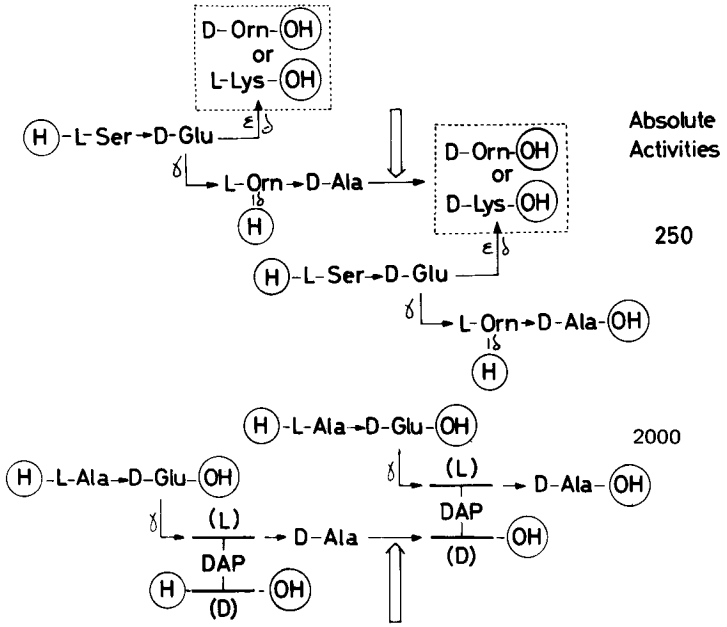


Figure 9. Substrate specificity of *Streptomyces* D-alanine carboxypeptidase. Action on D-Ala → D-R-OH linkages. Results and assays, see Fig. 8. Upper part: dimer from *B. rettgeri*; lower part: dimer from *E. coli*.

the influence of the R_3 residue, i.e. the residue immediately preceding the terminal D-Ala-D-R-OH sequence, seems to be of prime importance since the replacement of L-ornithine by *meso*-diaminopimelic acid results in a 80-fold increase of the rate of hydrolysis of the:

$$\text{D-Ala-D-R-OH linkages.}$$
$$\Big|$$
$$\text{X}$$

All the foregoing examples are consistent with the hypothesis that the presence at the R_3 position of an ω-amino group (N^{ϵ}-L-lysine or N^{δ}-L-ornithine) which is not a possible acceptor for a bridge closure transpeptidation reaction, makes the D-alanyl-D-alanine and more generally the D-Ala-D-R-OH linkages fairly resistant to the carboxypeptidase. Conversely, the introduction at the R_3 position of homoserine (i.e. a neutral amino acid not involved in the transpeptidation; in *C. poinsettiae* the peptide cross-linking involves the α-amino group of a D-ornithine residue which substitutes the α-COOH of D-glutamic acid, see Fig. 5) or of an

α-amino group that can be utilized in the bridge closure reaction, considerably enhances the sensitivity of the substrates to the carboxypeptidase.

To all appearances and from the scanty information so far available (Izaki and Strominger, 1968), the *E. coli* carboxypeptidase has substrate specificities similar to those of the *Streptomyces* enzyme. Particularly, UDP-MurNAc-L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala-D-Ala is again a much better substrate for the *E. coli* enzyme than N^{α}-(UDP-MurNAc-L-Ala-γ-D-Glu)-L-Lys-D-Ala-D-Ala. Moreover, the Michaelis constants of both the *E. coli* and the *Streptomyces* enzymes for UDP-MurNAc-L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala-D-Ala are virtually identical (6 and 8×10^{-4} M respectively). From the foregoing, one can visualize both enzymes as being able to act either as carboxypeptidases (or seemingly, as endopeptidases), i.e. catalyzing a reaction which involves water and results in hydrolysis, when they are in a solubilized form (as they were isolated), or as transpeptidases, i.e. catalyzing a reaction which involves a specific acceptor in place of water and results in α-peptide linkage synthesis, when, possibly, they are bound *in situ* to some lipoprotein constituents at the exterior of the plasma membrane.

The *Streptomyces* carboxypeptidase was isolated from an organism very resistant to penicillins and related antibiotics. *Streptomyces* growth inhibition required at least 500 μg/ml of Cephalothin, 1000 μg/ml of Penicillin G, and 10,000 μg/ml of Oxacillin. *In vitro* assays under the conditions described in Fig. 8 and using N^{α}-[GlcNAc-MurNAc-L-Ala-γ-D-Glu (amide], N^{ϵ}-$(Gly)_5$-L-Lys-D-Ala-D-Ala as substrate, showed that a 50% inhibition of the carboxypeptidase activity was performed by cephalothin and penicillin G at molar ratios, antibiotic to substrate, of 15 and 70 to 1, respectively. Oxacillin at a molar ratio of 70 to 1, did not exhibit any inhibitory effect at all. Since the selected antibiotics are known to inhibit the transpeptidation reaction, the above parallelism between the *in vivo* and *in vitro* inhibition tests strengthens the hypothesis that the carboxypeptidase is indeed the transpeptidase which has undergone solubilization. All in all, however, much remains to be done to definitely ascertain our proposal.

In contrast to the *Streptomyces* enzyme, the *E. coli* carboxypeptidase is exceedingly sensitive to penicillins. Though they exhibit similar, perhaps identical, catalytic activities, the *Streptomyces* and the *E. coli* carboxypeptidases present quite different affinities for penicillins and related antibiotics. The fact that the *Streptomyces* "DD carboxypeptidase-transpeptidase" system is not inhibited by penicillins seems to be at variance with the idea (Tipper and Strominger, 1965) that an analogy between penicillins and the conformation of acyl-D-alanyl-D-alanine would be the molecular basis of the antibacterial action of these antibiotics. It shows, at least, that such an analogy is not universal among bacteria. (For a full discussion; see Leyh-Bouille *et al.*, 1970b).

SUMMARY

The peptidoglycan physically protects the membrane against deleterious influences. The membrane is involved in peptidoglycan synthesis and in other wall regulation mechanisms. The possible physiological function of a soluble carboxypeptidase active on acyl-D-alanyl-D-R-OH substrates and its relation to the membrane-bound transpeptidase involved in peptidoglycan synthesis are discussed.

ACKNOWLEDGMENTS

This investigation was supported by grant ER-E4-10-2 from the U.S. Department of Agriculture under Public Law 480 and by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium Contrats no. 515 and 1000.

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