Peptidoglycan Carboxypeptidase and Endopeptidase Activities of *Bacillus coagulans* NCIB 9365

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The D,D-carboxypeptidase of *Bacillus coagulans* was exclusively associated with the proto-plast membrane. The enzyme had a pH optimum of 4.9 and was sensitive to thiol reagents. The D,D-carboxypeptidase was stimulated by most divalent cations, Pb^{2+} and Cd^{2+} providing the greatest degree of activation, but it was inhibited by Hg^{2+} and Fe^{2+} . A particulate L,D-carboxypeptidase was demonstrated in membrane preparations which were also able to catalyse a simple transpeptidation reaction employing D-alanine as the carboxyl acceptor. An endopeptidase activity capable of liberating D-alanyl-D-alanine from UDP-N-acetylmuramyl-L-alanyl- γ -D-glutamyl-meso-2,6-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide) was present in the cytoplasm. This appeared to be the reverse reaction of the D-alanyl-D-alanine-adding enzyme responsible for the generation of the peptidoglycan precursor UDP-MurNAc-pentapeptide.

INTRODUCTION

D-Alanyl-D-alanine carboxypeptidases (D,D-carboxypeptidase, carboxypeptidase I) catalyse the liberation of the terminal D-alanine residue from UDP-N-acetylmuramyl-L-alanyl- γ -D-glutamyl-meso-2,6-diaminopimelyl-D-alanyl-D-alanine (abbreviated to UDP-MurNAc-L-Ala-D-Glu-ms-A₂pm-D-Ala-D-Ala and also designated UDP-MurNAc-pentapeptide) and related substrates. Like other activities involved in the final stages of peptidoglycan biosynthesis, namely transpeptidases and endopeptidases, these enzymes represent specific sites of action of β -lactam antibiotics.

D,D-Carboxypeptidases have been studied in a wide variety of bacterial systems (reviewed by Blumberg & Strominger, 1974; Ghuysen *et al.*, 1974; Ghuysen, 1977) and in the majority of these the enzyme activity is associated with the cytoplasmic membrane. However, in some strains of *Escherichia coli* much of the enzyme has been found in the cytoplasm (Tamura *et al.*, 1976), while the streptomycetes are apparently unique in that the D,D-carboxypeptidase/transpeptidase is excreted into the growth medium (Ghuysen *et al.*, 1974).

Most membrane preparations have additionally been shown to perform L,D-carboxypeptidase (carboxypeptidase II) reactions, measured as the removal of D-alanine from UDP-MurNAc-L-Ala-D-Glu-ms-A₂pm-D-Ala generated by the prior action of D,D-carboxypeptidase (Blumberg & Strominger, 1974). In some instances transpeptidation reactions of varying degrees of complexity ('natural', 'natural model' and 'unnatural model' transpeptidases) have also been demonstrated (Izaki *et al.*, 1968; Reynolds, 1971; Wickus & Strominger, 1972; Ghuysen *et al.*, 1974; Nguyen-Distèche *et al.*, 1974; Reynolds & Barnett, 1974; Yocum *et al.*, 1974; Marquet *et al.*, 1976; Shepherd *et al.*, 1977).

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The present paper describes investigations of the peptidase activities associated with the facultatively thermophilic micro-organism *Bacillus coagulans* NCIB 9365 with particular reference to the D,D-carboxypeptidase.

METHODS

Bacterial strain and growth conditions. *Bacillus coagulans* NCIB 9365 (ATCC 7050) was obtained from the National Collection of Industrial Bacteria. The growth medium contained (per litre): 10 g tryptone (Difco Bacto), 5 g yeast extract (Difco Bacto), 2.5 g K_2HPO_4 , 1 g glucose; the pH was adjusted to 7.2. The organisms were cultured in 2 l Erlenmeyer flasks containing 1 l medium, in an orbital incubator (250 rev. min⁻¹) at 55 °C.

Substrates. The following substrates were used: (a) D-[¹⁴C]Ala-D-[¹⁴C]Ala; (b) UDP-MurNAc-L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala; (c) UDP-MurNAc-L-Ala-D-Glu-*ms*-A₂pm-D-Ala-D-Ala; (d) UDP-MurNAc-L-Ala-D-Glu-*ms*-A₂pm-D-Ala-D-[¹⁴C]Ala; (e) L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala. With the exception of substrate (d), which was a gift from Professor H. R. Perkins (University of Liverpool), all substrates were prepared according to previously published procedures (Reynolds, 1971; Barnett, 1973; Nguyen-Distèche *et al.*, 1974).

Preparation of enzyme fractions. Bacteria in the exponential phase of growth were harvested and washed twice with 0.05 M-Tris/HCl, 10 mM-MgCl₂, pH 7.2 (TM buffer). Particulate and supernatant fractions were prepared from them by two alternative methods.

(i) Protoplast membranes. Bacteria were resuspended at a density of 10 mg dry wt ml⁻¹ in TM buffer, treated with lysozyme (250 µg ml⁻¹) and incubated at 37 °C for 15 min. Deoxyribonuclease (5 µg ml⁻¹) and ribonuclease (10 µg ml⁻¹) were added and the incubation was continued for a further 15 min. All subsequent steps were performed at 4 °C.

Any remaining whole organisms and large debris were removed by centrifugation at 8000 g for 10 min; the membrane fraction was then sedimented from the supernatant fraction by centrifugation at 38000 g for 20 min. The protoplast membranes were washed twice by resuspension in TM buffer with the aid of a Potter-Elvehjem tissue homogenizer and centrifugation at 38000 g for 20 min. The washed membranes were then resuspended at a protein concentration of 2 to 15 mg ml⁻¹ in the appropriate buffer conditions required for each experiment. Such preparations could be stored at -20 °C for at least 18 months without any loss of D,D-carboxypeptidase activity.

Protoplast membranes in 0.05 M-Tris/HCl, pH 7.2 were treated with EDTA (50 mM final concentration) at 20 °C for 30 min and then dialysed exhaustively against the appropriate buffer at 4 °C to obtain the cation-depleted enzyme.

(ii) Sonication. Washed organisms were resuspended at a density of 10 mg dry wt ml⁻¹ in TM buffer and subjected to sonication for 3 min in a Mullard E7590B Sonicator at 500 W. Removal of whole organisms and the subsequent preparation of the particulate and supernatant enzymes was as described above.

Assay of D,D-carboxypeptidase activity. The standard assay mixture contained 20 mM-sodium acetate/acetic acid, pH 4.9, 15 mM-MnCl₂, 2.16 mM-substrate and particulate enzyme, in a total volume of 25 µl. Samples were incubated at 37 °C for 30 min and the reaction was terminated by heating at 100 °C for 2 min. All assays were performed in duplicate. Reaction products were separated by chromatography in solvent A (substrates *b* and *d*) or by electrophoresis at pH 6.5 (substrate *e*). Thereafter enzyme activity was assessed as described by Barnett (1973). Enzyme activities are defined in terms of International Units (U), where one unit is equivalent to the liberation of 1 µmol D-alanine min⁻¹.

Protein estimation. Protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Sodium dodecyl sulphate (1%, w/v) was added to the stock solutions to prevent precipitate formation during assay in the presence of detergents (Yocum *et al.*, 1974).

Paper chromatography. Solvent mixtures employed were: (A) isobutyric acid/1 M-NH₄OH (5:3, v/v); (B) 2-propanol/acetic acid/H₂O (75:10:15, by vol.); (C) 2-picoline/25% (v/v) NH₄OH/H₂O (70:2:28, by vol.).

High voltage electrophoresis. Buffers for electrophoresis were: pH 2.0, acetic acid/formic acid/H₂O (8:2:90, by vol.); pH 6.5, pyridine/acetic acid/H₂O (800:24:7200, by vol.). Reaction products were separated by electrophoresis on Whatman 3MM paper at 60 V cm⁻¹ for 60 min.

Chemicals. Bovine serum albumin was obtained from Armour Pharmaceuticals, Eastbourne, Sussex. Lysozyme and ribonuclease IA were from Sigma, deoxyribonuclease I from Miles Seravac, Maidenhead, Berks, and D-[¹⁴C]alanine from The Radiochemical Centre, Amersham.

Fig. 1. Procedure for cellular fractionation of *B. coagulans*.

RESULTS

Detection of endopeptidase activity

In initial experiments with UDP-MurNAc-L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala (substrate *b*) using sonicated cell supernatants as a source of D,D-carboxypeptidase activity, we found, in addition to D-[¹⁴C]alanine, a further radioactive product (*R*_{Ala} 1.2) when chromatograms were developed in solvent A. This radioactive material was removed from chromatograms by overnight elution with distilled water and subjected to electrophoresis at pH 2.0, two-dimensional descending paper chromatography in solvents B and C, and rechromatography in solvent A. It consisted of a single component with mobilities identical to those of a D-[¹⁴C]alanyl-D-[¹⁴C]alanine standard. Hydrolysis of the eluate (6 M-HCl for 16 h at 105 °C) followed by similar chromatographic and electrophoretic procedures gave rise to a single product with the mobility of D-[¹⁴C]alanine. This endopeptidase activity therefore appears to catalyse the removal of D-alanyl-D-alanine from UDP-MurNAc-pentapeptide.

Cellular location of carboxypeptidase activities

Bacillus coagulans was fractionated according to the scheme in Fig. 1. D,D-Carboxypeptidase activity was negligible in the dialysed culture filtrate, the dialysed lysozyme digest and the protoplast lysate; essentially all the activity remained associated with the protoplast membrane fraction. However, endopeptidase activity was located exclusively in the cytoplasm.

L,D-Carboxypeptidase activity and the linearity of D-alanine release

More than 1 mol D-[¹⁴C]alanine was released per mol substrate (*b*) by protoplast membrane preparations. This implied that some of the penultimate D-alanine was released as a

Fig. 2. Release of D- $[^{14}\text{C}]$ alanine from doubly- or terminally-labelled UDP-MurNac-pentapeptide. Substrates UDP-MurNac-L-Ala-D-Glu-*ms*-A₂pm-D- $[^{14}\text{C}]$ Ala-D- $[^{14}\text{C}]$ Ala (b) or UDP-MurNac-L-Ala-D-Glu-*ms*-A₂pm-D-Ala-D- $[^{14}\text{C}]$ Ala (d) were incubated with protoplast membranes (64 μg protein). Results are expressed as the percentage of the total available D-alanine released as D- $[^{14}\text{C}]$ alanine. Maximum release of radioactivity corresponded to 103.2 and 56.6 nmol D- $[^{14}\text{C}]$ alanine for substrates (b) and (d) respectively.

result of L,D-carboxypeptidase activity in addition to the D,D-carboxypeptidase. The difficulty of obtaining UDP-MurNac-L-Ala-D-Glu-*ms*-A₂pm-D- $[^{14}\text{C}]$ Ala prevented its use as a substrate for the assay of L,D-carboxypeptidase alone; thus although this activity is undoubtedly present in the membrane it might also occur in other cellular fractions.

The concomitant liberation of the penultimate D-alanine by L,D-carboxypeptidase could interfere with the estimate of D,D-carboxypeptidase, and hence the effect of the former enzyme was studied in greater detail (Fig. 2). The release of radioactivity from either the doubly-labelled substrate (b) or the terminally-labelled substrate (d) was linear to approximately 30% of the total available D-alanine. The curves for the release of D- $[^{14}\text{C}]$ alanine from substrates (b) and (d) were also coincident for the linear portion of the graph indicating the absence of L,D-carboxypeptidase activity under these conditions, and that only D,D-carboxypeptidase was being measured. In fact, release of the penultimate D-alanine residue was negligible by the time the D,D-carboxypeptidase reaction approached completion, and only about 60% of the total D- $[^{14}\text{C}]$ alanine had been released from substrate (b) after 16 h incubation.

A linear release of 30% of the total D-alanine was also found with L-Ala-D-Glu-*ms*-A₂pm-D- $[^{14}\text{C}]$ Ala-D- $[^{14}\text{C}]$ Ala (substrate e) and was valid for substrate concentrations up to at least 7.0 mM, and with final membrane concentrations up to at least 6.5 mg protein ml⁻¹.

The initial rates of reaction of the D,D-carboxypeptidase alone could therefore be determined by ensuring that less than 25% of the available D-alanine of the substrate was released in each incubation.

Experiments on the possible function of the endopeptidase

The time course (Fig. 3) of the release of D- $[^{14}\text{C}]$ alanine and D- $[^{14}\text{C}]$ alanyl-D- $[^{14}\text{C}]$ alanine from substrate (b) by a sonicated cell supernatant showed that the release of D-alanyl-D-alanine quickly reached a maximum but the amount of product decreased with continued incubation. This could result from the hydrolysis of D-alanyl-D-alanine by the D,D-carboxypeptidase, although such hydrolysis was not detected when the dipeptide was used as the sole source of substrate in control incubations (240 min). The maximum release of D-alanyl-D-alanine was about 30% of the total D-alanine of the substrate; thus the peak of D-alanyl-D-alanine release was unlikely to have been due to substrate limitation.

To investigate possible requirements for the enzyme, a sonicated cell supernatant was initially dialysed overnight against 0.05 M-Tris/HCl, pH 7.2, and was then used as an enzyme source. Endopeptidase activity was estimated after incubation at 37 °C for 10 min; D,D-carboxypeptidase activity was negligible during this time.

Fig. 3. Time course of the concurrent release of D-alanyl-D-alanine and D-alanine from UDP-MurNAC-pentapeptide. Incubation mixtures contained 0.05 M-Tris/HCl, pH 7.2, 10 mM-MgCl₂, 1.7 mM-UDP-MurNAC-L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala (substrate *b*) and sonicated cell supernatant (80 µg protein), in a total volume of 25 µl. Samples were incubated at 37 °C for the times indicated. Results are expressed as the percentage of the total D-alanine released as either D-[¹⁴C]-alanyl-D-[¹⁴C]alanine (●) or D-[¹⁴C]alanine (○).

Enzyme activity was stimulated considerably when supplied with 2.5 mM-ADP or 5 mM-Mg²⁺ (seven- and fivefold, respectively), and stimulated slightly by 5 mM inorganic phosphate (twofold). An almost additive increase in activity was found when any or all of these compounds were present in combination. Co²⁺ (twofold stimulation) was not as effective as Mg²⁺. In order that this activity should not interfere in subsequent investigations, protoplast membranes were used as the source of D,D-carboxypeptidase.

pH and ionic strength optima and kinetic parameters of D,D-carboxypeptidase

The effect of pH on the reaction rate in the presence or absence of 5 mM-Mg²⁺ was determined between pH 3.7 and 8.9 (pH 3.7 to 5.6, sodium acetate/acetic acid; pH 5.0 to 7.4, sodium cacodylate/HCl; pH 7.1 to 8.9, Tris/HCl). An optimum at pH 4.9 was found in both instances. The values of enzyme activity obtained in the overlapping buffer ranges were comparable, indicating that none of the buffers were inhibitory relative to one another. Fresh protoplast membranes gave identical activity profiles; the pH optimum was therefore unaffected by the depletion procedure. The ionic strength optimum for the sodium acetate buffer was 5 to 10 mM; enzyme activity was inhibited slightly at higher buffer concentrations.

The kinetic parameters of the membrane-bound D,D-carboxypeptidase for the substrates UDP-MurNAC-L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala (*b*) and L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala (*e*) under standard assay conditions were determined by Lineweaver-Burk plots. *K_m* values were 4.8 and 10 mM for substrates (*b*) and (*e*) respectively, while *V* values were 15.2 and 13.3 mU (mg protein)⁻¹.

Metal ion requirements

The effect of a wide range of cations on the activity of the membrane-bound D,D-carboxypeptidase was investigated. All cations (10 mM final concentration) were supplied in the chloride form except Fe²⁺, Cd²⁺, Pb²⁺ and Al³⁺ which were present as NO₃⁻, CH₃COO⁻, NO₃⁻ and SO₄²⁻, respectively. The D,D-carboxypeptidase did not demonstrate an absolute requirement for added cations [activity in their absence being 1.54 mU (mg protein)⁻¹], but a number of metal ions caused considerable stimulation. Surprisingly, the most effective cation tested was Pb²⁺ which stimulated activity sevenfold; this stimulation was up to two-fold greater than that of any other cation tested. A fivefold activation was found with Cd²⁺ and Cu²⁺, while Mn²⁺, Co²⁺, Ni²⁺ and Zn²⁺ led to a three- to fourfold increase in enzyme

Fig. 4. Time course of the unnatural model transpeptidation reaction. Particulate membranes were incubated with donor, UDP-MurNAC-L-Ala-D-Glu-*ms*-A₂pm-D-Ala-D-Ala (0.5 mM), and acceptor, D-[¹⁴C]alanine [2.5 mM (○) or 8.16 mM (●)], for various times. The extent of hydrolysis of the donor by D,D-carboxypeptidase activity was determined by incubating UDP-MurNAC-L-Ala-D-Glu-*ms*-A₂pm-D-[¹⁴C]Ala-D-[¹⁴C]Ala in the absence of acceptor (■).

activity. Lesser degrees of stimulation were obtained with Mg²⁺, Ca²⁺, Ba²⁺ and Sn²⁺, while the activity was inhibited 90% by Hg²⁺ and Fe²⁺. Although the monovalent cations tested (Li⁺, Na⁺, K⁺ and NH₄⁺) had little effect on activity, stimulatory effects were not confined to divalent compounds; Al³⁺ caused a three- to fourfold stimulation in activity although Fe³⁺, like the divalent form, was inhibitory at the concentration employed.

The stimulation by Pb(NO₃)₂ was not due to the anionic component, as comparable values were obtained with Pb(CH₃COO)₂. Similarly no significant differences in stimulation were obtained between the chloride and nitrate salts of Zn²⁺ or Co²⁺. Maximal activity for each divalent cation was reached at a concentration of about 15 mM.

Thermal stability of the membrane-bound D,D-carboxypeptidase

Membrane preparations were incubated at the appropriate temperature for 30 min prior to the assay of enzyme activity at 37 °C. No significant reduction in activity was observed after preincubation at temperatures of up to 50 °C. Although the enzyme was still quite stable up to 55 °C where more than 70% of the activity was retained, above this temperature there was a steep decline in thermal stability. The particulate D,D-carboxypeptidase was considerably more thermostable than the enzyme in the mesophilic organism *Bacillus megaterium* (Diaz-Mauriño *et al.*, 1974; Marquet *et al.*, 1976).

Effect of thiol reagents

The effect of several reagents which react with thiol groups was examined. The particulate D,D-carboxypeptidase was not significantly inhibited by 0.1 M-*N*-ethylmaleimide, although a similar concentration of iodacetate resulted in almost total inactivation. The enzyme was, however, inhibited considerably by lower concentrations of 5,5'-dithiobis(2-nitrobenzoic acid) (96% inhibition at 18 mM) and *p*-hydroxymercuribenzoate (63% inhibition at 2.5 mM).

Transpeptidation reactions

Particulate membrane preparations were unable to catalyse either natural transpeptidation or natural model transpeptidation reactions when assayed by the methods of Reynolds (1971) or Nguyen-Distèche *et al.* (1974), respectively. However, protoplast membranes

contained an unnatural model transpeptidase (Fig. 4). The extent of product formation was dependent on the acceptor concentration. The amount of transpeptidated product increased initially with time but then slowly decreased as the radioactive nucleotide pentapeptide was degraded; by implication such degradation would have been due to D,D-carboxypeptidase activity. Transpeptidation was, however, extremely low relative to D,D-carboxypeptidase activity assayed under identical conditions in the absence of acceptor.

DISCUSSION

Four peptidase activities were found in *Bacillus coagulans*: D,D- and L,D-carboxypeptidases and an unnatural model transpeptidase, which were membrane-bound, and a soluble endopeptidase.

The D,D-carboxypeptidase displayed properties resembling those found in other bacterial systems, especially those in other *Bacillus* species (Umbreit & Strominger, 1973; Barnett, 1973; Yocum *et al.*, 1974; Diaz-Mauriño *et al.*, 1974). Thus the enzyme demonstrates a low pH optimum, similar K_m values, inhibition by thiol reagents and an unnatural model transpeptidase activity. In most instances where this latter activity has been reported it appears to be an additional reaction catalysed by the D,D-carboxypeptidase (Nguyen-Distèche *et al.*, 1974; Reynolds & Barnett, 1974; Ghuysen *et al.*, 1974; Yocum *et al.*, 1974; Tamura *et al.*, 1976; Marquet *et al.*, 1976).

The majority of D,D-carboxypeptidases, although not displaying an absolute requirement for metal ions, are stimulated by divalent cations. However, the degree of stimulation afforded by a particular cation shows considerable species specificity except that, where it has been tested, Hg^{2+} is always a strong inhibitor. The maximal activation observed with Pb^{2+} is of significance. In the only other instance where Pb^{2+} has been tested on a D,D-carboxypeptidase, that of *B. megaterium* (Diaz-Mauriño *et al.*, 1974; Marquet *et al.*, 1976), the enzyme was inhibited some 25% with respect to controls lacking cations, and like other heavy metals, Pb^{2+} at high concentrations inactivates most enzymes. Previous reports of possible enzyme activation by Pb^{2+} are few (Stickland, 1949; Vallee & Ulmer, 1972; Farkas, 1975; Sabbioni *et al.*, 1976), and can mostly be explained by either a partial reactivation of enzyme activity after removal of an essential cation, or an activation totally dependent on the presence of another cation. It has been reported that no metal with an atomic number greater than 55 can activate enzymes by itself (Dixon & Webb, 1964).

Studies on the peptidoglycan composition of *B. coagulans* NCIB 9365 (H. A. I. McArthur, unpublished results; O. Kandler, personal communication) have shown it to contain the amino acids D-glutamic acid, alanine and *meso*-diaminopimelic acid in the ratio 1.00:1.40:0.99. Only 0.4 mol of the alanine was the D-isomer, while about 40% of the *meso*-diaminopimelic acid was involved in cross-linkage. This indicates that all the D-alanine residues of the peptidoglycan which are not involved in cross-linkages have been removed by peptidase activity. In *B. coagulans* this can, in theory, occur by the combined activities of the D,D- and L,D-carboxypeptidases, or in a single step by the endopeptidase. The main objection to the latter reaction operating *in vivo* is its cytoplasmic location. This would apparently preclude peptidoglycan from being the native substrate of the enzyme on purely spatial grounds.

Reactions catalysed by several amino acid-adding enzymes involved in the synthesis of the peptidoglycan precursor UDP-MurNAc-pentapeptide are reversible (Nathenson *et al.*, 1964; Mizuno & Ito, 1968; Carpenter & Neuhaus, 1972; Egan *et al.*, 1973; Oppenheim & Patchornik, 1974) and catalyse the general reaction:

Thus endopeptidase activities from *Bacillus subtilis* (Egan *et al.*, 1973) and *Staphylococcus aureus* and *Streptococcus faecalis* (Oppenheim & Patchornik, 1974) which liberate

D-alanyl-D-alanine from UDP-MurNAc-pentapeptide were found to be associated with the ligase which catalysed the addition of the dipeptide to UDP-MurNAc-tripeptide; although in *Escherichia coli* K12 (Gondré *et al.*, 1973) these reactions were separate. It is likely that the endopeptidase of *B. coagulans* was also not concerned with the formation of tripeptide subunits in the peptidoglycan but was a manifestation of the reversibility of the D-alanyl-D-alanine-adding enzyme. Thus the rapidly reached peak of D-alanyl-D-alanine release in time course experiments (Fig. 3) could be due to the formation of an equilibrium between the hydrolytic and synthetic reactions according to the scheme:

Gradual removal of the pentapeptide by the concomitant activity of the D,D-carboxypeptidase would alter this equilibrium in favour of the biosynthetic reaction and account for the decrease in D-alanyl-D-alanine with continued incubation.

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REFERENCES

- BARNETT, H. J. (1973). D-Alanine carboxypeptidases of *Bacillus stearothermophilus*: solubilization of particulate enzymes and mechanism of action of penicillin. *Biochimica et biophysica acta* **304**, 332–352.
- BLUMBERG, P. M. & STROMINGER, J. L. (1974). Interaction of penicillin with the bacterial cell: penicillin binding proteins and penicillin-sensitive enzymes. *Bacteriological Reviews* **38**, 291–335.
- CARPENTER, C. V. & NEUHAUS, F. C. (1972). Enzymatic synthesis of D-alanyl-D-alanine. Two binding modes for product on D-alanine:D-alanine ligase (ADP). *Biochemistry* **11**, 2594–2598.
- DIAZ-MAURIÑO, T., NIETO, M. & PERKINS, H. R. (1974). Membrane-bound D,D-carboxypeptidases from *Bacillus megaterium* KM. *Biochemical Journal* **143**, 391–402.
- DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd edn, pp. 421–422. London: Longmans Green & Co.
- EGAN, A., LAWRENCE, P. J. & STROMINGER, J. L. (1973). Enzymatic synthesis of the peptide in bacterial uridine nucleotides. V. Co^{2+} -dependent reversal of peptide bond formation. *Journal of Biological Chemistry* **248**, 3122–3130.
- FARKAS, W. R. (1975). The effects of plumbous ion on protein biosynthesis in reticulocytes. *Research Communications in Chemical Pathology and Pharmacology* **10**, 127–148.
- GHUYSEN, J. M., LÉYH-BOUILLE, M., FRÈRE, J. M., DUSART, J., MARQUET, A., PERKINS, H. R. & NIETO, M. (1974). The penicillin receptor in Streptomyces. *Annals of the New York Academy of Sciences* **235**, 236–266.
- GHUYSEN, J. M. (1977). The concept of the penicillin target from 1965 until today. *Journal of General Microbiology* **101**, 13–33.
- GONDRE, G., FLOURET, B. & VAN HEIJENOORT, J. (1973). Release of D-alanyl-D-alanine from the precursor of the cell wall peptidoglycan by a peptidase of *Escherichia coli* K12. *Biochimie* **55**, 685–691.
- IZAKI, K., MATSUHASHI, M. & STROMINGER, J. L. (1968). Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions in strains of *Escherichia coli*. *Journal of Biological Chemistry* **243**, 3180–3192.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MARQUET, A., NIETO, M. & DIAZ-MAURIÑO, T. (1976). Membrane-bound D,D-carboxypeptidase and transpeptidase activities from *Bacillus megaterium* KM at pH 7. *European Journal of Biochemistry* **68**, 581–589.
- MIZUNO, Y. & ITO, E. (1968). Purification and properties of uridine diphosphate N-acetylmuramyl-L-alanyl-D-glutamate: meso-2,6-diaminopimelate ligase. *Journal of Biological Chemistry* **243**, 2665–2672.
- NATHENSON, S. G., STROMINGER, J. L. & ITO, E. (1964). Enzymatic synthesis of the peptide in bacterial uridine nucleotides. IV. Purification and properties of D-glutamic acid-adding enzyme. *Journal of Biological Chemistry* **239**, 1773–1776.
- NGUYEN-DISTÈCHE, M., GHUYSEN, J. M., POLLOCK, J. J., REYNOLDS, P. E., PERKINS, H. R., COYETTE, J. & SALTON, M. R. J. (1974). Enzymes involved in wall peptide cross-linking in *Escherichia coli* K12, strain 44. *European Journal of Biochemistry* **41**, 447–455.

- OPPENHEIM, B. & PATCHORNIK, A. (1974). Formation of D-alanyl-D-alanine and D-alanine from UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala by extracts of *Staphylococcus aureus* and *Streptococcus faecalis*. *FEBS Letters* **45**, 172-175.
- REYNOLDS, P. E. (1971). Peptidoglycan synthesis in bacilli. I. Effect of temperature on the *in vitro* system from *Bacillus megaterium* and *Bacillus stearothermophilus*. *Biochimica et biophysica acta* **237**, 239-254.
- REYNOLDS, P. E. & BARNETT, H. J. (1974). Transpeptidases and D,D-carboxypeptidases in bacilli. *Annals of the New York Academy of Sciences* **235**, 269-282.
- SABBIONI, E., GIRARDI, F. & MARAFANTE, E. (1976). Replacement of metal in metalloenzymes. A lead-alkaline phosphatase. *Biochemistry* **15**, 271-276.
- SHEPHERD, S. T., CHASE, H. A. & REYNOLDS, P. E. (1977). The separation and properties of two penicillin-binding proteins from *Salmonella typhimurium*. *European Journal of Biochemistry* **78**, 521-532.
- STICKLAND, L. H. (1949). The activation of phosphoglucomutase by metal ions. *Biochemical Journal* **44**, 190-197.
- TAMURA, T., IMAE, Y. & STROMINGER, J. L. (1976). Purification to homogeneity and properties of two D-alanine carboxypeptidases I from *Escherichia coli*. *Journal of Biological Chemistry* **251**, 414-423.
- UMBREIT, J. N. & STROMINGER, J. L. (1973). D-Alanine carboxypeptidase from *Bacillus subtilis* membranes. I. Purification and characterization. *Journal of Biological Chemistry* **248**, 6759-6766.
- VALLEE, B. L. & ULMER, D. D. (1972). Biochemical effects of mercury, cadmium, and lead. *Annual Review of Biochemistry* **41**, 91-128.
- WICKUS, G. G. & STROMINGER, J. L. (1972). Penicillin-sensitive transpeptidation during peptidoglycan biosynthesis in cell-free preparations from *Bacillus megaterium*. I. Incorporation of free diaminopimelic acid into peptidoglycan. *Journal of Biological Chemistry* **247**, 5297-5306.
- YOCUM, R. R., BLUMBERG, P. M. & STROMINGER, J. L. (1974). Purification and characterization of the thermophilic D-alanine carboxypeptidase from membranes of *Bacillus stearothermophilus*. *Journal of Biological Chemistry* **249**, 4863-4871.