**The Specificity of Enzymes
Adding Amino Acids in the Synthesis of the Peptidoglycan
Precursors of *Corynebacterium poinsettiae* and
*Corynebacterium insidiosum***

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SUMMARY

A soluble extract from *Corynebacterium poinsettiae* able to synthesize the nucleotide precursor of its peptidoglycan was prepared. This extract contained all the enzymes necessary for the synthesis of the peptide side-chain. The specificity of these enzymes was determined and compared with the specificity of similar enzymes extracted from the closely related *Corynebacterium insidiosum*. In both organisms, addition of the third amino acid of the peptide side-chain was specific for the amino acid and nucleotide dipeptide involved in peptidoglycan synthesis in the parent organism. L-Diaminobutyric acid, which is found as the acetyl derivative in the precursor nucleotide and in the completed peptidoglycan of *C. insidiosum*, was added as the free amino acid and not as the acetylated compound.

INTRODUCTION

The peptidoglycan of bacterial walls is synthesized from UDP-*N*-acetylglucosamine and another precursor nucleotide having the general composition UDP-*N*-acetylmuramyl-X-D-iso-glutamyl-Y-D-alanyl-D-alanine (for a review of peptidoglycan structures see Schleifer & Kandler, 1972). The sequential synthesis of the nucleotide precursor UDP-MurNac-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala (where MurNac stands for *N*-acetylmuramic acid) was first described with enzymes from *Staphylococcus aureus* (Ito & Strominger, 1962*a, b*). L-Alanine, D-glutamic acid and L-lysine are added individually in sequence and enzymes for the addition of these amino acids have been purified (Mizuno, Yaegashi & Ito, 1973; Ito & Strominger, 1964; Nathenson, Strominger & Ito, 1964). Two molecules of D-alanine are first converted by a synthetase to the dipeptide D-Ala-D-Ala and this is then added to the UDP-MurNac-tripeptide by the action of a ligase. Both the synthetase and ligase have been studied in *Streptococcus faecalis* (Neuhaus *et al.* 1969).

The specificities of the L-lysine- and *meso*-diaminopimelic acid-adding enzymes have been studied by Ito & Strominger (1973). These authors showed that extracts from bacteria containing L-lysine in their walls could add L-lysine but not *meso*-diaminopimelic acid to UDP-MurNac-L-Ala-D-Glu and those bacteria with *meso*-diaminopimelic acid could add that amino acid but not L-lysine. Addition of D-Ala-D-Ala was less specific for the presence of L-lysine or *meso*-diaminopimelic acid as amino acid residue 3 (the residues of the peptide side-chain will be referred to numerically as 1 to 5, number 1 being that amino acid attached to the muramic acid residue). Specificity for addition of L-lysine or *meso*-diaminopimelic acid occurs in *Bacillus sphaericus*, where vegetative cultures contain an L-lysine-adding

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enzyme which cannot utilize *meso*-diaminopimelic acid whereas sporulating ones have *meso*-diaminopimelic acid-adding enzyme (Tipper & Pratt, 1970).

The walls of the plant-pathogenic *Corynebacterium poinsettiae* contain glycine and L-homoserine as amino acid residues 1 and 3 of the peptide side-chain (Perkins, 1965, 1971). The present work describes the extraction of enzymes capable of sequentially synthesizing the nucleotide precursor UDP-MurNAc-Gly-D-isoGlu-L-Hsr-D-Ala-D-Ala (where Hsr stands for homoserine) and investigates the specificity of these enzymes for both the added amino acids and the nucleotide substrates. Preparations from a closely related organism, *Corynebacterium insidiosum*, are also compared for their specificity of addition to UDP-Mur-NAc-Gly-D-Glu.

METHODS

Materials. All the chemicals used were analytical-reagent grade. ATP, alkaline phosphatase type I, and D-cycloserine were purchased from Sigma, chloroamphenicol (Chlormycetin) was from Parke-Davis, Hounslow, Middlesex, and novobiocin (Vulcamycin) from Le Petit, Milan, Italy. The following radioactive amino acids were purchased from the Radiochemical Centre, Amersham, Buckinghamshire: [2-³H]glycine (3.8 Ci/mmol), [2-¹⁴C]glycine (51 mCi/mmol), L-[U-¹⁴C]alanine (159 mCi/mmol), D-[U-¹⁴C]alanine (37 mCi/mmol), D,L-[1-¹⁴C]glutamic acid (29 mCi/mmol), L-[4,5-³H]lysine (250 mCi/mmol), and L-[U-¹⁴C]-homoserine (29 mCi/mmol). Diamino [1-(7)-¹⁴C]pimelic acid (11.2 mCi/mmol) was purchased from Calbiochem Ltd, London W.1, and D,L-2,4-diamino [4-¹⁴C]butyric acid (19 mCi/mmol) from Schwarz/Mann, Orangeburg, New York, U.S.A. D-Ala-D-[U-¹⁴C]Ala (30 mCi/mmol) was synthesized as described by Nieto *et al.* (1973).

Analytical methods. Protein was determined according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. *N*-acetylhexosamine was determined by the method of Reissig, Strominger & Leloir (1955) and amino acids were determined with a Beckmann-Spinco automatic amino acid analyser after hydrolysis of the sample in 6 M-HCl at 104 °C for 16 h.

Radioactivity on paper strips (after electrophoresis or chromatography) was located by using a Packard Radiochromatogram Scanner (model 7201) and the appropriate areas were cut out and counted in a Packard Tri-Carb liquid scintillation spectrometer using a toluene-based scintillant [toluene containing 2,5-diphenyloxazole (0.4 %) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (0.01 %)]. Radioactivity in liquid samples was counted in a dioxan-based scintillant [dioxan containing naphthalene (10 %), 2,5-diphenyloxazole (1 %) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (0.06 %)].

Electrophoresis was carried out at 90 V/cm on Whatman No. 3 paper at pH 3.5 in buffer containing pyridine-acetic acid-water (1:10:989, by vol.).

Organisms and cultural conditions. *Staphylococcus aureus* H and *C. poinsettiae* NCPPI77 were grown at 35 °C and *C. insidiosum* NCPPI110 was grown at 25 °C in shaken-flask cultures in Hedley-Wright broth (Wright, 1933) containing 1 % (w/v) of glucose.

A culture of TOF33, a mutant of *S. aureus* 655HT isolated by Good & Pattee (1970), was kindly given to us by Dr D. J. Tipper. It was grown at 30 °C in shaken-flask cultures in a medium containing Bacto-tryptone (1 %), Bacto-yeast extract (1 %) (both from Difco), K₂HPO₄ (0.5 %) and glucose (1 %).

Preparation of enzymically active extracts. Exponential-phase cultures of *C. poinsettiae* were harvested at 4 °C and the bacteria washed with cold 0.025 M-tris-HCl buffer pH 7.9 containing 1 mM-β-mercaptoethanol (buffer A). All further manipulations were carried out at 4 °C. The washed bacteria were resuspended in buffer A (40 ml buffer/l culture) and were disrupted in a pre-cooled French pressure cell. The broken suspension was centrifuged at

39000 g for 45 min and the supernatant collected for $(\text{NH}_4)_2\text{SO}_4$ fractionation. Protein insoluble at 45 % saturation of $(\text{NH}_4)_2\text{SO}_4$ was discarded, and the fraction precipitating between 45 and 75 % saturation of $(\text{NH}_4)_2\text{SO}_4$ was collected. This was dissolved in buffer A to a final protein concentration of 10 to 20 mg/ml. The extract obtained in this way was dispensed into several portions and stored at -20°C . Such enzyme preparations retained 'adding activity' for several months.

Extracts were prepared from *C. insidiosum* in exactly the same way, but the $(\text{NH}_4)_2\text{SO}_4$ fractions were collected at 45 to 55 %, 55 to 65 % and 65 to 75 % of saturation. These are referred to as *C. insidiosum* fractions I, II and III, respectively.

Preparation of substrates. Cultures of TOF33 accumulate nucleotide precursors of wall peptidoglycan when grown at 30°C . UDP-MurNAc is the major nucleotide accumulated by this mutant (Dr D. J. Tipper, personal communication). Bacteria from an overnight culture of TOF33 were harvested and the nucleotide extracted and purified on a column of Dowex-1 essentially as described by Good & Tipper (1972). UDP-MurNAc was further purified by descending chromatography on Whatman No. 3 paper (previously washed extensively with 1 M-ammonium acetate and water) in a solvent system containing ethanol-1 M-ammonium acetate pH 7.5 (5:2, v/v). The nucleotide was located under u.v. light and eluted from the paper. Amino acid analysis showed that it contained muramic acid and no amino acids.

UDP-MurNAc-L-Ala was isolated from cultures of *S. aureus* after incubation with novobiocin (Wishnow *et al.* 1965). Amino acid analysis of the product isolated showed it contained muramic acid and alanine. Measurement of the ratio of bound *N*-acetylhexosamine to alanine gave a ratio of 3:1. Novobiocin inhibition of *S. aureus* is known to give an accumulation of UDP-MurNAc and other nucleotides in addition to UDP-MurNAc-L-Ala (Wishnow *et al.* 1965), and since UDP-MurNAc and UDP-MurNAc-L-Ala do not separate in the purification systems used, i.e. chromatography on Dowex-1 followed by paper chromatography in ethanol-1 M-ammonium acetate pH 7.5 (5:2, v/v), the product was a mixture of these two nucleotides. This material was used as a substrate in experiments to determine the addition of other amino acids to UDP-MurNAc-L-Ala, its concentration being determined from the L-alanine content.

Radioactive UDP-MurNAc-Gly was prepared by enzymic addition of $[2\text{-}^3\text{H}]$ glycine to UDP-MurNAc (prepared as described above) with an enzyme preparation from *C. poinsettiae* (see above). The reaction mixture contained tris-HCl buffer pH 8.9 (50 mM), neutralized ATP (5 mM), MnCl_2 (11 mM), KCl (10 mM), UDP-MurNAc (475 nmol), $[2\text{-}^3\text{H}]$ glycine (2 μmol , 10 $\mu\text{Ci}/\mu\text{mol}$) and the enzymically active extract from *C. poinsettiae* (8 mg protein) in a final volume of 1 ml. After incubation for 4 h at 37°C the reaction was terminated by heating at 100°C for 2 min. The precipitated protein was centrifuged and the pellet washed three times with water (1 ml). The washings were combined with the supernatant and concentrated by evaporation *in vacuo*. The UDP-MurNAc- $[2\text{-}^3\text{H}]$ Gly was then desalted on a column (2 $\times$ 67 cm) of Sephadex G-25 which was eluted with water. Fractions containing radioactivity which were eluted in the exclusion volume were pooled and concentrated and the radioactivity measured. The concentration of the UDP-MurNAc- $[2\text{-}^3\text{H}]$ Gly was then calculated from the known specific activity of the $[2\text{-}^3\text{H}]$ glycine used. UDP-MurNAc- $[2\text{-}^3\text{H}]$ Gly-D-Glu was prepared by enzymic synthesis exactly as described above for UDP-MurNAc- $[2\text{-}^3\text{H}]$ Gly except that the incubation mixture also contained D-glutamic acid (4 μmol).

UDP-MurNAc-L-Ala-D-Glu was a gift from Dr J. B. Ward and had been prepared from lysine-deprived *S. aureus* H (Strominger & Threnn, 1959).

UDP-MurNAc-L-Ala-D-isoGlu-L-Lys was accumulated in cultures of *S. aureus* H inhibited with D-cycloserine and chloramphenicol as described by Ito *et al.* (1966).

UDP-MurNAc-L-Ala-D-isoGlu-meso-A₂pm (where A₂pm stands for diaminopimelic acid) was a gift from Dr J. B. Ward and had been prepared from cultures of *Bacillus licheniformis* Lyt⁻3 (Ward, 1974).

UDP-MurNAc-Gly-D-isoGlu-L-Hsr was accumulated in cultures of *C. poinsettiae* inhibited with D-cycloserine (240 µg/ml) and chloramphenicol (200 µg/ml) essentially as described by Ito *et al.* (1966).

Preparation of γ-acetyl-L-2,4-diaminobutyric acid. This compound was synthesized essentially as described for ε-acetyl-L-lysine by Greenstein & Winitz (1961). The radioactive material was prepared by similar treatment of diamino-[4-¹⁴C]butyric acid, the final product being purified by paper electrophoresis in 0.25 M-formic acid. The band of radioactivity corresponding to a marker of the unlabelled product was eluted and concentrated.

Assay of 'adding enzyme' activities. Extracts were assayed for their ability to add a particular amino acid to a given nucleotide substrate. Incubation mixtures all contained tris-HCl buffer pH 8.9 (50 mM), neutral ATP (5 mM), MnCl₂ (11 mM), KCl (10 mM), substrate (20 to 40 nmol), radioactive amino acid (20 to 40 nmol, 1 µCi/µmol) and enzyme (200 to 400 µg protein) in a final volume of 50 µl. Incubation was at 37 °C for 4 h for the enzyme from *C. poinsettiae* and at 25 °C for 2 h for the enzyme from *C. insidiosum*. All enzyme reactions were terminated by heating (100 °C, 2 min) and the entire contents of the incubation tubes applied to Whatman No. 3 paper for electrophoresis at pH 3.5. The amino acids and the reaction products were well separated after electrophoresis for 20 min and were located and counted (see Analytical methods, above). All experiments included controls in which the nucleotide substrate was omitted from the incubation mixture.

Identification of free amino groups. The Dnp-derivatives of nucleotide-peptides containing radioactivity from labelled diaminobutyric acid were made as follows: the samples were dissolved in 1 % NaHCO₃ (30 µl) and mixed with 50 µl fluorodinitrobenzene (0.5 %, v/v, solution in ethanol). After storage overnight at 25 °C in the dark, the mixtures were acidified and untreated fluorodinitrobenzene was extracted into ether. The aqueous layer was dried, hydrolysed in 6 M-HCl (104 °C, 16 h) and finally applied to Whatman No. 3 paper for electrophoresis at pH 3.5, with markers of γ-Dnp-diaminobutyric acid and free L-diaminobutyric acid.

RESULTS

Corynebacterium poinsettiae 'adding enzyme'

An extract prepared from disrupted *C. poinsettiae* was assayed for its ability to synthesize sequentially the UDP-MurNAc-pentapeptide contained in the peptidoglycan of this organism, UDP-MurNAc-Gly-D-isoGlu-L-Hsr-D-Ala-D-Ala. In addition, the specificities of these adding enzymes for addition of other amino acids in particular positions of the peptide side-chain were examined.

Addition of amino acid residue 1. The addition of glycine, the first amino acid in the side-chain of peptidoglycan in this organism, was compared with the ability of the same preparation to add L-alanine, which is the first amino acid in almost all other bacteria (Table 1). Under the assay conditions used, formation of UDP-Mur-NAc-Gly reached a maximum after 2 h and then remained constant for up to 16 h incubation. Very little L-alanine was added.

Addition of amino acid residue 2. D-Glutamic acid occurs as second amino acid in the side-chain in all bacterial peptidoglycans. The extract from disrupted *C. poinsettiae* was tested for its ability to synthesize from the appropriate precursors, either its homologous

Table 1. Addition to UDP-MurNAc-peptides by enzymes extracted from *C. poinsettiae*

Conditions as indicated in Methods. All incubations at 37 °C for 4 h.

Substrate	Amino acid	Amount added (nmol/mg protein)
UDP-MurNAc (0.6 mM)	Glycine (0.8 mM)	54.5
	L-Alanine (0.8 mM)	0.8
UDP-MurNAc-Gly (0.3 mM)	D-Glutamic acid (0.5 mM)	32.7
UDP-MurNAc-L-Ala (0.4 mM)	D-Glutamic acid (0.8 mM)	41.5
UDP-MurNAc-Gly-D-Glu (0.5 mM)	L-Homoserine (0.8 mM)	38.0
	L-Diaminobutyric acid (0.8 mM)	ND
	L-Lysine (0.8 mM)	ND
	meso-Diaminopimelic acid (1.2 mM)	ND
UDP-MurNAc-L-Ala-D-Glu (0.4 mM)	L-Homoserine (0.8 mM)	1.0
	L-Diaminobutyric acid (0.8 mM)	ND
	L-Lysine (0.8 mM)	ND
	meso-Diaminopimelic acid (1.2 mM)	ND
UDP-MurNAc-Gly-D-isoGlu-L-Hsr (0.4 mM)	D-Alanyl-D-alanine (0.6 mM)	8.9
UDP-MurNAc-L-Ala-D-isoGlu-L-Lys (0.4 mM)	D-Alanyl-D-alanine (0.6 mM)	5.5
UDP-MurNAc-L-Ala-D-isoGlu- meso-A ₂ pm (0.4 mM)	D-Alanyl-D-alanine (0.6 mM)	11.0

ND, Not detectable.

nucleotide dipeptide, UDP-MurNAc-Gly-Glu, or the most common nucleotide dipeptide, UDP-MurNAc-L-Ala-D-Glu. Table 1 shows that both UDP-MurNAc-Gly and UDP-MurNAc-L-Ala are good substrates for the D-glutamic acid-adding enzyme. The glutamic acid occurring as amino acid residue 2 of the muramyl pentapeptide chain of all bacterial peptidoglycans is present in the D-configuration (Schleifer & Kandler, 1972) and the specificity of the D-glutamic acid adding enzyme from various bacteria has been established by Ito & Strominger (1962*a*, 1973). In the present experiments, D-glutamic acid (40 nmol) containing 170000 d.p.m. ¹⁴C-labelled D,L-isomer was used. Calculations of the adding activity assumed that only the D-isomer was incorporated into dipeptide.

Addition of amino acid residue 3. The peptidoglycans of most bacteria contain either L-lysine or diaminopimelic acid (L,L or meso isomer) as amino acid residue 3 of the peptide side-chain, but the walls of certain plant-pathogenic corynebacteria contain L-homoserine or L-diaminobutyric acid (Perkins, 1965, 1971). The enzyme preparation from *C. poinsettiae* added L-homoserine to UDP-MurNAc-Gly-D-Glu to form the nucleotide-tripeptide UDP-MurNAc-Gly-D-isoGlu-L-Hsr, an intermediate in the synthesis of the peptidoglycan of this organism (Table 1). No addition of L-lysine, meso-diaminopimelic acid or L-diaminobutyric acid could be detected when UDP-MurNAc-Gly-D-Glu was the substrate. When UDP-MurNAc-L-Ala-D-Glu was used as the substrate a very small amount of L-homoserine-adding activity was detected, but the enzyme preparation from *C. poinsettiae* could not form tripeptide from UDP-MurNAc-L-Ala-D-Glu with L-lysine, meso-diaminopimelic acid or L-diaminobutyric acid (Table 1).

Addition of amino acid residues 4 and 5. UDP-MurNAc-pentapeptide is formed by the addition of D-Ala-D-Ala to UDP-MurNAc-tripeptide. The enzyme preparation from *C. poinsettiae* was able to synthesize UDP-MurNAc-pentapeptide by addition of D-Ala-D-Ala to its homologous tripeptide UDP-MurNAc-Gly-D-isoGlu-L-Hsr or to UDP-MurNAc-L-Ala-D-isoGlu-L-Lys or UDP-MurNAc-L-Ala-D-isoGlu-meso-A₂pm (Table 1). To confirm

Table 2. *Addition to UDP-MurNAc-peptides by enzymes extracted from C. insidiosum*

Conditions as indicated in Methods. All incubations at 25 °C for 2 h.

Substrate	Amino acid	(NH ₄) ₂ SO ₄ fraction	Amount added (nmol/mg protein)
UDP-MurNAc (0.4 mM)	Glycine (0.8 mM)	I	24.3
		II	22.7
		III	ND
UDP-MurNAc-Gly (0.1 mM)	D-Glutamic acid (0.5 mM)	I	12.1
		II	0.07
UDP-MurNAc-Gly-D-Glu (0.3 mM)	L-Diaminobutyric acid (0.5 mM)	I	18.8
		II	1.9
	γ -Acetyl-L-diaminobutyric acid* (0.5 mM)	I	9.0
		II	0.3
	L-Homoserine (0.5 mM)	I	ND
		II	ND

ND, Not detectable.

* γ -Acetyl-L-diaminobutyric acid is first de-acetylated and the free L-diaminobutyric acid formed is then added to UDP-MurNAc-Gly-D-Glu (see Results).

that the enzyme preparation from *C. poinsettiae* was capable of synthesizing its homologous UDP-MurNAc-pentapeptide from UDP-MurNAc and glycine, D-glutamic acid, L-homoserine and D-Ala-D-Ala, a preparative scale incubation was set up as follows: UDP-MurNAc (475 nmol), glycine (1.5 μ mol), D-glutamic acid (1.5 μ mol), L-homoserine (1.5 μ mol), D-Ala-D-[U-¹⁴C]Ala (150 nmol) (588 000 c.p.m.) and enzyme (8 mg protein), in 1 ml of buffer containing tris-HCl pH 8.9 (50 mM), neutral ATP (5 mM), MnCl₂ (11 mM) and KCl (10 mM). After incubation at 37 °C for 4 h the reaction was terminated by heating (100 °C, 2 min) and the precipitated protein was removed by centrifugation. The pellet was washed three times with water and the combined supernatant and washings were concentrated and fractionated on a column (2 $\times$ 67 cm) of Sephadex G-25. Radioactive material collected in the exclusion volume of the effluent was concentrated (1 ml) and counted. The concentration of product was calculated from the radioactivity as 96 nmol/ml. On electrophoresis at pH 3.5 all the radioactivity moved in the same position as authentic UDP-MurNAc-Gly-D-isoGlu-L-Hsr-D-Ala-D-Ala. Further evidence of the authenticity of the UDP-MurNAc-pentapeptide came from its reaction with vancomycin at neutral pH. After mixing 2 nmol of the radioactive UDP-MurNAc pentapeptide with 20 nmol vancomycin, a product was obtained which did not move from the origin on electrophoresis at pH 6.5 in pyridine-acetic acid-water buffer (25:1:474, by vol.). Such combination is dependent upon the presence of a D-Ala-D-Ala terminal (Perkins, 1969). D-Alanyl-D-alanine synthetase was not detectable in the assay system used, since no pentapeptide formation was seen when D-[U-¹⁴C]alanine was incubated with UDP-MurNAc-Gly-D-isoGlu-L-Hsr and enzyme.

Enzyme preparation from C. insidiosum

All three fractions collected from ammonium sulphate precipitation of the extract from *C. insidiosum* were tested for their ability to add glycine to UDP-MurNAc. Fractions I and II were both equally active in adding glycine but fraction III was inactive (Table 2).

D-Glutamic acid-adding activity was found mainly in fraction I, with very little in fraction II (Table 2).

The peptidoglycan precursor nucleotide of *C. insidiosum*, accumulated in the presence of vancomycin, contains in its pentapeptide the γ -acetyl derivative of L-diaminobutyric acid (Perkins, 1971). Hence *C. insidiosum* enzyme fractions I and II were tested for their ability to add L-diaminobutyric acid, γ -acetyl-L-diaminobutyric acid or L-homoserine to UDP-MurNAc-Gly-D-Glu. Radioactivity from both L-diaminobutyric acid and γ -acetyldiaminobutyric acid was incorporated into the nucleotide in the presence of fraction I, incorporation from the amino acid itself being about twice as much as from its γ -acetyl derivative (Table 2). However, control incubations showed that in the absence of substrate, the γ -acetyl derivative was converted into free diaminobutyric acid (presumably by the action of an endogenous acylase); during a 2 h incubation with the *C. insidiosum* fraction I, 9.3 nmol of free diaminobutyric acid were formed from 25 nmol of the γ -acetyl derivative. It thus became important to determine whether the addition product of γ -acetyldiaminobutyric acid and UDP-MurNAc-Gly-D-Glu contained free diaminobutyric acid or its acetyl derivative.

Incubations were set up for the addition of γ -acetyldiaminobutyric acid or free L-diaminobutyric acid. After electrophoresis at pH 3.5, nucleotide tripeptides were eluted from the paper and dried *in vacuo*. The Dnp-derivatives of the nucleotide products were then prepared, hydrolysed and examined by paper electrophoresis at pH 3.5. Both nucleotide samples gave a single peak of radioactivity corresponding to the Dnp-derivative, with 70 % recovery of the initial radioactivity. γ -Dnp-diaminobutyric acid could only have been formed if the initial nucleotide tripeptide contained L-diaminobutyric acid with a free γ -amino group; if the nucleotide tripeptide had contained γ -acetyl diaminobutyric acid this would not have formed a Dnp-derivative but the radioactivity would have appeared as free L-diaminobutyric acid after the hydrolysis. Hence the *C. insidiosum* fraction I must have first deacetylated the γ -acetyldiaminobutyric acid and then used the free diaminobutyric acid formed for addition to UDP-MurNAc-Gly-D-Glu. *Corynebacterium insidiosum* fraction II showed similar behaviour with very much lower activities.

C. insidiosum enzyme and L-homoserine

In a series of more than ten experiments, whenever the enzyme preparation from *C. insidiosum* was incubated with L-[U- 14 C]homoserine either in the presence or absence of UDP-MurNAc-Gly-D-Glu, radioactivity appeared in a product which was highly negatively charged on electrophoresis at pH 3.5. In a typical experiment 24 nmol of L-homoserine gave rise to 10 nmol of product, and in any given experiment there was no difference in the amount of product formed in the presence or absence of UDP-MurNAc-Gly-D-Glu. This product could not have been UDP-MurNAc-Gly-D-isoGlu-L-Hsr, since it was formed in the absence of any nucleotide substrate, but it moved in the same position on electrophoresis at pH 3.5.

An enzyme capable of converting homoserine to O-phosphohomoserine is present in yeast (Watanabe & Shimura, 1956). O-phosphohomoserine would move as a very negatively charged molecule on electrophoresis at pH 3.5. The product of the reaction between L-homoserine and the *C. insidiosum* enzyme preparation was identified as O-phosphohomoserine from the following results: (i) Chromatography in butan-1-ol-acetic acid-water (50:15:35, by vol.) showed a radioactive spot in the position expected for O-phosphohomoserine (published R_f in this solvent for O-phosphohomoserine is 0.13). (ii) Formation of the dinitrophenyl derivative indicated the presence of a free amino group. (iii) Incubation (30 min at 37 °C) with alkaline phosphatase (1.9 units activity) at pH 8.9 gave complete conversion to a product moving in the same electrophoretic position as homoserine.

To determine whether any L-homoserine was added to UDP-MurNAc-Gly-D-Glu when incubated with the enzyme from *C. insidiosum*, it was necessary to separate *O*-phosphohomoserine and UDP-MurNAc-Gly-D-isoGlu-Hsr. These substances did not separate on electrophoresis at pH 3.5 or on chromatography on a column (2 × 67 cm) of Sephadex G-25. Separation was, however, achieved on electrophoresis for 2 h at 10 V/cm in 0.25 M-formic acid (pH 1.9), with *O*-phosphohomoserine and nucleotide tripeptide moving 3.2 and 4.9 cm, respectively, towards the anode. The products of the reaction between L-[U-¹⁴C]-homoserine and the enzyme from *C. insidiosum* in the presence and absence of UDP-MurNAc-[³H]Gly-D-Glu were isolated and subjected to electrophoresis in 0.25 M-formic acid. In each case all the ¹⁴C was recovered in the *O*-phosphohomoserine peak and no ¹⁴C could be detected in the peak containing the ³H-labelled nucleotide. Thus it was confirmed that the enzyme from *C. insidiosum* does not add L-homoserine to UDP-MurNAc-Gly-D-Glu.

DISCUSSION

The sequential synthesis of UDP-MurNAc-Gly-D-isoGlu-L-Hsr-D-Ala-D-Ala has been achieved by using a mixture of soluble enzymes isolated from *C. poinsettiae*, and the specificity of these enzymes for both the amino acids added and the UDP-MurNAc-peptide substrates has been investigated.

Addition of glycine to UDP-MurNAc occurred very readily and in the same system a small but definite amount of L-alanine addition could also be detected (Table 1). Addition of small amounts of glycine or D,L-serine to UDP-MurNAc occurs with the L-alanine ligase purified from *S. aureus* (Mizuno *et al.* 1973) and addition of glycine to UDP-MurNAc has also been shown in an enzyme preparation from *B. subtilis* (Hishinuma, Izaki & Takahashi, 1970). It would seem, therefore, that the ligase for the first amino acid of the peptide side-chain is highly specific for the particular amino acid that occurs in that position in the peptidoglycan of the parent organism, but that to a small extent L-alanine can substitute for glycine and vice versa. This is consistent with the experiments of Hammes, Schleifer & Kandler (1973) who studied the inhibitory effects of high concentrations of glycine on bacterial growth. In three bacterial species they found that high concentrations of glycine in the growth medium sufficient to cause growth inhibition and to damage wall assembly, led to replacement of L-alanine by glycine as first amino acid of the peptide side-chain, accompanied by accumulation of UDP-MurNAc. They attributed this result to an inhibition of the UDP-MurNAc: L-alanine ligase by glycine similar to that found *in vitro* in *B. subtilis* by Hishinuma, Izaki & Takahashi (1971).

D-Glutamic acid is the only amino acid found as second amino acid in the peptide side-chain. The extract from *C. poinsettiae* showed no nucleotide substrate specificity for addition of D-glutamic acid at this position, with UDP-MurNAc-Gly and UDP-MurNAc-L-Ala both being good substrates (Table 1).

The specificity for addition of the third amino acid of the peptide side-chain was absolute, the extract from *C. poinsettiae* being capable only of adding L-homoserine to UDP-MurNAc-Gly-D-Glu; there was no detectable addition of L-lysine, *meso*-diaminopimelic acid or L-diaminobutyric acid to this substrate (Table 1). A small but measurable amount of L-homoserine was added to the heterologous nucleotide-dipeptide UDP-MurNAc-L-Ala-D-Glu, but L-lysine, *meso*-diaminopimelic acid and L-diaminobutyric acid were not added to this substrate either.

Corynebacterium insidiosum is closely related to *C. poinsettiae* (in the taxonomic scheme of Schleifer & Kandler, 1972, the organisms are placed in classes B2γ and B2β, respectively),

both peptidoglycan precursors having glycine as first amino acid of the nucleotide peptide side-chain but the former having the γ -acetyl derivative of diaminobutyric acid as third amino acid instead of L-homoserine (Perkins, 1971). Correspondingly, the enzyme preparation from *C. insidiosum* was also only able to use L-diaminobutyric acid and not L-homoserine for nucleotide tripeptide synthesis (Table 2). The addition of the 'correct' (homologous) amino acid as amino acid residue 3 of nucleotides having a substituted (heterologous) amino acid as residue 1 is consistent with the composition of the nucleotides accumulated by several strains in the presence of high glycine concentration (Hammes *et al.* 1973). Only *Lactobacillus cellobiosus* precursors showed a partial substitution at amino acid residue 3 of L-ornithine by L-lysine. Similar conclusions with regard to the addition of L-lysine or meso-diaminopimelic acid to UDP-MurNAc-L-Ala-D-Glu were reached for enzyme preparations from organisms containing one or other of these amino acids in their cell walls (Tipper & Pratt, 1970; Ito & Strominger, 1973).

Since in *C. insidiosum* the peptidoglycan and its precursor both contain the γ -acetyl derivative of L-diaminobutyric acid (Perkins, 1971), this compound was tested with the enzyme from *C. insidiosum* for addition to UDP-MurNAc-Gly-D-Glu. However, the extract prepared from *C. insidiosum* first removed the γ -acetyl group and then utilized the free L-diaminobutyric acid for tripeptide formation by addition to UDP-MurNAc-Gly-D-Glu, and no nucleotide tripeptide with an acetylated γ -amino group of L-diaminobutyric acid was detected. These results suggest that the pathway of peptidoglycan precursor synthesis in *C. insidiosum* involves the addition as amino acid residue 3 of free L-diaminobutyric acid, which is subsequently acetylated either before or after the addition of the terminal D-alanine dipeptide.

The extract prepared from *C. poinsettiae* could add D-Ala-D-Ala to all three nucleotide tripeptides tested with little difference in the efficiency of addition (Table 1). This observation agrees with those of Ito & Strominger (1973), who also found low substrate specificity for the D-Ala-D-Ala-adding enzymes present in the bacterial extracts that they studied.

Synthesis of UDP-MurNAc-pentapeptide by a mechanism involving step-wise addition of amino acids to the peptide side-chain attached to UDP-MurNAc is now well established. The experiments described clearly demonstrate that the enzymes responsible for addition at a given position of the peptide side-chain are directly related to the peptidoglycan of the parent organism. D-Glutamic acid and D-Ala-D-Ala are common to all UDP-MurNAc-pentapeptides and the enzymes that add these moieties to the peptide side-chain show little specificity with regard to the nucleotide-peptide substrate so long as it contains the correct number of residues. The amino acids that occur as residues 1 and 3 are characteristic for a given bacterial peptidoglycan and the enzymes for addition of these residues have specific amino acid and nucleotide-peptide substrate requirements. Closely related bacteria such as *C. insidiosum* and *C. poinsettiae* which differ in the amino acid residue 3 of the peptide side-chain have enzymes capable only of synthesizing the homologous nucleotide pentapeptide. Thus the composition of the primary peptide chain of the peptidoglycan, so important in bacterial taxonomy (Schleifer & Kandler, 1972), is closely controlled by the high specificities of the enzymes that add the first and third amino acid residues.

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