A DIRECTLY CROSS-LINKED L-ORNITHINE-CONTAINING PEPTIDOGLYCAN IN CELL WALLS OF *SPIROCHAETA STENOSTREPTA*

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1. Introduction

The location of the peptidoglycan as a thin electron-dense layer adjacent and external to the cytoplasmic membrane in cells of *Spirochaeta stenostrepta* and *Spirochaeta litoralis* has recently been demonstrated [1]. The morphology and chemical composition of the spirochetal peptidoglycan [1–4] is similar to that of many Gram-negative bacteria [5, 6] with the exception that L-ornithine [1] occurs as the diamino acid instead of either lysine or diaminopimelic acid. Since a new type may occur in these organisms [1], we have elucidated the primary structure of the peptidoglycan of *S. stenostrepa* ATCC 25803. The evidence for a directly cross-linked, L-ornithine-containing peptidoglycan is presented in this paper.

2. Methods

The peptidoglycan of *S. stenostrepta* ATCC 25083 was isolated as previously described [1]. The purity of the peptidoglycan preparation was determined by electron microscopy and amino acid analysis. Total hydrolysis of the peptidoglycan and peptides was carried out in 4 N HCl at 100°C for 16 hr.

Isolation of peptides from partial acid hydrolysates of the peptidoglycan and determination of the amino acid sequence of these peptides were performed as described previously by Schleifer and Kandler [7]. Dinitrophenylation of the peptidoglycan was carried out according to Primosigh et al. [8]; N-terminal amino acids of peptides were assayed for by the method of Ghuysen et al. [9]. Determination of *C*-terminal amino acids was performed by hydrazinolysis with anhydrous hydrazine as described by Braunitzer [10]. The configuration of amino acids was determined enzymatically; that of alanine as described by Larson et al. [11]; that of glutamic acid as described by Niebler et al. [12] and that of ornithine as described by Jones [13].

3. Results

The quantitative amino acid and amino sugar composition, and molar ratios of the peptidoglycan components are listed in table 1. The peptidoglycan was composed solely of glucosamine, muramic acid, ornithine, glutamic acid and alanine. Ammonia release occurred only after total hydrolysis of peptidoglycan (less than 0.2 moles/mole of glutamic acid), indicating that the α-carboxyl group of glutamic acid was probably not amidated. Only the alanine content varied (1.31–1.8 moles/mole glutamic acid) depending on the peptidoglycan preparation used. A possible explanation for this diversity will be given later.

Ornithine was present only as the L-isomer, whereas glutamic acid occurred solely as D-isomer. The ratio of L-alanine to D-alanine was 1.0:0.28. Following dinitrophenylation and total acid hydrolysis δ-DNP-ornithine was the only dinitrophenylated amino acid

Table 1
Amino acid and amino sugar composition of the peptidoglycan of *S. stenostrepta*[a].

	μmole/mg[b]	Molar ratio[c]
Muramic acid	0.515	0.99
Glucosamine	0.492	0.95
Glutamic acid	0.521	1.0
Alanine	0.682	1.31
Ornithine	0.531	1.02
Ammonia	0.063	0.12

[a] Hydrolysis was in 4 N HCl at 100°C for 16 hr. Analysis was performed on a Beckman 120C amino acid analyzer.
[b] μmole/mg of peptidoglycan.
[c] Molar ratio relative to glutamic acid.

found. Of the total ornithine, 68% contained an N-terminal δ-amino group and thus 68% of the ornithine residues were not involved in the cross-linkage. In addition, ornithine was the only C-terminal amino acid in the intact peptidoglycan. At least 60% of the total ornithine possessed an unsubstituted carboxyl group. Two-dimensional chromatography of a partial acid hydrolysate of the peptidoglycan was used to determine the amino acid sequence (fig. 1, table 2). The occurrence of the peptides muramyl-L-alanine, L-alanyl-D-glutamic acid, γ-D-glutamyl-L-ornithine and L-ornithyl-D-alanine established the following sequence in the peptide subunit: muramyl-L-alanyl-γ-D-glutamyl-L-ornithyl-D-alanine. The occurrence of the peptide N^5-D-alanyl-L-ornithine indicated that the peptide subunits were directly cross-linked between the δ-amino group of L-ornithine and the carboxyl group of D-alanine of an adjacent peptide subunit. The occurrence of polymerized peptide subunits was unlikely as the peptides D-alanyl-L-alanine and D-alanyl-L-alanyl-D-glutamic acid which would be hydrolysis products of such a sequence were not detected.

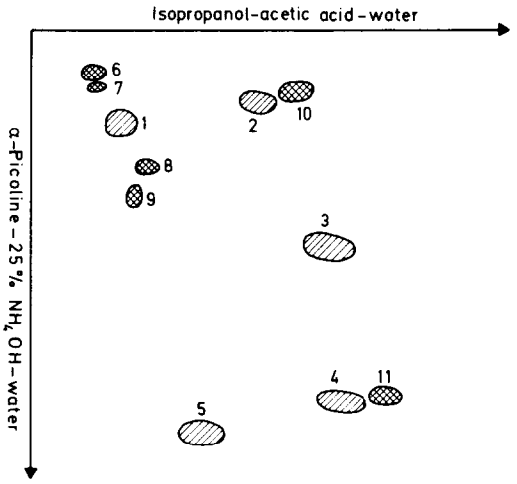

Fig. 1. Two-dimensional paper chromatogram of a partial acid hydrolysate (4 N HCl, 100°C, 45 min) of the peptidoglycan of *S. stenostrepta*. Development was ascending in: i) isopropanol–acetic acid–water (75:10:15, v/v/v) and ii) α-picoline–25% NH_4OH–water (70:2:28, v/v/v); Single hatch, amino acids; cross hatch, peptides; (1) ornithine; (2) glutamic acid; (3) alanine; (4) muramic acid; (5) glucosamine; (6) γ-D-glutamyl-L-ornithine; (7) L-alanyl-D-glutamyl-L-ornithine; (8) L-ornithyl-D-alanine; (9) N^5-D-alanyl-L-ornithine; (10) L-alanyl-D-glutamic acid; (11) muramyl-L-alanine.

Only about 30% of the L-ornithine residues were involved in the cross-linkage because 68% of the ornithine residues contained an unsubstituted δ-amino group. This low rate of cross-linkage was also in agreement with the low content of D-alanine (0.3 moles/mole glutamic acid). The lack of C-terminal D-alanine residues indicated that all of the D-alanine was involved in cross-linkages. This, in turn, suggested that very potent D-alanyl-D-alanine and D-alanine carboxypeptidases occurred in *S. stenostrepta*. Because of this the content of D-alanine, and thus the total content of alanine and the degree of cross-linkage may vary in different peptidoglycan preparations [1].

A typical fragment of the proposed primary structure of the peptidoglycan of *S. stenostrepa* is depicted in fig. 2. As in almost all other peptidoglycans the amino groups of muramic acid and glucosamine were acylated since neither DNP-muramic acid nor DNP-glucosamine were detected in total hydrolysates of dinitrophenylated peptidoglycan. At least 50% of the peptide subunits must consist of the tripeptide *N*-acyl-muramyl-L-alanyl-γ-D-glutamyl-L-ornithine because of the low amount of cross-linkage (30%).

4. Discussion

The peptidoglycan of *S. stenostrepta* and possibly that of *S. litoralis* too, are the first known examples of a directly cross-linked peptidoglycan containing L-ornithine as diamino acid. For a long time the direct

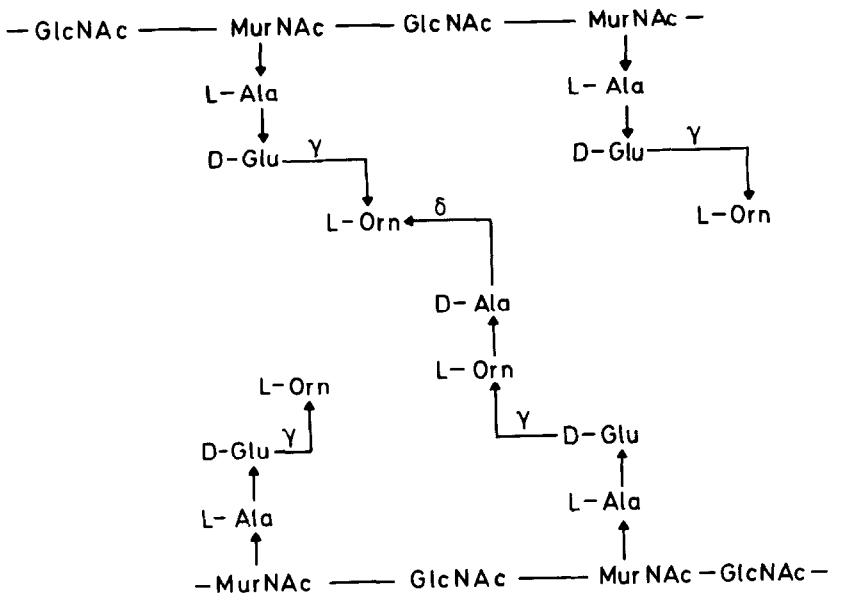

Fig. 2. Proposed primary structure of the peptidoglycan of *S. stenostrepta*.

cross-linkage in which the peptide subunits were connected without an insertion of additional interpeptide bridges seemed to occur only in peptidoglycans containing meso-diaminopimelic acid. Very recently, however, Nakel et al. [14] have shown that the same type of peptide bridging also occurs in peptidoglycans containing L-lysine. In addition to the ultrastructural similarity [1], the chemical structure of the peptidoglycan of *S. stenostrepta* is similar to that in many Gram-negative eubacteria. The peptide subunits are directly cross-linked in both cases, only the diamino acids being different. This monolayered peptidoglycan is rather different from the multilayered arrangement of the peptidoglycan in Gram-positive bacteria. Thus, the actual difference between the peptidoglycan of *S. stenostrepta* (and probably of other spirochetes) and that of Gram-negative *Eubacteriales* appears only to be in the diamino acid.

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Table 2
Amino acid composition and sequence of the peptides of the peptidoglycan of *S. stenostrepta*.

Peptide no.	Molar ratio				DNP amino acids	Peptide structure
	Ala	Glu	Orn	Mur		
6	–	1.0	0.9	–	DNP–Glu, δ-DNP–Orn	γ-D-Glu–L–Orn
7	0.88	1.0	0.95	–	DNP–Ala, δ-DNP–Orn	L-Ala– -D-Glu–L-Orn
8	1.1	–	1.0	–	di-DNP–Orn	L-Orn–D-Ala
9	1.04	–	1.0	–	δ-DNP–Orn, DNP–Ala	N^5-D-Ala–L-Orn
10	0.95	1.0	–	–	DNP–Ala	L-Ala–D-Glu
11	1.0	–	–	1.1	–	Mur–L-Ala

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