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Peptidoglycan Precursor from *Fusobacterium nucleatum* Contains Lanthionine

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***Fusobacterium nucleatum* was grown in the presence of [^{14}C]UDP. By means of sequential precipitation and chromatographic separation of the cytoplasmic content, a peptidoglycan [^{14}C]UDP pentapeptide containing lanthionine was isolated. This finding indicates that lanthionine is synthesized and incorporated as such during the assembly of the peptidoglycan.**

Fusobacterium nucleatum is an anaerobic gram-negative oral bacterium which is associated with periodontal disease (1). Lanthionine, the monosulfur analog of diaminopimelic acid, has been shown to be a natural constituent of the peptidoglycan of *F. nucleatum*, replacing the *meso*-diaminopimelic acid in this bacterium (7, 9). The lanthionine of one stem peptide cross-links with the D-alanine of a neighboring stem peptide (6).

The biosynthesis of this lanthionine is not understood. It has been shown that lanthionine can be made by alkali treatment of certain proteins (3). A cystine disulfide bridge is broken, yielding cysteine and dehydroalanine (2-aminopropenoic acid). When these two intermediates react, lanthionine is formed and is found as such after acid hydrolysis of the peptide bonds (10). Thus, there is a possibility that lanthionine can be formed as a result of a cross-linking reaction, e.g., between cysteine residues in the peptidoglycan monomer unit. Counting against this assumption is the fact that lanthionine is found also in un-cross-linked peptidoglycan (6).

Cysteine or cystine is apparently a precursor for lanthionine in *F. nucleatum* because the peptidoglycan contains radioactive lanthionine when the cells are grown in the presence of [^{35}S]cysteine (6, 7, 9). On the other hand, radioactive alanine or serine in the medium did not result in labeled lanthionine (unpublished data). To rule out the possibility that lanthionine is formed as a result of cross-linking or other modifying reactions of (mature) peptidoglycan, we have isolated and characterized a peptidoglycan precursor. This was found to contain UDP-muramyl-alanyl-glutamyl-lanthionyl-alanyl-alanine, which shows that lanthionine is formed as such in the cell and incorporated in the peptidoglycan precursors.

F. nucleatum Fev 1 was grown anaerobically in the presence of 250 μCi of [^{14}C]UDP (Amersham) (7) and harvested in the mid-exponential phase of growth. The cells were washed in 50 mM Tris hydrochloride (pH 7.4) containing 5 mM $MgCl_2$, resuspended in the same buffer, and opened by passage through a French pressure cell (3,000 lb/in^2). Unopened cells and envelope fragments were pelleted at 30,900 × *g* for 1 h. To the supernatant were added 0.1 volume of 3 M sodium acetate (pH 7) and 3 volumes of ice-cold 96% ethanol. The mixture was kept at −80°C for 20 min or at −20°C overnight before sedimentation, which was performed at 10,400 × *g* for 20 min. Acetone was then added to the supernatant (60% final concentration), and the mixture was kept on ice for 2 h before centrifugation at 12,000 × *g* for 30 min. The supernatant was concentrated to 3 ml on a rotary evaporator. Desalting was achieved on a Sephadex G-10 column (2.1 by 34 cm) which was both equilibrated and eluted with 10 mM NH_4OH at a flow rate of 0.48 ml/min; 2.9-ml fractions were collected. The material with $K_{av} = 0$ containing about 45% of the radioactivity was concentrated and applied to a column of Sephadex G-25 Fine (1.45 by 115 cm). To take advantage of the weak ion-exchange properties of the Sephadex matrix, equilibration and elution (flow rate, 0.26 ml/min; fractions, 3.6 ml each) were performed with water (5). The major radioactive peak (Fig. 1, Ia) was concentrated and spotted on a silica thin-layer plate with fluorescence indicator (Merck, Darmstadt, Federal Republic of Germany) and run in isobutyric acid–10 mM NH_4OH (5:3, vol/vol). UDP-peptidoglycan precursors were detected as

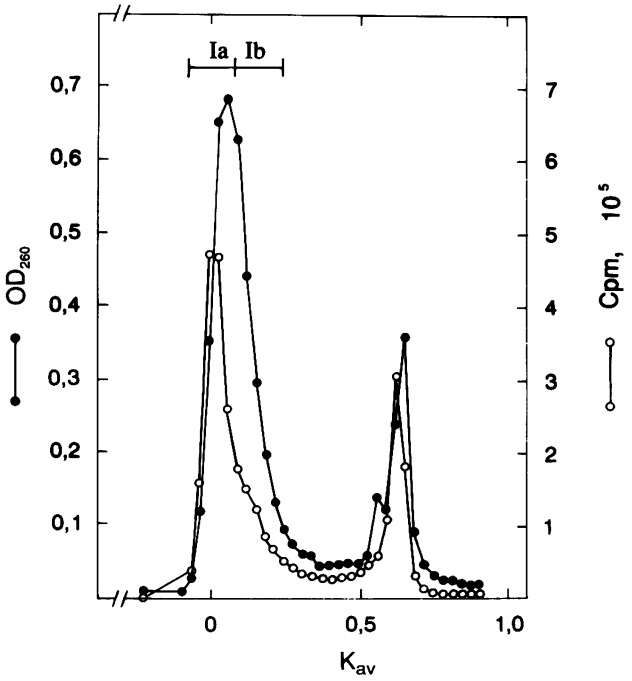

FIG. 1. Separation of desalted ^{14}C-UDP-labeled extract of *F. nucleatum* on Sephadex G-25 Fine. OD_{260}, Optical density at 260 nm.

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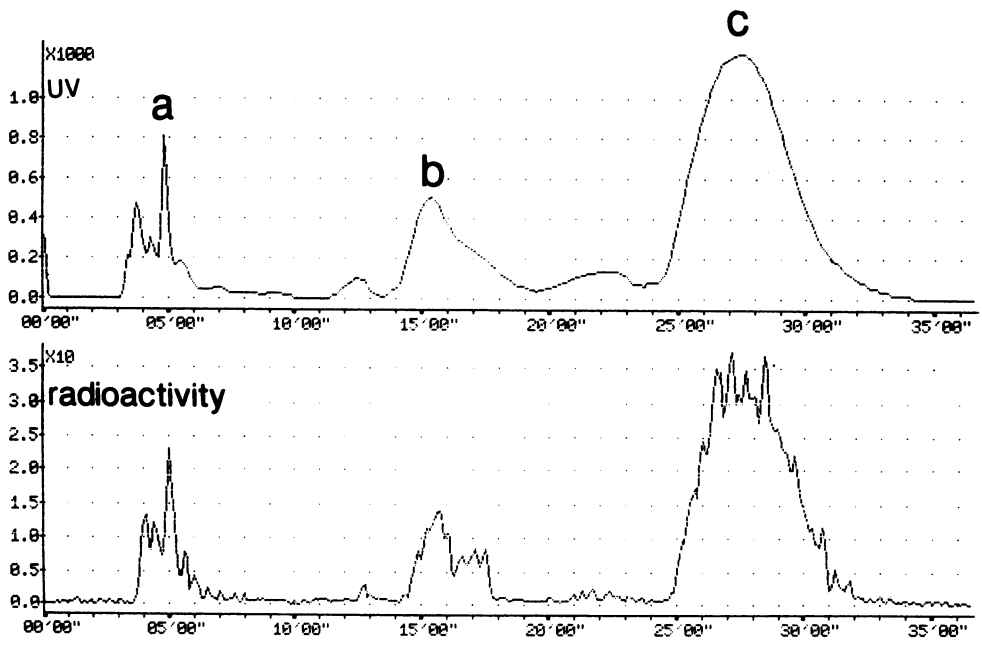

FIG. 2. Elution pattern of ^{14}C-UDP-peptidoglycan precursors separated by high-performance chromatography. The elution profile was serially traced with UV light at 260 nm and a flow scintillation spectrophotometer.

both UV-absorbing (254 nm) and radioactive spots; the latter were detected with an IM-3000 Radio-TLC-Analyzer (Isomess, Münster, Federal Republic of Germany).

The precursor material with approximately the same R_f value as the standard, UDP-MurNAc-alanyl-glutamyl-diaminopimelic acid (gift from D. Mirelman, Weizmann Institute of Science, Rehovot, Israel), was eluted from the thin-layer chromatography plate with 10 mM NH_4OH and purified further by high-performance liquid chromatography (2) with a C-18 column (250 by 4.6 mm; Particil; Whatman, Maidstone, England) using 50 mM ammonium phosphate, pH 3.65. The samples were serially traced both for UV-absorbing material at 260 nm (ISCO UV absorbance detector; ISCO, Lincoln, Nebr.) and for radioactivity with a RAMONA-D flow scintillation spectrophotometer (Isomess) equipped with a 100-μl yttrium cell. Three major UV-absorbing and radioactive peaks were detected (Fig. 2, peaks a, b, and c). Peaks b and c were concentrated and desalted on a Sephadex G-10 column (0.7 by 28 cm) and subjected to acid hydrolysis (4 N HCl at 105°C for 16 h in a vacuum). Quantitative amino acid and amino sugar analysis was performed as previously described (8). The results are presented in Table 1. It appears that both peaks b and c contain UDP-MurNAc-alanyl-glutamyl-lanthionyl-alanyl-alanine. The difference in retardation time in high-performance liquid chromatography indicates modifications not detected by the amino acid analysis. Because of the small amounts available, such possible modifications were not further investigated. The purification yielded 27.6 nmol of pentapeptide precursor per g (dry weight) of bacteria. This amount is about 5% of that isolated from *Escherichia coli* (4). Because of the lack of specific methods for detection of nucleotide precursors, it is impossible to estimate losses during purification.

Various antibiotics have been used in order to achieve accumulation of peptidoglycan precursors in *F. nucleatum*. Preliminary experiments showed, however, that vancomycin and cycloserine did not have any inhibiting effect on the growing bacteria and that cefotaxime caused lysis of the cells.

The present results show that lanthionine is incorporated as such into the growing stem peptide of the final UDP-MurNAc-pentapeptide before the assembly of peptidoglycan. The synthesis of the *F. nucleatum* peptidoglycan is therefore in this respect similar to, e.g., *meso*-diaminopimelic acid-containing peptidoglycans.

TABLE 1. Amino acid analysis of ^{14}C-UDP precursors after high-performance liquid chromatography[a]

Compound	Peak b		Peak c	
	Amt (nmol/sample)	Amt relative to glutamine	Amt (nmol/sample)	Amt relative to Glu
Muramic acid	2.8	1.2	5.1	1.0
Glutamic acid	2.4	1.0	5.2	1.0
Alanine	7.0	2.9	17.7	3.4
Lanthionine	3.4	1.4	6.5	1.3

[a] Samples taken from the peaks indicated in Fig. 2.

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