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Demonstration of Lanthionine as a Natural Constituent of the Peptidoglycan of *Fusobacterium nucleatum*

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Peptidoglycan was purified from the oral bacterium *Fusobacterium nucleatum* strain Fev 1, using boiling sodium dodecyl sulfate and pronase. The composition of this peptidoglycan was found to be similar to that of other gram-negative bacteria, except that it lacked diaminopimelic acid. Lanthionine, the monosulfur analog of diaminopimelic acid, was identified as the diaminodicarboxylic acid of this peptidoglycan. It is assumed that lanthionine replaced diaminopimelic acid. Thus, the peptidoglycan of *F. nucleatum* Fev 1 is one of the few known sources of naturally occurring lanthionine.

The components that normally constitute the peptidoglycan of gram-negative bacteria are: *N*-acetylglucosamine, *N*-acetyl muramic acid, alanine, glutamic acid, and a diamino acid. Although a few exceptions have been reported (23), the diamino acid is usually *meso*-diaminopimelic acid (Dpm). Thus, Dpm together with muramic acid have been used as markers for these peptidoglycans. We report here the purification of the peptidoglycan of the obligate anaerobic organism *Fusobacterium nucleatum* strain Fev 1. It is shown that a thioether diaminodicarboxylic acid, identified as lanthionine, is present in this peptidoglycan. Lanthionine, the monosulfur analog of Dpm, is found in the peptide antibiotics subtilin (1) and nisin (5). It is also present in insect hemolymph (16), locust muscle protein (26), chicken embryo (25), and plant pollen (19). Recently, it has been detected in human urine (27). Lanthionine, however, is often thought to represent an artefact formed from cystine due to the influence of alkali on proteins (10). To our knowledge, this is the first time lanthionine has been demonstrated to exist as a natural constituent of a gram-negative bacterium.

MATERIALS AND METHODS

Pronase (101,000 proteolytic units/g) was from Calbiochem, La Jolla, Calif. DL-Lanthionine and *meso*-lanthionine were from EGA Chemie, Steinheim/Albach, W. Germany. Radioactive materials were from the Radiochemical Centre, Amersham, England. Cell walls from *Ampullariella regularis* containing *meso*-2,6-diamino-3-hydroxypimelic acid (m-HyDpm) was a gift from H. R. Perkins, Liverpool, England. Peptidoglycan from *Escherichia coli* B cells containing *N*-acetyl muramic acid, N-acetylglucosamine, glutamic acid, alanine, and Dpm was prepared as described earlier (11). All other chemicals were of reagent grade.

Bacteria and growth conditions. *F. nucleatum* strain Fev 1 (6) was kindly provided by S. E. Mergenhagen, Bethesda, Md. Cultures were grown in 0.5-liter screw-cap bottles (9) filled to the top with the following (in grams per liter): tryptone (Oxoid Ltd., London), 15; NaCl, 5; KH_2PO_4, 1.5; $Na_2HPO_4 \cdot 2H_2O$, 3.5; $NaHCO_3$, 0.5; yeast extract (Oxoid), 3.0; L-cysteine-hydrochloride, 1; and glucose, 2.5; pH 7.0. Cultures were grown for 48 h and washed twice with phosphate-buffered saline (pH 7.4). When not used immediately, the cells were stored at −21°C. In some experiments, ascorbic acid (final concentration, 0.1%) replaced L-cysteine-hydrochloride as the reducing agent in the medium.

Purification of the peptidoglycan. The cells were added to boiling 4% sodium dodecyl sulfate (SDS) (21) under constant stirring. After 1 h, the suspension was brought to room temperature and sonicated (low energy, 1 min), and the insoluble material was sedimented by centrifugation (27,000 × *g*, 30 min, 15°C). The pellet was suspended in distilled water and sedimented at least six times. The preparation obtained is called sacculi. Sacculi were incubated with pronase (50:1, wt/wt, 10 mM Tris-hydrochloride, pH 7.4, 37°C, 16 h), followed by the addition of SDS (final concentration, 4%) and placed in a boiling-water bath for 15 min. SDS-insoluble material was sedimented and washed as described above. The peptidoglycan thus obtained was lyophilized before further analysis.

To omit the use of boiling SDS, cell walls were also prepared by a rapid screening method (20). The cells (500 mg, wet weight) were suspended in 10 ml of 10% trichloroacetic acid and placed in a boiling-water bath for 20 min with occasional stirring. The suspension was sonicated and centrifuged as described above. The pellet was carefully rinsed with distilled water and suspended and sedimented in 0.1 M Tris-hydrochloride (pH 7.4) before it was incubated with pronase as described above.

Radioactive labeling of cells. *F. nucleatum* Fev 1 was grown in the presence of 455 μCi of L-[^{35}S]-

cysteine-hydrochloride (74 mCi/mmol) in 0.5 liters of medium. L-Cysteine was added to the medium to a final concentration of 0.1%. Growth was permitted for 48 h, and the peptidoglycan was prepared as described above. The sacculi contained 0.3% of the total radioactivity, and after pronase digestion the specific radioactivity was approximately 50,000 cpm/mg of peptidoglycan.

Amino acid and amino sugar analysis. Hydrolyzed samples (4 N HCl, 105°C, 16 h, in vacuum) were dried over NaOH pellets in vacuum. Standards were treated under the same conditions. Quantitative analyses were performed in a Beckmann 120B amino acid analyzer. The conditions used in the long column (25; D. H. Spackman, Fed. Proc. Am. Chem. Soc. **22**:244) have been modified as follows to allow muramic acid to elute before glutamic acid and Dpm to elute after methionine. The samples were dissolved in pH 2.2 buffer, and the elution was started with pH 3.1 buffer at 45°C. The temperature was raised to 55°C after 60 min, and the buffer change was set at 85 min.

Chromatography. Samples from acid hydrolysates were analyzed on cellulose thin-layer chromatography (TLC) plates (E. Merck AG, Darmstadt, Germany). Whatman 3MM paper was used for preparative purposes. The following solvent systems were used: solvent I (butanol/pyridine/glacial acetic acid/water, 60:40:3:30, vol/vol/vol/vol), solvent II (methanol/pyridine/12 N HCl/water, 80:10:2:18, vol/vol/vol/vol), solvent III (methanol/pyridine/98% formic acid/water, 80:10:1:19, vol/vol/vol/vol), solvent IV (methanol/glacial acetic acid/12 N HCl/water, 80:10:2:18, vol/vol/vol/vol).

Electrophoresis. Electrophoresis on cellulose TLC plates at 10 V/cm for 1 h was performed at pH 6.4 (pyridine/glacial acetic acid/water, 100:4:900, vol/vol/vol) and pH 1.9 (2.5% formic acid).

Detection of radioactivity. Samples were dried on glass filters and counted in a toluene-based fluid by using a liquid scintillation counter. Radioactive spots on TLC plates were revealed by exposure to X-ray film for 7 days.

Isolation of lanthionine. Acid hydrolysates of *F. nucleatum* Fev 1 peptidoglycan were applied to prewashed Whatman 3MM paper (30 mg/sheet) and developed in solvent III for 20 h. Lanthionine was the slowest moving compound and was well separated from the other amino acids. Elution from the paper was performed with water (8), and complete elution was verified with ninhydrin. Due to its poor solubility in water, the lyophilized compound was made 1 mg/ml in 0.01 N HCl before further analysis.

Ninhydrin reaction at pH 0.9. Diamino acids dissolved in 0.01 N HCl (final volume, 0.25 ml) were heated in capped tubes with 0.25 ml of glacial acetic acid and 0.25 ml of ninhydrin reagent (2.5% [wt/vol]) ninhydrin in a mixture of glacial acetic acid and 0.6 M phosphoric acid, 3:2, vol/vol at 100°C for 5 min (28). The mixture was rapidly cooled and diluted with glacial acetic acid (final volume, 2.5 ml), and the optical density was read against a reagent blank in a Zeiss PMQ II spectrophotometer.

Peroxide oxidation. Dried acid hydrolysates of peptidoglycan (1 mg) and amino acid standards (0.1 mg) were dissolved in water and oxidized with peroxide (final concentration, 1%) for 4 h at 25°C in the presence of 0.002 M ethylenediaminetetraacetic acid (EDTA) (7). The volume of the reaction mixture was 0.6 ml. Analysis of the oxidation products was performed in the amino acid analyzer as described above.

Detection of sulfur-containing amino acids. The modified iodoplatinate reagent (14) was used to visualize sulfur-containing amino acids after TLC by using solvent IV. The reagent consisted of 0.1 ml of chloroplatinic acid (10%), 7 ml of potassium iodide solution (1.1%), and 7 ml of water, to which 80 ml of 0.5% starch solution was added immediately before use. The chromatograms were extensively dried in a stream of cold air before being sprayed with the reagent.

Modification with 1-fluoro-2,4-dinitrobenzene. Saturated $NaHCO_3$ was added to 100-μl portions of isolated lanthionine from acid hydrolysates of *F. nucleatum* Fev 1 peptidoglycan and commercial lanthionine until the pH reached 10. A 10-μl amount of a freshly prepared 10% 1-fluoro-2,4-dinitrobenzene solution in absolute ethanol was added, and the mixture was incubated for 30 min at 60°C. Unreacted reagent was extracted twice with ether, and the mixture was acidified with 6 N HCl. Any di-dinitrophenyl (di-DNP) derivative was extracted (twice) with ether, and the water phase was again extracted (twice) with butanol to obtain the mono-DNP derivative. The extracts were dried by using a rotary evaporator and dissolved in a small volume of 96% ethanol.

Other techniques. The anthrone test (22) was performed to test the peptidoglycan preparation for its sugar content, using D-glucose as standard. The absorbance was read at 625 nm.

RESULTS

Preparation and chemical composition of the peptidoglycan. An average of 7 to 10 mg of peptidoglycan was obtained from a 500-ml culture of *F. nucleatum* Fev 1. The sugar content was estimated to be 3% (wt/wt). TLC of acid hydrolysates (solvent I) of the peptidoglycan (Fig. 1a) revealed a pure preparation, apparently containing the same constituents as the peptidoglycan from *E. coli*: *N*-acetyl muramic acid, *N*-acetylglucosamine, alanine, glutamic acid, and Dpm. The amino acid analyzer, however, failed to demonstrate Dpm.

Due to their role in cross-linking adjacent peptide subunits, diamino acids are important constituents of peptidoglycans. The possibility that Dpm might be replaced by an amino acid with similar properties was therefore investigated.

The slowly moving component with an R_f nearly identical to Dpm in solvent I (Fig. 1a) was thus compared with several of the amino acids reported to replace Dpm in the peptide chain of peptidoglycans of gram-positive cells (20) and other naturally occurring diamino acids

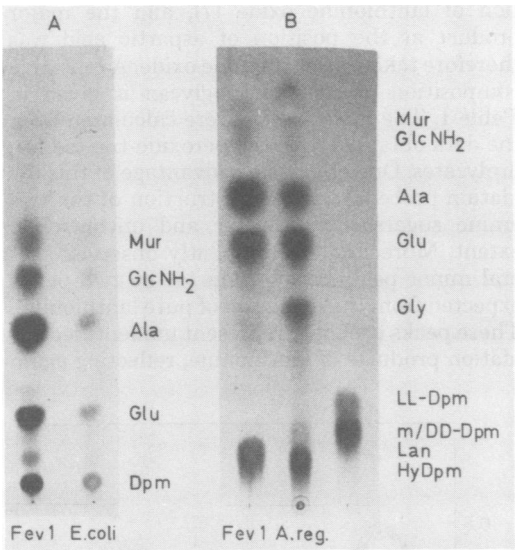

FIG. 1. *TLC of acid hydrolysates of peptidoglycans in solvent I (A) and solvent II (B). Fev 1, F. nucleatum Fev 1; E. coli, E. coli B; A. reg., A. regularis; Lan, lanthionine; Mur, muramic acid.*

(28). In solvent II, which is especially useful for the separation of slowly moving amino acids, including isomers of Dpm (18) and HyDpm (15), the slowly moving component had an R_{Dpm} of 0.71 (Fig. 1B), and was clearly separated from HyDpm. In addition, it did not show the characteristic greenish color turning to permanent yellow when sprayed with ninhydrin which is observed with Dpm and HyDpm when run in this solvent. On the basis of its chromatographic behavior in solvents I and II, it was concluded that the slowly moving component of the Fev 1 peptidoglycan was not any of the amino acids tested, with the possible exception of lanthionine. This amino acid migrated identically to the component that migrated slowly in either solvent I or II. It also showed the same color reaction with ninhydrin, namely, grayish purple when run in solvent I and grayish brown when run in solvent II. The same slowly moving component was found in the cell wall preparations obtained by using trichloroacetic acid instead of SDS extraction, and in peptidoglycan prepared from *F. nucleatum* Fev 1 cells grown in the media in which ascorbic acid replaced L-cysteine as the reducing agent.

Identification of the slowly moving component as lanthionine: (i) Demonstration of sulfur. Sulfur-containing amino acids give bright blue color with the iodoplatinate reagent containing starch (14). To avoid bleaching of the reagent with pyridine (2), the samples were chromatographed in solvent IV. The isolated slowly moving component and lanthionine had identical R_f values and color reactions. Quantities of 5 μg were clearly visible, in agreement with an earlier report (14). The presence of sulfur in the peptidoglycan was also established through the labeling of the cell wall with L-[^{35}S]cysteine. An equivalent of 20,000 cpm of the peptidoglycan was hydrolyzed and subjected to TLC in solvent II. As revealed by autoradiography, the radioactivity was located at the position of lanthionine (Fig. 2) with no trace of cysteine. Traces of radioactivity were detected at the position of cystine.

(ii) Electrophoretic mobility. Samples of the isolated, slowly moving component were subjected to electrophoresis on cellulose TLC plates. At pH 6.4, a single neutral ninhydrin-positive spot was revealed which at pH 1.9 moved toward the cathode (Fig. 3). Coelectrophoresis of the slowly moving compound and commercial lanthionine at pH 1.9 revealed one spot only. Both the mono- and di-DNP derivatives moved toward the cathode at pH 6.4, and

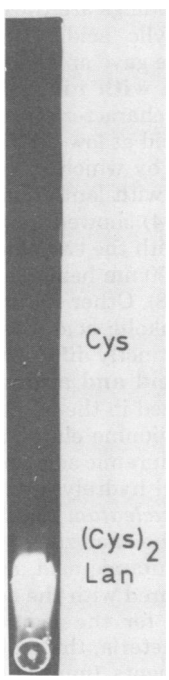

FIG. 2. *Negative print of a TLC autoradiogram of acid hydrolysate of peptidoglycan of F. nucleatum Fev 1. The peptidoglycan was isolated from cells grown in the presence of L-[^{35}S]cysteine-hydrochloride (see text); 20,000 cpm was applied and chromatographed with solvent II. The film was exposed for 7 days. Lan, lanthionine.*

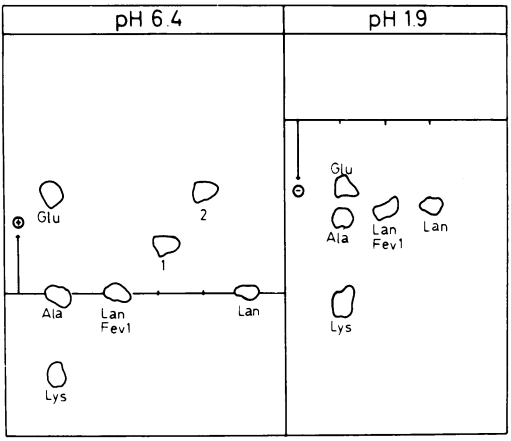

FIG. 3. *Electrophoresis on cellulose TLC plates of amino acids and DNP derivatives at pH 6.4 and pH 1.9. The electrophoresis was carried out for 60 min at 10 V/cm. The mono- and di-DNP derivative of this amino acid are indicated by 1 and 2, respectively. Lan, commercial lanthionine; Lan Fev 1, lanthionine from F. nucleatum Fev 1.*

only the mono-DNP derivative reacted with ninhydrin. These findings are those expected for a diaminodicarboxylic acid. Commercially prepared lanthionine gave an identical reaction.

(iii) Reaction with ninhydrin at pH 0.9. Ninhydrin gives characteristic absorption spectra to diamino acid at low pH (28). This offered a further means by which to compare the isolated compound with lanthionine. The absorption curves (Fig. 4) showed the two compounds to be identical, with the two characteristic maxima at 360 and 450 nm being in agreement with earlier reports (28). Other sulfur-containing diamino acids (djenkolic acid, cystathionine, and cystine) have distinctly different spectra (28).

(iv) Amino acid and amino sugar analysis. When analyzed in the amino acid analyzer, commercial lanthionine eluted as two peaks at the positions of muramic acid and glutamic acid, respectively. Acid hydrolysates of the peptidoglycan from *F. nucleatum* Fev 1 gave only four major peaks on the analyzer, at the positions of muramic acid, glutamic acid, alanine, and glucosamine. Compared with the composition that is usually found for the peptidoglycans from gram-negative bacteria, the molar ratios of the first two components (muramic acid and glutamic acid) were higher than expected when related to alanine. Analysis of the peroxide oxidation products of commercial lanthionine and isolated lanthionine revealed a major peak (90 to 95%) eluting at the position of aspartic acid, and a minor peak (5 to 10%) at the position of cysteic acid. The mild oxidation conditions and the presence of EDTA should favor the formation of lanthionine oxide (7), and the major product at the position of aspartic acid was therefore taken as lanthionine oxide. A tentative composition of the peptidoglycan is given in Table 1. The molar ratios were calculated from the data obtained with the peroxide-treated hydrolysates. One obvious disadvantage of this oxidation procedure is the destruction of the two amino sugars to a variable and uncontrolled extent. Moreover, we frequently observed several minor peaks in addition to the two peaks expected from the oxidation of pure lanthionine. These peaks probably represent unidentified oxidation products of lanthionine, reflecting again

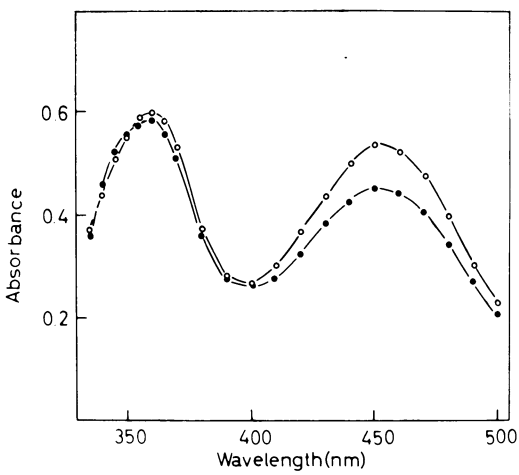

FIG. 4. *Reaction of ninhydrin at pH 0.9 with lanthionine. The reaction mixture was incubated at 100°C for 5 min and diluted with glacial acetic acid (final volume, 2.5 ml; see text). Symbols: ○, commercial lanthionine (20 μg); ●, lanthionine from F. nucleatum Fev 1 (18 μg).*

TABLE 1. *Tentative composition of the peptidoglycan of F. nucleatum* Fev 1[a]

Component	Ratio to glutamic acid
Amino acid	
Glutamic acid	1
Alanine	1.81
Lanthionine[b]	0.77
Amino sugar[c]	
Muramic acid	0.80
Glucosamine	1.06

[a] Subsequent to hydrolysis (4 N HCl, 105°C, 16 h), all samples were treated with 1% (final concentration) peroxide for 4 h at 25°C.

[b] The amount of lanthionine was determined as the combined peaks eluting at the place of cysteic acid and aspartic acid by using the same factor, and it was corrected for 37% loss during the treatment.

[c] The amount of both muramic acid and glucosamine was corrected for the 47 and 62% losses due to the treatment, respectively.

the imperfection of the method used for the quantitation of this amino acid.

DISCUSSION

We have obtained an apparently pure peptidoglycan by treating *F. nucleatum* Fev 1 with boiling SDS and pronase. Beside *N*-acetyl muramic acid, *N*-acetylglucosamine, alanine, glutamic acid, and a diamino acid, the polymer contained only 3% sugar and only traces of other ninhydrin positive material. This diamino acid, however, is not Dpm, which is ubiquitously distributed in the peptidoglycans of facultative gram-negative bacteria (20). The sulfur-containing diaminodicarboxylic acid lanthionine replaces Dpm in this organism (Fig. 5). This is contradictory to earlier reports on the composition of undigested cell walls of fusobacteria in which the authors reported the presence of Dpm (4, 13).

In one instance (13), the discrepancy may be due to the analytical conditions used on the amino acid analyzer which do not distinguish between Dpm and methionine. Our results, on the other hand, are supported by those of Baboolal (3), who was not able to demonstrate Dpm in the cell wall of five strains of *F. nucleatum*.

That lanthionine was not artificially formed from cystine, which might be the natural constituent of the peptidoglycan, was ruled out by replacing the alkali (pH 9.0) boiling-SDS treatment with a shorter treatment, using trichloroacetic acid, that was followed by pronase digestion (20). In the rather crude preparation thus obtained, lanthionine was easily demonstrated in normal amounts.

The labeling experiment with L-[^{35}S]cysteine indicates that L-cysteine is a precursor of lanthionine. It was thus a possibility that the high concentration of L-cysteine (0.1%) in the medium favored the incorporation of the Dpm analog, lanthionine, into the peptidoglycan. When L-cysteine was replaced by ascorbic acid as a reducing agent in the medium, lanthionine was still present and no trace of Dpm was detected.

It has been shown that the presence of lanthionine in the medium of Dpm auxotrophic mutans of *E. coli* permits growth and prevents lysis at suboptimal concentrations of Dpm (12, 17). Under these conditions, however, the shape of the *E. coli* cells was not normal, and they tended to form spheroplasts (12). With the growth medium used in our experiments, the morphology of *F. nucleatum* Fev 1 seemed normal and did not differ from that of other strains of *F. nucleatum*.

The uniformity of the peptidoglycans of gram-negative bacteria has not so far permitted the use of the composition of this cellular entity in the classification of these bacteria (20). If present in other strains or species of *Fusobacterium*, lanthionine might serve as a taxonomic marker for this genus. This particular possibility is under investigation in our laboratory.

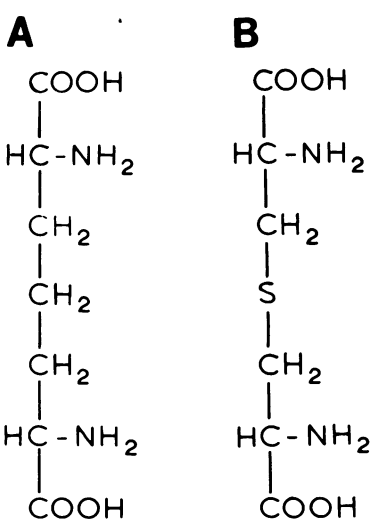

FIG. 5. *Structure of (A) Dmp and (B) meso-lanthionine.*

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