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Lanthionine as an Essential Constituent of Cell Wall Peptidoglycan of *Fusobacterium nucleatum*

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Abstract. Lanthionine, a sulfur-containing diamino acid which had not previously been reported as one of the main amino acids of any bacterial cell wall peptidoglycan, was demonstrated in *Fusobacterium nucleatum* peptidoglycan isolated by sodium dodecyl sulfate extraction and protease digestion. Lysine, diaminopimelic acid, and ornithine were absent. Lanthionine seems to be an essential dibasic amino acid, involved in cross-linkages between stem peptide subunits in *F. nucleatum*.

A large number of studies on chemical structure of bacterial cell wall peptidoglycans have been summarized in recent reviews of Schleifer and Kandler [12], Ghuysen and Schockman [5], and Tipper [14]. However, information is very limited on chemical characteristics of peptidoglycans of anaerobic bacteria. In the present study, analyses have been made of amino acid and amino sugar compositions of peptidoglycan of *Fusobacterium nucleatum*, which was isolated by boiling whole cells in 4% sodium dodecyl sulfate (SDS) and by digestion of SDS-extracted residue with proteases. Analyses with an amino acid analyzer on the peptidoglycan demonstrated the presence of an amino acid that had never been reported as a main component of cell wall peptidoglycans. This amino acid was shown to be lanthionine.

Materials and Methods

Organisms. *Fusobacterium nucleatum* ATCC 25586 was grown in a medium (pH 7.2) consisting of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Maryland), 15 g; yeast extract (Difco), 5 g; sodium chloride, 2.5 g; and L-cysteine hydrochloride, 0.75 g per liter; at 37°C for 5 days under anaerobic conditions in a mixture of nitrogen, hydrogen, and carbon dioxide (80:10:10, vol/vol).

Isolation of cell envelope and peptidoglycan. The cell envelope and peptidoglycan fractions were prepared principally by the method described by Heilman [6] and Yanai et al. [15], with minor modifications as follows.

Step 1. Nine grams of lyophilized cells were suspended in 450 ml of 4% SDS and boiled for 1 h with constant stirring. A pellet obtained by centrifugation of the suspension at 58,000 × *g* for 90 min was washed twice with 6 M urea solution and three times with distilled water. To remove some coarse debris, the washed pellet suspended in water was centrifuged at 1,500 × *g* for 10 min and then lyophilized (820 mg, a cell envelope fraction).

Step 2. A cell envelope (800 mg) was suspended in 160 ml of 0.01 M Tris-hydrochloride buffer, pH 7.4, containing 40 mg of Pronase P (Kakken Kagaku Ltd., Tokyo; 750,000 tyrosine U/g) and incubated at 37°C for 18 h with stirring. The pellet recovered by centrifugation of the incubation mixture, after being washed once with water, was resuspended in 0.01 M Tris-hydrochloride buffer, pH 8.2, containing 40 mg of crystalline trypsin (Trypsilin, Mochida Pharmaceutical Co. Ltd., Tokyo; 100,000 U/mg) and was subjected to digestion at 37°C for 2 h with stirring. The protease-treated cell envelope was lyophilized, after washing three times with water (213 mg, a peptidoglycan fraction).

Homogeneity and purity of the peptidoglycan fraction was shown to be in satisfactory condition for chemical analyses by examination with a Hitachi electron microscope (model HU-12A; Hitachi Ltd., Tokyo).

Chemical analyses. For amino acid analysis, specimens were hydrolyzed in 6N HCl at 100°C for 14 h, in a sealed tube filled with nitrogen. Hydrolysates were dried in a vacuum over pellets of sodium hydroxide to remove hydrogen chloride, and examined with an amino acid analyzer (model KLA-3B; Hitachi Ltd. Tokyo). The temperature of an analyzer column was maintained in most analyses at 55°C, but in a part of the analyses the temperature of the column was shifted to 45°C to obtain good separation of muramic acid from glutamic acid.

Paper chromatography was performed by use of Whatman filter paper no. 3 (W. & R. Balston Ltd., England) and *n*-butanol–pyridine–water–acetic acid (60:40:30:3, vol/vol), according to the conventional method.

Optical configurations of glutamic acid and alanine were determined by the enzymatic method, as described by Kotani et al. [9]. Hydrolyzed specimens were treated with either L-glutamic acid decarboxylase or D-amino acid oxidase, and residual amounts of glutamic acid and alanine were estimated by the amino acid analyzer, respectively.

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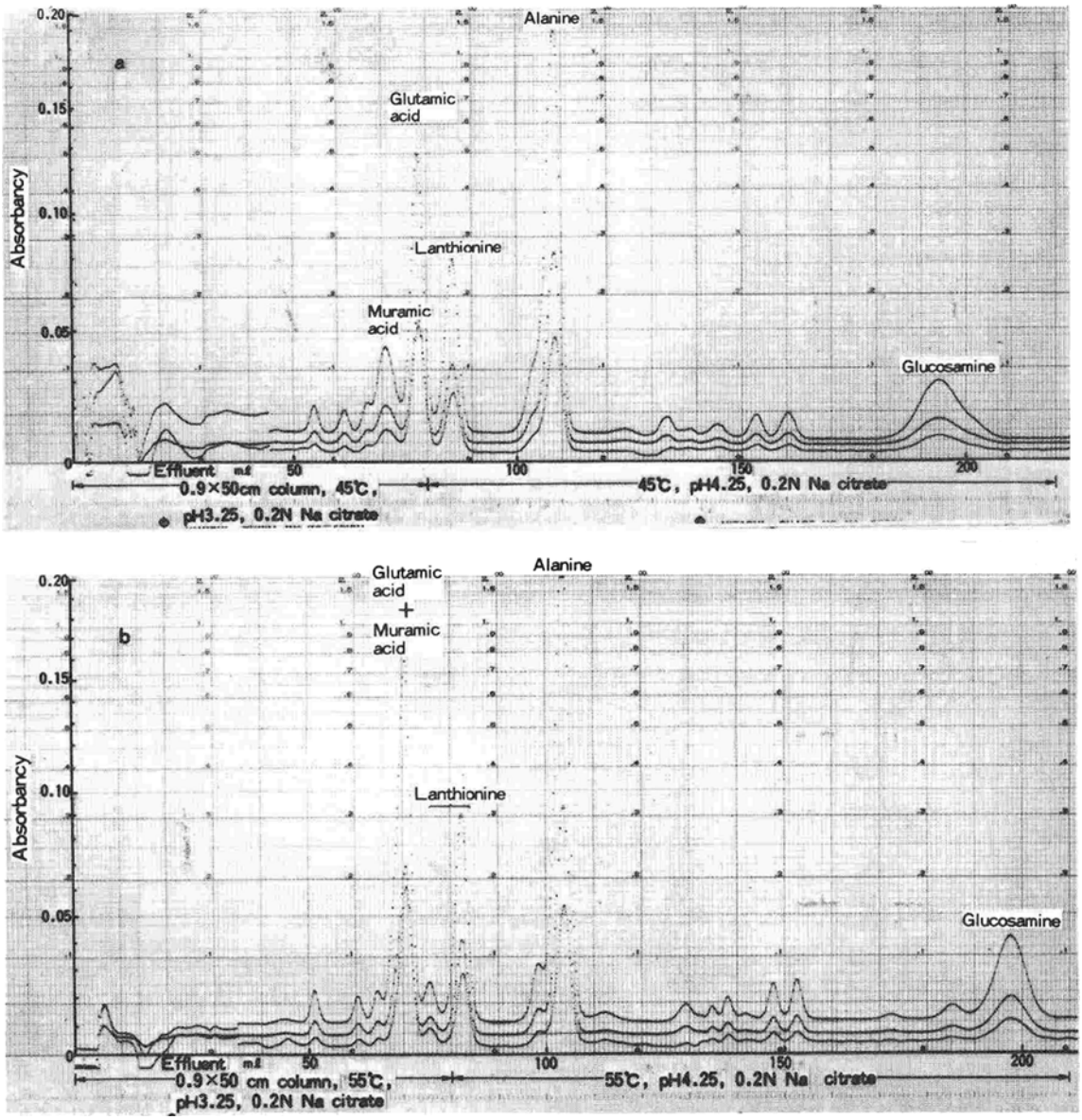

Fig. 1. Elution profiles of a hydrolysate of *Fusobacterium nucleatum* cell wall peptidoglycan on automatic amino acid analyzer. Temperature of the resin column was 45°C (a) and 55°C (b).

N- and C-terminal amino acids were quantitatively determined as described previously [7]. The amino acid composition of test specimens was determined before and after dinitrophenylation, and a decreased amount of constituent amino acid by dinitrophenylation was calculated as N-terminal amino acid. The amino acids released by hydrazinolysis, on the other hand, were determined with the amino acid analyzer and were taken as C-terminal amino acids.

Results

Lanthionine as an essential component of *Fusobacterium nucleatum* peptidoglycans. Elution profiles of the acid hydrolysate of *F. nucleatum* peptidoglycan in amino acid (and amino sugar) analyses are shown in Fig. 1a (resin column temperature adjusted to 45°C) and Fig. 1b (55°C). These indicate the existence of one or more amino acids which have never before been reported as constituents of bacterial cell wall peptidoglycans. Identity of this compound as lanthionine was established by comparing the retention time in the amino acid analyzer (Fig. 2) and the R_f value in paper chromatography (Fig. 3) of an authentic specimen of DL-lanthionine (Wako Chemical Industries Ltd., Osaka) and L(+)*meso*-lanthionine (Nakarai Chemicals Ltd., Kyoto) with those from *F. nucleatum* peptidoglycan.

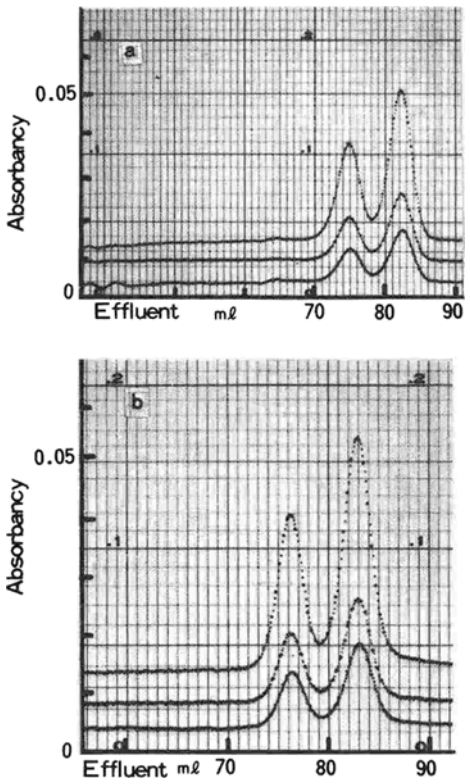

Fig. 2. Elution profiles of an authentic DL-lanthionine or L(+)*meso*-lanthionine on automatic amino acid analyzer. The program of elution with citrate buffer was the same as shown in Fig. 1. Temperature of the resin column was 45°C (a) and 55°C (b).

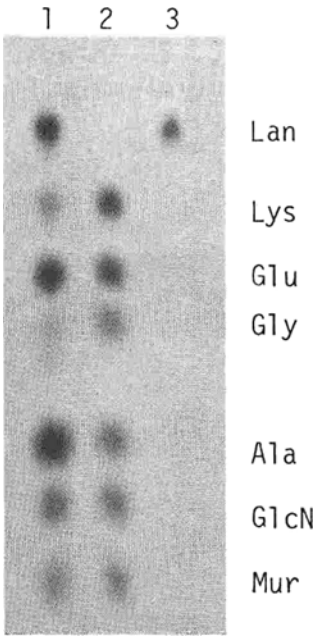

Fig. 3. Paper chromatogram of a hydrolysate of the peptidoglycan of *Fusobacterium nucleatum* and authentic amino acids and amino sugars. Spots were developed with ninhydrin reagent, and the lanthionine spot was separately identified with iodine solution containing sodium azide [13]. (1) Hydrolysate of *F. nucleatum* peptidoglycan, (2) a mixture of authentic amino acids and amino sugars (but lacking lanthionine), (3) authentic DL-lanthionine.

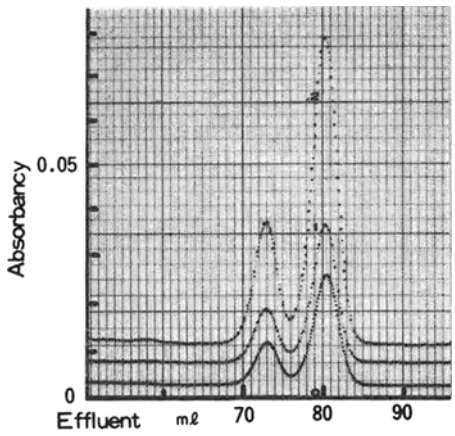

Fig. 4. Elution profile on automatic amino acid analyzer of an eluate from a spot—having an R_f value identical with authentic lanthionine—given by a hydrolysate of *Fusobacterium nucleatum* peptidoglycan. The program of elution with citrate buffer was the same as shown in Fig. 1. Temperature of the resin column was 45°C.

The authentic lanthionine samples and the lanthionine contained in the hydrolysate of *F. nucleatum* peptidoglycan all gave two, clearly separated peaks in the amino acid analyses (Figs. 1 and 2). However, in the analyses of the hydrolysates at a temperature of 45°C, the more rapidly eluted peak of lanthionine overlapped that of glutamic acid.

It may be added here that an eluent with water of the spot given by the test hydrolysate and having an R_f value identical with lanthionine in paper chromatography also gave two peaks coinciding with those of authentic lanthionine (Fig. 4).

Amino acid and amino sugar composition of *F. nucleatum* peptidoglycans. Table 1 shows the amino sugar and amino acid composition of *F. nucleatum* peptidoglycan as compared with those of a cell envelope fraction. Main constituent amino sugars and amino acids were glucosamine, muramic acid, glutamic acid, lanthionine, and alanine in a molar ratio of 0.66 : 0.72 : 1.00 : 0.68 : 1.74. The content of lanthionine was estimated on the basis of the results obtained by analyses at 55°C, and that of glutamic acid was calculated by subtracting a value shared by overlapping lanthionine from a value (at 45°C) of an apparent glutamic acid. In the latter calculation, a molar ratio of two peaks given by lanthionine at 55°C analysis was taken to be 0.09 : 1.00 based on the result illustrated in Fig. 1b, assuming that the coefficients of ninhydrin color development are not different between the two. The quantity of glutamic acid was unchanged on treatment with L-glutamic acid

Table 1. Amino acid and amino sugar composition of cell envelope and peptidoglycan of *Fusobacterium nucleatum* ATCC 25586.

Component[a]	Cell envelope		Peptidoglycan	
	nmol/mg	molar ratio	nmol/mg	molar ratio
Glucosamine	—[b]		510[c]	0.66
Lysine	556	0.71	122	0.16
Arginine	218	0.28	37	0.05
Histidine	81	0.10	tr	
Aspartic acid	466	0.59	46	0.06
Threonine	222	0.28	32	0.04
Serine	181	0.23	23	0.03
Muramic acid	—[b]		560[c]	0.72
Glutamic acid	785	1.00	775	1.00
Lanthionine	207	0.26	44 532	0.06 0.68
Glycine	402	0.51	125	0.16
Alanine	994	1.27	1,349	1.74
Valine	418	0.53	32	0.04
Methionine	92	0.12	23	0.03
Isoleucine	269	0.34	45	0.06
Leucine	423	0.54	60	0.08
Tyrosine	108	0.14	12	0.02
Phenylalanine	248	0.32	32	0.04

[a] Hexose and methylpentose were detected, but only in small amounts. Neither pentose nor phosphorus was found.
[b] Not detected
[c] Value was corrected for destruction during hydrolysis in 6 N HCl at 100°C. The values obtained after 0.5, 1, 2, 4, and 8 h were plotted against the time of hydrolysis and the line was extrapolated to zero time.

Table 2. Determination of N- and C-terminal amino acids of *Fusobacterium nucleatum* peptidoglycan by dinitrophenylation and hydrazinolysis, respectively.

Component	Before treatment	After dinitrophenylation	Recovered by hydrazinolysis
D-Glutamic acid	1.00	1.00	
L- and D-Alanine	1.71	1.88	0.16
Lanthionine	0.62	0.31	0.03

decarboxylase and about 50% of alanine disappeared by treatment with D-amino acid oxidase, indicating that all of the glutamic acid residue had D-configuration and the ratio of D-alanine to L-alanine residues was about 1:1.

N- and C-terminal amino acids of *F. nucleatum* peptidoglycans. Quantitative analyses of the N- and C-terminal amino acids have revealed that about half of the lanthionine residue located at the N-terminal had disappeared after dinitrophenylation, and 0.16 mol and 0.03 mol per mol of glutamic acid residue of alanine and lanthionine were recovered as C-terminal amino acids by hydrazinolysis, respectively. These results strongly suggest that one-half of the lanthionine was involved in the cross-linkages.

Discussion

In this study, we demonstrated that lanthionine, a sulfur-containing diamino acid, was one of the major constituents of *Fusobacterium nucleatum* cell wall peptidoglycan. No other diamino acids—such as lysine, diaminopimelic acid, and ornithine—which have been reported as essential components of other bacterial peptidoglycans were found. N- and C-terminal amino acid analyses have shown that half of the lanthionine residues participating in cross-linkages between the neighboring stem peptide subunits consists of D-glutamic acid, D- and L-alanine, and lanthionine. However, the recovery of C-terminal amino acid by hydrazinolysis was not satisfactory in a quantitative sense. This estimate may need further evidence; for instance, by isolation and identification of some fragments of a cross-linked portion of the peptidoglycan obtained by use of a lytic enzyme or by partial hydrolysis.

With regards to the origin of lanthionine, one possibility may be that the L-cysteine in the medium used for cultivation of *F. nucleatum* is utilized for biosynthesis of lanthionine. This mechanism does not seem to be probable, however, because we also find lanthionine in the peptidoglycan of *F. nucleatum* ATCC 25586 isolated from the cells grown in the medium without added L-cysteine hydrochloride. It may be noted, in this connection, that Knüsel et al. [8] described incorporation of ^{35}S-lanthionine in the cell walls of a mutant of *Escherichia coli* (which required diaminopimelic acid as one of the essential amino acids) when it was grown in a medium with radiolabeled lanthionine added in place of diaminopimelic acid.

Lanthionine and β-methyl-lanthionine have also been reported as components of antibiotics of bacterial origin, such as subtilin, nisin, and cinamycin [1,2,3,10,11].

We could not determine an optical configuration of *F. nucleatum* lanthionine because only a limited quantity of the compound was available. However, the elution profile in amino acid analysis suggested that the lanthionine occurring in the *F. nucleatum* peptidoglycan was a mixture of the *meso* (or D) isomer and the L isomer in an approximate molar ratio of 11.5 : 1. This view is strengthened by the paper by Blackburn and Lee [4], which reported that L-lanthionine emerged before *meso*-lanthionine when a

mixture of L- and *meso*-lanthionine was applied to a column of Dowex 50 and eluted with a buffer solution of pH 3.2.

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