JOURNAL OF BACTERIOLOGY, Apr. 1981, p. 49-53
0021-9193/81/040049-05$02.00/0

Vol. 146, No. 1

Chemical Structure of Peptidoglycan in *Selenomonas ruminantium*: Cadaverine Links Covalently to the D-Glutamic Acid Residue of Peptidoglycan

YOSHIYUKI KAMIO,* YOSHIFUMI ITOH, AND YOSHIRO TERAWAKI

Department of Bacteriology, Shinshu University School of Medicine, Matsumoto 390, Japan

Received 21 November 1980/Accepted 17 January 1981

The peptidoglycan of *Selenomonas ruminantium*, a strictly anaerobic bacterium, contains cadaverine (Y. Kamio, Y. Itoh, Y. Terawaki, and T. Kusano, J. Bacteriol. **145**:122-128, 1981). This report describes the chemical structure of the peptidoglycan of this bacterium. The [^{14}C]cadaverine-labeled peptidoglycan was degraded with the lytic enzymes prepared from *Streptomyces albus* G into three small fragments including a major fragment (band A compound). Band A compound was composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, D-alanine, and cadaverine in the molar ratio 0.98:1.0:1.0:0.98:0.97. Diaminopimelic acid, L-alanine, and cadaverine were N-terminal residues in band A compound. When the [^{14}C]cadaverine-labeled band A compound was subjected to partial acid hydrolysis, two peptide fragments were obtained. One of them consisted of diaminopimelic acid and D-alanine; diaminopimelic acid was the N-terminal amino acid, and the other fragment was composed of L-alanine, D-glutamic acid, and cadaverine, of which L-alanine and cadaverine were N-terminal. These results led us to conclude that the primary peptide structure of band A compound is L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine and that cadaverine links covalently to the D-glutamic acid residue.

Our previous study (6) revealed that cadaverine exists as a component of the peptidoglycan of *Selenomonas ruminantium* subsp. *lactilytica*, a strictly anaerobic, gram-negative strain, and that one of the two amino groups of cadaverine links covalently to the peptidoglycan but the other is free. In addition, we found (6) that the peptidoglycan of this strain is composed of L-alanine, D-alanine, D-glutamic acid, *meso*-diaminopimelic acid (*meso*-DAP), cadaverine, muramic acid, and glucosamine in the molar ratio 1.0:1.0:1.0:1.1:0.9:1.0. These findings led us to study which component of the peptidoglycan cadaverine links covalently. We previously obtained (6) spot A compound, which contains muramic acid, glucosamine, alanine, glutamic acid, *meso*-DAP, and cadaverine, by digesting the peptidoglycan with lysozyme. However, the yield of the compound was too low to allow us to examine its chemical structure. Therefore, in the present study, we used lytic enzymes prepared from *Streptomyces albus* G to examine the primary structure of the peptidoglycan. The enzyme preparation contained glycosidase, *N*-acetylmuramyl-L-alanine amidase, and endopeptidase (K. Kato, personal communication). As a result, we succeeded in obtaining the peptide subunit containing cadaverine in high yield. This report shows that cadaverine linked covalently to the D-glutamic acid residue of the peptidoglycan and further shows the chemical structure of the peptidoglycan of *S. ruminantium*.

MATERIALS AND METHODS

Bacterial strain. *S. ruminantium* subsp. *lactilytica*, described previously (6), was used.

Medium and culture conditions. *S. ruminantium* was grown in a yeast extract-glucose medium (7) supplemented with 0.01% sodium *n*-valerate at 37°C under anaerobic conditions (7).

Preparation of [^{14}C]cadaverine-labeled cells. The yeast extract-glucose medium (20 liters) containing *n*-valerate and [1,5-^{14}C]cadaverine (50 μCi, 0.11 μM) was inoculated with 80 ml of an overnight culture of *S. ruminantium* and incubated at 37°C for 4 h. The cells were collected and used for preparation of the peptidoglycan.

Preparation of peptidoglycan. The peptidoglycan was prepared by the procedure described previously (6).

Solvent systems. The following solvent systems were used for paper and cellulose thin-layer chromatography and for paper electrophoresis: system A contained *n*-butanol-acetic acid-water-pyridine (915:3:12:10, by volume); system B contained phenol-water (250:75, wt/wt) in an NH_3 atmosphere; system C was an upper phase of *n*-butanol-acetic acid-water (4:1:5, by volume); and system D contained 0.1 M citric acid-

NaOH buffer (pH 3.6).

Digestion of the peptidoglycan with enzymes from *S. albus* G. [^{14}C]cadaverine-labeled peptidoglycan (10 mg) was incubated at 37°C with *S. albus* G enzymes (500 μg of protein), which contain glycosidase, *N*-acetylmuramyl-L-alanine amidase, and endopeptidase (kindly donated by K. Kato, Department of Microbiology and Oral Bacteriology, Osaka University), for 20 h. Digestion with the enzyme was done in 5 ml of 0.01 M sodium phosphate buffer (pH 8.0) containing 50 μl of toluene. The reaction mixture was examined by descending paper chromatography on Whatman 3MM paper at room temperature for 50 h with solvent system C. A major band (designated band A compound; Fig. 1) was eluted with distilled water, lyophilized, and subjected to chemical analysis.

Acid hydrolysis of band A compound. Band A compound (500 μg) was hydrolyzed with 1 ml of 6 N HCl at 110°C for 18 h in a sealed tube for complete hydrolysis. Partial hydrolysis of the compound was performed by heating 500 μg of the compound in either 1 ml of 2 N HCl at 100°C for 2 h or 1 ml of 0.03 N HCl at 100°C for 12 h. After HCl was removed in vacuo in the presence of solid NaOH at room temperature, the hydrolysate was examined by paper chromatography and paper electrophoresis.

Analytical procedure. The amount of amino acids and amino sugars in the acid hydrolysate was determined quantitatively by using a Hitachi 835 automatic amino acid analyzer or by paper chromatography according to the method of Primosigh et al. (8), with alanine, glutamic acid, DAP, glucosamine, and muramic acid as the standards. Cadaverine was determined quantitatively by the method described previously (6). Determination of the *N*-acetylglucosamine was carried out by paper chromatography with solvent system C.

The optical configuration of alanine was determined according to the method described previously (6).

Carboxyl-terminal amino acid was determined by hydrazinolysis as described by Yanai et al. (9). One milligram of the peptidoglycan and 1.5 μmol of L-alanyl-L-alanyl-L-alanyl-L-alanine were hydrazinolyzed with 500 μl of freshly distilled anhydrous hydrazine in vacuum-sealed tubes at 100°C for 8 h. After almost all of the hydrazine was removed in vacuo in the presence of concentrated H_2SO_4, the preparations were dissolved in 1 ml of water and the remaining hydrazine was extracted with distilled benzaldehyde. After benzaldehyde was extracted with ethyl ether, the amount of each amino acid was determined by the ninhydrin reaction after 100 μl of the solution was applied to paper chromatography, using solvent system C. The amount of C-terminal amino acid was corrected on the basis of the yield of tetraalanine.

N-terminal amino acids were determined by subtracting the amino acid contents of dinitrophenylated samples from those of intact samples. Dinitrophenylation was carried out by the method of Yanai et al. (9).

Radioactivity was quantitated with a Packard 3255 liquid scintillation spectrometer with Bray scintillation fluid (2). Radioactive peptide fragments were detected by radioautography or by an Irigaku TRM 1-B radioscanner equipped with a windowless gas flow counter.

Sephadex G-15 column chromatography. The [^{14}C]cadaverine-labeled band A compound was dissolved in 50 mM NaCl and applied to a Sephadex G-15 column (1.6 by 90 cm) in 50 mM NaCl. The column was eluted with the same solution at room temperature.

Materials. D-Amino acid oxidase, peroxidase, and tetraalanine were purchased from Sigma Chemical Co., St. Louis, Mo. Cadaverine dihydrochloride and dinitrofluorobenzene were from Wako Chemical Industries Ltd., Tokyo, Japan. Hydrazine was from Nakarai Chemicals, Ltd., Kyoto, Japan. [1,5-^{14}C]cadaverine dihydrochloride was purchased from New England Nuclear Corp., Boston, Mass. The other chemicals used were of the best grade commercially available.

Cellulose thin-layer plates were purchased from E. Merck AG (Darmstadt, West Germany). For paper chromatography and electrophoresis, Whatman 3MM filter paper from W. & R. Balstom Ltd., Maidstone, Kent England, was used. Sephadex G-15 was from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

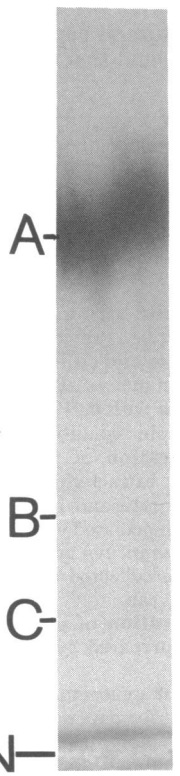

FIG. 1. *Radioautogram of the degradation products of the [^{14}C]cadaverine-labeled peptidoglycan after S. albus G enzymes digestion. (A) Band A; (B) and B; (C) band C.*

RESULTS

N- and C-terminal components of the peptidoglycan. Quantitative analyses of N-

and C-terminal components of the peptidoglycan revealed that cadaverine and half of the DAP had disappeared after dinitrophenylation (Table 1), and about one-fourth of the alanine was recovered as C-terminal amino acid. These results indicate that one-half of the DAP is involved in the cross-linkage to alanine and that cadaverine does not participate in the cross-linkage.

Treatment of the [^{14}C]cadaverine-labeled peptidoglycan with the enzymes from *S. albus* G. To elucidate the amino acid sequence of the peptidoglycan and the amino acid which links covalently to cadaverine, we tried to obtain the peptide subunit from the peptidoglycan. If the D-lactyl group of the *N*-acetylmuramic acid residue in the glycan strand is substituted by the L-alanine of the peptide subunit, this amide linkage should be degraded with *N*-acetylmuramyl-L-alanine amidase in the enzymes. The *S. albus* G enzymes hydrolyzed insoluble peptidoglycan of *S. ruminantium* into soluble fragments. Figure 1 shows a paper chromatogram of the degradation products. Approximately 80% of the total radioactivity incorporated into the peptidoglycan was found to be recoverable in band A compound. Trace amounts of radioactivity were present in bands B and C (Fig. 1). No radioactive band except at the origin was detected in the sample without enzyme treatment. Band A compound was eluted with distilled water from the paper and was purified by paper chromatography with solvent system A and by paper electrophoresis with solvent system D.

Chemical composition of band A compound. Band A compound was hydrolyzed in 6 N HCl and was subjected to automatic amino acid analysis and paper electrophoresis. Glutamic acid, alanine, DAP, and cadaverine, but not muramic acid or glucosamine, were found. These results clearly indicate that the L-alanine residue links covalently to -COOH of the lactyl group of *N*-acetylmuramic acid.

Quantitative analysis of amino acid and cadaverine in band A compound. Band A compound was found to consist of glutamic acid, alanine, DAP, and cadaverine in amounts of 1.76, 3.46, 1.78, and 1.70 μmol/mg (dry weight), respectively. These results give the purity of band A compound as 100%. Based on these results and our previous findings (6), the molar ratio of L-alanine, D-alanine, *meso*-DAP, and cadaverine to D-glutamic acid in band A compound was calculated as 0.98:0.98:1.01:0.97.

Elution profile of band A compound on Sephadex G-15 gel column chromatography. The radioactive peak of band A compound was detected at a position slightly later than that of NAD, whose molecular weight is 663. One can calculate the molecular weight of band A compound to be 545, assuming that the compound is a monomer.

N-terminal components of band A compound. DAP and cadaverine were completely dinitrophenylated, and one-half of the alanine was dinitrophenylated (Table 2). From these results and the elution profile on Sephadex G-15, it can be concluded that the compound in band A is a peptide monomer.

Partial acid hydrolysis of band A compound. Band A compound labeled with [^{14}C]-cadaverine was partially acid hydrolyzed, and the degradation products were separated by paper chromatography. Five ninhydrin-positive spots were obtained (Fig. 2). Radioactivity was detected in spots II and III. No ninhydrin-positive and radioactive spot corresponding to glutamic acid was detected. The compounds corresponding to spots I, II, III, IV, and V were eluted with distilled water from the paper on which the ninhydrin reagent was not sprayed. They were used for determination of the peptide sequence. Spots I and IV were identified as alanine and DAP, respectively, by cellulose thin-layer chromatography with solvent system B. Spot V was found to be intact band A compound which was not degraded.

Identification of spots II and III. (i) Purity of spots II and III. Both spots II and III gave a single spot on electrophoresis. Spot II migrated faster to the cathode than intact band A com-

TABLE 1. *Determination of N-terminal components of S. ruminantium peptidoglycan by dinitrophenylation*

Component	Content	
	Before treatment	After dinitrophenylation
Glutamic acid	100[a]	99
Alanine	199	200
DAP	102	51
Cadaverine	100	0

[a] Glutamic acid content of intact peptidoglycan is referred to as 100.

TABLE 2. *Determination of N-terminal components of band A compound*

Component	Content	
	Before treatment	After dinitrophenylation
Glutamic acid	100[a]	100
Alanine	200	100
DAP	101	0
Cadaverine	98	0

[a] Glutamic acid content of intact band A compound A is referred to as 100.

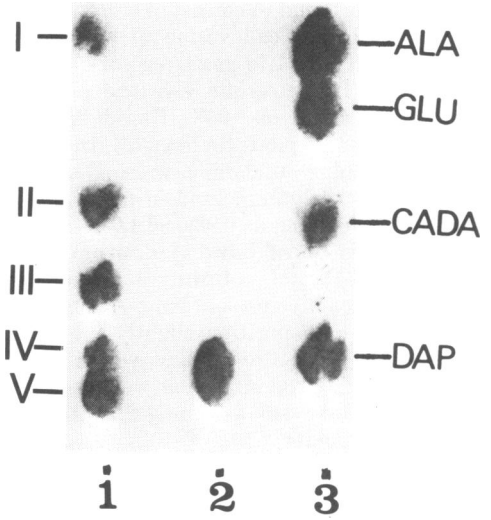

FIG. 2. *Paper chromatogram of the peptide fragments from band A compound after partial acid hydrolysis. Spots were detected by spraying with ninhydrin reagent. (1) Degradation products from band A compound after partial acid hydrolysis; (2) intact band A compound; (3) authentic alanine (ALA), glutamic acid (GLU), cadaverine (CADA), and diaminopimelic acid (DAP).*

pound and was free of free alanine, glutamic acid, and cadaverine. Spot III migrated at the positions of alanine and DAP (data not shown).

(ii) Chemical composition of spots II and III. Spot II and spot III compounds were hydrolyzed with 6 N HCl in sealed tubes at 110°C for 18 h, and the amino acids and cadaverine in the compounds were examined by paper chromatography. Spot II was composed of alanine, glutamic acid, and cadaverine (Fig. 3). Spot III consisted of DAP and alanine. Alanine in spots II and III were determined to be of L and D configurations, respectively.

(iii) N-terminal residue(s) of spots II and III. Alanine and cadaverine were N-terminal in spot II, and DAP was N-terminal in spot III. From these results, the chemical structures of spots II and III were determined to be L-alanyl-D-glutamyl-cadaverine and *meso*-diaminopimelyl-D-alanine, respectively. Cadaverine was found to link covalently to glutamic acid residue. It is well known that the γ-glutamyl linkage is more labile than the α linkage. Only spots II and III were obtained by partial hydrolysis with 0.03 N HCl at 100°C for 12 h, and no other fragments were detected. These results indicate that cadaverine is bound to glutamic acid with a α-peptide linkage and that the linkage between D-glutamic acid and DAP is γ.

DISCUSSION

We propose that the primary structure of the peptidoglycan of *S. ruminantium* is as shown in Fig. 4 based on the following evidence: (i) the peptidoglycan of this strain consisted of *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-glutamic acid, *meso*-DAP, cadaverine, and D-alanine; (ii) protein components corresponding to the bound form of the lipoprotein of Braun and Rehn (1) did not exist in the peptidoglycan (5, 6); (iii) the peptidoglycan was di-

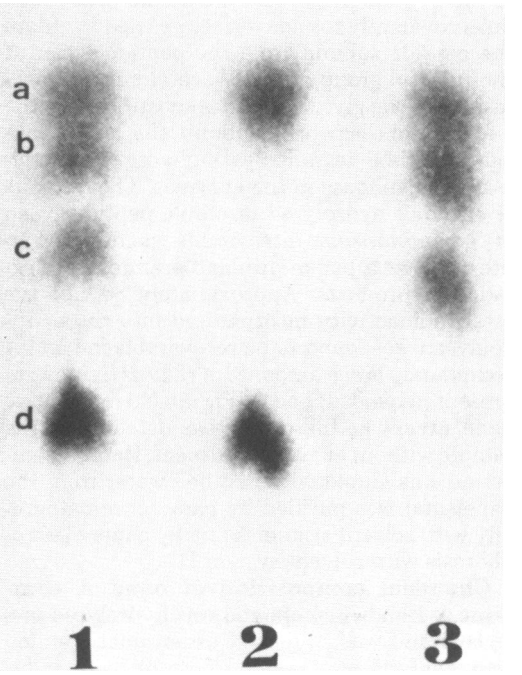

FIG. 3. *Paper chromatogram of the components of spots II and III. Spots were detected by spraying with ninhydrin reagent. Solvent system C was used. (1) Authentic alanine (a), glutamic acid (b), cadaverine (c), and DAP (d); (2) acid hydrolysate of spot III compound; (3) acid hydrolysate of spot II compound.*

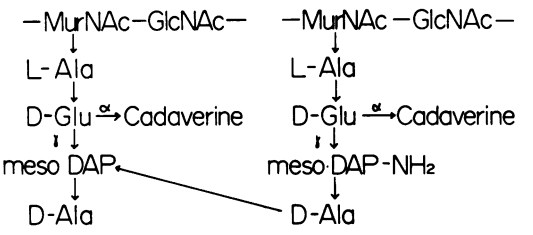

FIG. 4. *Proposed primary structure of the peptidoglycan of S. ruminantium. MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine. Arrows indicate the CO→NH direction of the linkages.*

gested with lysozyme, indicating that the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the glycan strand is β-1,4; (iv) one-half of the DAP residue was N terminal; (v) one of the two amino groups of cadaverine linked covalently to the C_1-carboxyl group of D-glutamic acid; (vi) peptide monomer was obtained by digesting the peptidoglycan with *S. albus* G enzymes; and (vii) the covalent linkage between D-glutamic acid and *meso*-DAP was labile to treatment with 2 N HCl at 100°C for 2 h.

The tetrapeptide backbone of *S. ruminantium* shown in Fig. 4 was identical to that of *Escherichia coli*, i.e., L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine. The C-terminal D-alanine residue of the tetrapeptide unit was involved in bridging to the DAP residue of the second tetrapeptide unit. Based on the finding that endopeptidase from *S. albus* G hydrolyzes the D-alanyl-(D)-*meso*-DAP linkage in the peptidoglycan of various bacteria (3), the linkages between D-glutamic acid and *meso*-DAP and between *meso*-DAP and D-alanine participating in the cross-linkage of *S. ruminantium* were considered to be D-glutamic acid-(L)-*meso*-DAP and *meso*-DAP-(D)-D-alanine, respectively.

It is known (3) that the α-carboxyl group of D-glutamic acid is amidated with NH_3 in some *Micrococcaeae, Lactobacillaceae,* and *Streptococcaceae.* In *S. ruminantium,* the α-carboxyl group of D-glutamic acid is substituted with cadaverine. We have not examined whether the α-carboxyl group of DAP which is not involved in the peptide linkage is amidated.

At present, our studies are focused on the process by which cadaverine links covalently to the α-carboxyl group of the D-glutamic acid residue in *S. ruminantium.* It is known that D-cycloserine inhibits peptidoglycan synthesis and causes the cells to accumulate the uridine nucleotide tripeptide (UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP) in *Bacillus cereus* (4). We found that D-cycloserine inhibited cell growth in *S. ruminantium* and that the nucleotide tripeptide was accumulated in cells treated with the antibiotic. However, cadaverine was not found in the nucleotide peptide that accumulated. Moreover, cadaverine was not bound to UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP, added as an exogenous substrate in the presence of ATP, by the cell extract of *S. ruminantium* in vitro (data not shown). Therefore, it is possible to speculate that cadaverine links to the glutamic acid residue of "lipid intermediate" or cross-linked peptidoglycan.

ACKNOWLEDGMENTS

We are grateful to K. Kato of Osaka University and K. Izaki, of Tohoku University, for their generous supply of enzymes from *S. albus* G and UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP. We also thank A. Kikuchi, of Tohoku University, for her help in amino acid analysis. We also express our appreciation to H. Takahashi and K. Izaki, of Tohoku University, for their discussions.

LITERATURE CITED

1. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and function of lipoprotein (murein lipoprotein) of the *E. coli* wall: the specific effects of trypsin on the membrane structure. Eur. J. Biochem. **10**:426–438.
2. **Bray, G. A.** 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
3. **Ghuysen, J.-M., and G. D. Shockman.** 1973. Biosynthesis of peptidoglycan, p. 37–130. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, New York.
4. **Izaki, K., M. Matsuhashi, and J. L. Strominger.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reaction in strains of *Escherichia coli.* J. Biol. Chem. **243**:3180–3192.
5. **Kamio, Y., and H. Takahashi.** 1980. Outer membrane proteins and cell surface structure of *Selenomonas ruminantium.* J. Bacteriol. **141**:899–907.
6. **Kamio, Y., Y. Itoh, Y. Terawaki, and T. Kusano.** 1981. Cadaverine is covalently linked cadaverine to peptidoglycan in *Selenomonas ruminantium*: J. Bacteriol. **145**: 122–128.
7. **Kanegasaki, S., and H. Takahashi.** 1967. Function of growth factors for rumen microorganisms. I. Nutritional characteristics of *Selenomonas ruminantium.* J. Bacteriol. **93**:456–463.
8. **Primosigh, J., H. Pelzer, D. Mass, and W. Weidel.** 1961. Chemical characterization of mucopeptides released from the *E. coli* B cell wall by enzymatic action. Biochim. Biophys. Acta **46**:68–80.
9. **Yanai, A., K. Kato, T. Beppu, and K. Arima.** 1976. Peptidoglycan of *Pseudomonas aeruginosa.* Agric. Biol. Chem. **40**:1505–1508.