Cadaverine Is Covalently Linked to Peptidoglycan in
Selenomonas ruminantium

YOSHIYUKI KAMIO,1 * YOSHIFUMI ITOH,1 YOSHIRO TERAWAKI,1 AND TOMONOBU KUSANO2

Department of Bacteriology, Shinshu University School of Medicine, Matsumoto 390,1 and The Institute of
Physical and Chemical Research, Wako 351,2 Japan

Cadaverine was found to exist as a component of cell wall peptidoglycan of
Selenomonas ruminantium, a strictly anaerobic bacterium. [14C]cadaverine added
to the growth medium was incorporated into the cells, and about 70% of the total
radioactivity incorporated was found in the peptidoglycan fraction. When the
[14C]cadaverine-labeled peptidoglycan preparation was acid hydrolyzed, all of the
14C counts were recovered as cadaverine. The [14C]cadaverine-labeled peptidogly-
can preparation was digested with lysozyme into three small fragments which
were radioactive and were positive in ninhydrin reaction. One major spot, a
compound of the fragments, was composed of alanine, glutamic acid, diaminopimel-
ic acid, cadaverine, muramic acid, and glucosamine. One of the two amino
groups of cadaverine was covalently linked to the peptidoglycan, and the other
was free. The chemical composition of the peptidoglycan preparation of this
strain was determined to be as follows: L-alanine-D-alanine-D-glutamic acid-
messo-diaminopimelic acid-cadaverine-muramic acid-glucosamine (1.0:1.0:1.0:1.0:
1.1:0.9:1.0).

The cell wall of a gram-negative bacterium such as Escherichia coli and Salmonella
typhimurium consists of two layers: the outer membrane layer and the peptidoglycan layer. The outer membrane contains lipopolysaccharide, protein, phospholipid, and a free form of lipoprotein described by Inouye et al. (8). The peptidoglycan layer contains lipoprotein described by Braun and Rehn that is covalently linked to the
dependoglycan (bound form of lipoprotein) (1). The bound form of lipoprotein at least plays a role in the maintenance of the structural integ-
ity of the outer membrane of the cell envelope (21, 17). The lpo or mlp mutant of E. coli shows the physiological and morphological alterations of the cell envelope, such as cell lysis by EDTA and the formation of membrane blebs by Mg2+-
starvation (21, 17). Kamio and Takahashi reported previously (11) that the wild type of Selenomonas ruminantium subsp. lactilytica, a strictly anaerobic, gram-negative strain, contains no detectable protein component in the cell wall fraction corresponding to the lipoprotein in E. coli. However, no significant cell lysis of S. ruminantium occurs with a high level of EDTA (11). These results led us to examine the composition of the peptidoglycan of S. ruminanti-
tum chemically. In this paper, we report the presence of cadaverine, which is a component of the peptidoglycan of S. ruminantium, and de-
scribe the chemical composition of the peptidoglycan of this strain. (A preliminary account of this paper has appeared [9a].)

MATERIALS AND METHODS

Bacterial strain. Selenomonas ruminantium subsp. lactilytica, described in a previous paper (11),
was used.

Medium and cultural conditions. S. ruminant

tium was grown in a yeast extract-glucose medium

(11) supplemented with 0.01% sodium n-valerate at

37°C under anaerobic conditions (12).

Preparation of [14C]-amino acids or [14C]cadaverine-labeled cells. The yeast extract-glucose me-
dium (20 ml) containing n-valerate and either 50 µCi of an L-U-14C-amino acid mixture or 10 µCi of [1,5,
14C]cadaverine (4.5 µM) was inoculated with 0.1 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. Preparation of the peptidoglycan was done essentially by the proce-
dure of Yanai et al. (20) with the following exceptions. (i) During the preparation, α-amylase was used to de-
grade the high-molecular-weight glycogen (19) which contaminated the peptidoglycan fraction. (ii) Pronase or trypsin, used for release of the bound form of lipoprotein from the peptidoglycan of E. coli, was not used at any stage during the isolation procedure because of the absence of the bound or free form of lipoprotein in this strain. (iii) Finally, the peptidogly-
can fraction was treated with 3% cold perchloric acid. The typical protocol is shown in Fig. 1.

For preparation of peptidoglycan from [14C]-amino acids or [14C]cadaverine-labeled cells, a similar but
scaled-down procedure was employed.

Solvent systems. The following solvent systems were used for cellulose thin-layer chromatography, silica gel thin-layer chromatography, and paper chro-
matography: (i) ethanol-acetic acid-water (2:1:2 [vol/
PETIDOGLYCAN OF SELENOMONAS RUMINANTIUM

123

Analytical procedure. UV absorption of the peptidoglycan suspension was measured with a Shimazu (Kyoto, Japan) UV200 recording spectrophotometer.

The amount of amino acid and amino sugar in the acid hydrolysate of the peptidoglycan preparation was determined quantitatively by a Hitachi (Tokyo, Japan) 835 automatic amino acid analyzer with alanine, glutamic acid, dianaminopimelic acid, glucosamine, and muramic acid as standards. For quantitative determination of cadaverine, a portion of the acid hydrolysate of the peptidoglycan preparation was applied to paper for electrophoresis with cadaverine as the standard by the method of Inoue and Mizutani (7).

Optical configuration of glutamic acid was determined by the method of Kotani et al. (14). Glutamic acid from the peptidoglycan fraction and standard L- and D-glutamic acids were treated with L-glutamic acid decarboxylase, and the residual amount of glutamic acid was determined by ninhydrin reaction after a portion of the reaction mixture was paper chromatographed by using solvent system (ix). The amount of D-alanine was determined by a modification (16) of the method described by Johnson et al. (9). meso-Diaminopimelic acid was determined by the method of Bricas et al. (3).

2-Keto-3-deoxyoctulosonic acid and phospholipids were determined by methods described previously (10).

Radioactivity was quantitated with a Packard 3255 liquid scintillation spectrometer with the scintillation fluid of Bray (2).

Materials. Sodium dodecyl sulfate was obtained from BDH Chemical, Ltd., Poole, England. a-Amylase (4X, crystallized from Bacillus subtilis), egg white lysozyme, dianaminopimelic acid, muramic acid, and D-alanine oxidase were obtained from Sigma Chemical Co., St. Louis, Mo. Glucosamine hydrochloride, cadaverine dihydrochloride, and L-glutamic acid decarboxylase were from Wako Pure Chemical Industries Ltd., Tokyo, Japan. Cadaverine dihydrochloride was recrystallized in ethanol–water at 4°C. L- and D-amino acid mixtures and [1,2-14C]-cadaverine dihydrochloride were purchased from New England Nuclear Corp., Boston, Mass. The other chemicals used were of the best grade commercially available.

Cellulose thin-layer plates and Silica Gel 60 plates were purchased from E. Merck AG, Darmstadt, Germany. For paper chromatography, Whatman 3MM filter paper was obtained from W. & R. Balston Ltd., England.

RESULTS

Purity of the peptidoglycan preparation. The peptidoglycan suspension in water did not show any absorption peak between 250 and 350 nm. Neither protein, lipopolysaccharide, or phospholipid was detected in the peptidoglycan preparation. From these results, it was determined that the peptidoglycan preparation was free from nucleic acids, aromatic compounds, and membrane components.

Amino acid and amino sugar composition

Vol. 145, 1981

Dissolved in boiling 4% sodium dodecyl sulfate (boiled for 60 min)

Centrifuged at 240,000 × g for 60 min at room temperature

Washed twice with water and centrifuged at 240,000 × g for 60 min at room temperature

Boiled again in 4% sodium dodecyl sulfate for 30 min

Washed six times with water and centrifuged at 240,000 × g for 60 min at room temperature

Treated with α-amylase (10 µg/liter per mg of dried material) in 0.01 M sodium phosphate buffer (pH 7.0) at 37°C for 18 h

Boiled in 4% sodium dodecyl sulfate for 30 min

Washed several times with water

Treated with 3% cold perchloric acid

Washed four times with water

Peptidoglycan preparation

FIG. 1. Flow sheet of peptidoglycan preparation.
of *S. ruminantium* peptidoglycan. The elution profile of the acid hydrolysate of the peptidoglycan preparation is shown in Fig. 2. Glutamic acid, alanine, diaminopimelic acid, muramic acid, and glucosamine were found, but no other amino acid, especially lysine and arginine, which are representative amino acids of bacterial lipoprotein, was found. These results do not conflict with previous results (11) which revealed the absence of the protein corresponding to the bound form of lipoprotein of *E. coli* in this strain.

Cellulose thin-layer chromatography of the acid hydrolysate of the peptidoglycan preparation. A thin-layer chromatogram of the acid hydrolysate of the *14*C-amino acids-labeled peptidoglycan preparation is shown in Fig. 3. Four radioactive spots were detected. Spots a, b, and c had *R* values identical to those of authentic DL-glutamic acid, diaminopimelic acid, and DL-alanine, respectively. In addition, we found a unique radioactive spot (designated as spot d) which was positive in ninhydrin reaction.

Isolation and identification of the spot d compound. The purified peptidoglycan preparation (15 g) from the cells of a 2,000-liter culture was hydrolyzed. The hydrolysate was applied to a Dowex 50-X8 (type H) column (3 by 35 cm). After amino acids and amino sugars were eluted with 2 liters of 2 N NH₄OH, the column was washed with water and 1 N HCl until no ammonium ion was detected. Then, the spot d compound was eluted with 1 liter of 6 N HCl. Crystals were formed during the evaporation to remove HCl from the eluate. Recrystallization was performed in ethanol–water at 4°C. The crystals (650 mg) of the compound thus obtained were analyzed and determined to be cadaverine dihydrochloride from the following results. (i) The crystals had *R* values identical to those of authentic cadaverine dihydrochloride as determined on cellulose thin-layer plates by use of the solvent systems (i) to (v) described above. (ii) The crystals were subjected to elemental analysis and were found to contain the following: C, 34.3%; H, 9.22%; N, 15.9%; and Cl, 40.6%. Calculations for cadaverine dihydrochloride revealed the following: C, 34.3%; H, 9.21%; N, 16.0%; and Cl, 40.5%. (iii) The crystals were subjected to infrared spectrum and nuclear magnetic resonance analyses. The infrared spectrum of the crystals (Fig. 4A) was identical to that of authentic cadaverine dihydrochloride (Fig. 4B). The characteristically large absorption of −*NH₃*⁺ derived from the hydrochloride salt of the primary amine, which appeared between 2,900 and 3,200 cm⁻¹, was detected in both samples. The nuclear magnetic resonance spectrum of the crystals was also identical to that of authentic cadaverine dihydrochloride (Fig. 5A and B). The nuclear magnetic resonance spectrum of the crystals indicated the following: (i) the methyl group was absent, and all signals were from methylene group; and (ii) the carbon chain was straight and symmetrical. These results indicated that the crystals of the spot d compound were cadaverine dihydrochloride.

Determination of optical configurations of glutamic acid, alanine, and diaminopimelic acid. The quantity of glutamic acid was unchanged on treatment with L-glutamic acid.

![Fig. 2. Elution profile of the hydrolysate of S. ruminantium cell wall peptidoglycan on an automatic amino acid analyzer. (Mur) Muramic acid; (Glu) glutamic acid; (Ala) alanine; (DAP) diaminopimelic acid; (GlcN) glucosamine; (Lys) lysine; (Arg) arginine.](http://jb.asm.org/)
decarboxylase, and about 50% of the alanine disappeared after treatment with D-amino acid oxidase. These enzymatic analyses indicated that the glutamic acid residue had a D configuration and that the ratio of D-alanine to L-alanine residues was about one. To elucidate the optical configuration of dianinopimelic acid, the sample and authentic dianinopimelic acid (a mixture of D,D, L,L, and meso types) were treated with dinitrophenol and were silica gel thin-layer chromatographed by using solvent system (vi). Under these conditions, dinitrophenyl-D,D-dianinopimelic acid and dinitrophenyl-L,L-dianinopimelic acid have identical Rf values, and dinitrophenyl-meso-dianinopimelic acid gives an Rf value smaller than either of the former (3). As shown in Fig. 6, the dinitrophenylated sample had an Rf value identical to that of dinitrophenyl-meso-dianinopimelic acid, and no spot corresponding to the spot of dinitrophenyl-D,D-dianinopimelic acid and dinitrophenyl-L,L-dianinopimelic acid was detected. These results clearly indicate that the dianinopimelic acid residue of the peptidoglycan of this strain is a meso type.

Incorporation of labeled cadaverine into the peptidoglycan preparation. To determine the location of cadaverine in S. ruminantium, [1,5-14C]cadaverine was added to the growth medium of the cells. About 70% of the total radioactivity incorporated into the cells was recovered in the peptidoglycan fraction. The [14C]cadaverine-labeled peptidoglycan preparation was divided into two portions, one of which was hydrolyzed and cellulose thin-layer chromatographed. The other was digested with lysozyme and was paper chromatographed. When the [14C]cadaverine-labeled peptidoglycan fraction was acid hydrolyzed, all of the 14C counts were found to be recovered as cadaverine (Fig. 7). Three radioactive and ninhydrin-positive spots, including one major spot (designated "A") appeared in the paper chromatogram of the lysozyme digest (Fig. 8, sample 1), whereas no radioactive spot except at the origin was detected in the sample without lysozyme treatment (Fig. 8, sample 2). When the spot "A" compound was eluted with water from the paper, hydrolyzed in 6 N HCl, and applied to cellulose thin-layer chromatography with solvent systems (i) and (ii), alanine, dianinopimelic acid, glutamic acid, cadaverine, muramic acid, and glucosamine were detected. These results showed that cadaverine was one of the components of the peptidoglycan of this strain. The size of the spot "A" compound has not been determined yet.

Linkage of cadaverine to the peptidoglycan. [14C]cadaverine-labeled peptidoglycan preparation was dinitrophenylated and acid hydrolyzed. The hydrolysate was silica gel thin-layer chromatographed. As shown in Fig. 9, dinitrophenyl [14C]cadaverine migrated to the front, and [14C]cadaverine stayed at the origin on the silica gel plate. The hydrolysate of the dinitrophenylated peptidoglycan migrated to the area corresponding to an Rf value of 0.4. These results led us to the conclusion that one of two amino groups were covalently linked to the peptidoglycan, and the other was free.

![Infrared absorption spectra of the crystals (A) and authentic cadaverine dihydrochloride (B) (KBr).](http://jb.asm.org/)

*Fig. 4. Infrared absorption spectra of the crystals (A) and authentic cadaverine dihydrochloride (B) (KBr). The absorption spectra were recorded with a Hitachi 285 infrared spectrophotometer.*
D-glucose were calculated to be 1.0, 1.0, 1.0, 1.1, 0.9, and 1.0, respectively.

**DISCUSSION**

The occurrence, biosynthesis, and function of polyamines in a wide variety of organisms have been demonstrated (4, 18). However, no work...
concerning the presence of polyamines in cell wall peptidoglycan has been reported. Our present studies clearly demonstrated the presence of cadaverine as one of the components of the peptidoglycan in S. ruminantium and also confirmed the absence of the bound form of lipoprotein in this strain. Gmeiner showed in Proteus mirabilis (6) that although the exponentially growing cells do not contain any covalently linked lipoprotein, the stationary cells possess it in an amount similar to those found in E. coli and S. typhimurium during all growth phases. In S. ruminantium, the free and bound forms of lipoprotein were not detected in either exponential or stationary phases. To our knowledge, this is the first report which pointed out the presence of covalently linked polyamine to the peptidoglycan in bacteria. With regard to the origin of cadaverine, our recent studies revealed that cadaverine in S. ruminantium is synthesized from L-lysine by lysine decarboxylase in vitro (unpublished data).

In E. coli, the bound form of lipoprotein has an important role in the outer membrane assembly on peptidoglycan in vitro (5). Although the biological function(s) of the peptidoglycan-bound cadaverine in S. ruminantium remains to be elucidated, we propose that the cadaverine in peptidoglycan might associate with the outer membrane components, such as phospholipids or acidic proteins, by ionic interaction and might play a similar role to that of the bound form of lipoprotein in E. coli.

Kato et al. reported recently (13) the presence of lanthionine, a sulfur-containing diamino acid, in the peptidoglycan of Fusobacterium nucleatum, which is an anaerobic bacterium. They also showed that the lanthionine is an essential dibasic amino acid involved in cross-linkages between stem peptide subunits in F. nucleatum (13). In S. ruminantium, cadaverine seems not to be involved in cross-linkage because one of the two amino groups of cadaverine is free. From our preliminary data of N-terminal and C-terminal amino acid analyses, meso-diaminopimelic and residue seemed to participate in cross-linkage. To confirm this possibility, the isolation and identification of some fragments of a cross-linked portion of the peptidoglycan obtained by use of a lytic enzyme or partial hydrolysis should be performed.

At present, our studies are focused on how and where cadaverine is covalently linked to the peptidoglycan and on its biological function.
ACKNOWLEDGMENTS

We thank H. Takahashi and K. Izaki, of the Department of Agricultural Chemistry, Tohoku University for their discussions, and A. Kikuchi for the amino acid analysis. We also thank J. Uzawa and M. Uramoto of the Institute of Physical and Chemical Research for the nuclear magnetic resonance analysis and T. Furuchi of our laboratory for his help in large-scale peptidoglycan preparation.

LITERATURE CITED


