JOURNAL OF BACTERIOLOGY, Dec. 1984, p. 989–993
0021-9193/84/120989-05$02.00/0

Vol. 160, No. 3

Sensitivity of *Coxiella burnetii* Peptidoglycan to Lysozyme Hydrolysis and Correlation of Sacculus Rigidity with Peptidoglycan-Associated Proteins

KEN-ICHI AMANO† AND JIM C. WILLIAMS‡*

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, Hamilton, Montana 59840

Received 29 June 1984/Accepted 18 September 1984

The protease-resistant proteins associated with the peptidoglycan (PG) of the phase I small-cell variant *Coxiella burnetii* were either partially released from the PG by boiling the PG-protein complex (PG-PC) in sodium dodecyl sulfate containing 2-mercaptoethanol and EDTA or totally released by 1 N NaOH hydrolysis at 23°C. An 18,300-dalton protein was released from the PG-PC under reducing conditions, whereas 1 N NaOH treatment extracted PG-associated proteins without apparent dissolution of the PG. Purified PG was composed of muramic acid, glucosamine, glutamic acid, alanine, and *meso*-diaminopimelic acid in a molar ratio of 0.9:0.9:1.0:1.4:1.0. Lysozyme hydrolysis of cell walls, PG-PC, and purified PG caused an increase in reducing groups which correlated with roughly 60 to 100% digestion of disaccharides. There was no significant decrease in turbidity during lysozyme hydrolysis of cell walls and PG-PC; however, hydrolysis of purified PG caused about 90% decrease in turbidity. Approximately 60% of the *meso*-diaminopimelic acid groups of PG were not susceptible to dinitrophenylation, thus, demonstrating an apparent contribution of PG-associated proteins, rather than cross-linkage between peptides, to sacculus rigidity of cell wall and PG-PC. This association of PG and protease-resistant covalently bound proteins may be important structural and functional determiners of resistance to both environmental conditions and intracellular digestion of *C. burnetii* by eucaryotic cells.

Coxiella burnetii, the etiological agent of Q fever, has a typically eubacterial trilaminar cell envelope with a dense intermediate layer, presumably protein and peptidoglycan (PG) (5, 10, 15, 16). The chemical structure of PG in this organism has not been extensively investigated. Allison and Perkins (1) and Schramek et al. (21) reported the presence of muramic acid in several rickettsiae including *C. burnetii*, whereas Myers et al. (17) found diaminopimelic acid (DAP) in the cell walls of rickettsiae. Furthermore, Kravchenko et al. (13) described changes of the morphology of bacterial cells to cultivation of *C. burnetii* in the presence of penicillin. We have reported in a preceding paper (4) that partially purified PG of *C. burnetii* contains associated proteins which are resistant to hydrolysis by proteolytic enzymes. These results led us to examine the chemical composition and lysozyme sensitivity of purified PG of *C. burnetii*. The PG contains chemical components common to most gram-negative bacteria. The PG peptide subunits were approximately 40% cross-linked with *meso*-DAP, and the saccharide portion was sensitive to hydrolysis by lysozyme. The PG-associated proteins resistant to protease digestion apparently contributed to sacculus rigidity of cell walls and PG-PC after lysozyme hydrolysis.

MATERIALS AND METHODS

Organism. All procedures with phase I *C. burnetii*, Ohio isolate, were carried out as described previously (4).

Fractionation of bacteria. Subfractionation of *C. burnetii* into large-cell variant cell walls (LCV-CW), small-cell variant cell walls (SCV-CW), and PG-PC was carried out by previously described procedures (4).

Solubilization of PG. PG-PC was treated with various combinations of sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), glycerol, and EDTA to promote the dissociation of noncovalently associated or disulfide-linked proteins from PG as described previously (3). The PG-PC was suspended in each mixture, boiled for 10 min, cooled to room temperature, and centrifuged at 100,000 × *g* for 1 h at room temperature. The pellets were washed with distilled water and hydrolyzed for amino acid analysis.

EDTA treatment of PG-PC. Treatment of PG-PC with 27.2 mM EDTA was performed as described by Gray and Wilkinson (11). A 2-mg amount of PG-PC was suspended at a concentration of 0.5 mg/ml in 0.2 M borate buffer (pH 9.2) containing 27.2 mM EDTA. The mixture was kept at room temperature for 60 min and then centrifuged at 100,000 × *g* for 1 h. The precipitate was washed twice with distilled water and lyophilized.

SDS-PAGE. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (14), using a 5% spacer gel and 12.5% separating gel. Slab gels were 1.5 mm thick, and after electrophoresis of samples, the separated components were fixed with an aqueous solution of 25% isopropanol and 7.5% acetic acid. Slab gels were stained by the silver staining method described by Tsai and Frasch (23). Molecular weight markers were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Sodium hydroxide and hydrochloric acid treatments of PG-PC. PG-PC was treated with sodium hydroxide to promote the release of proteins noncovalently and covalently bound to the PG (3, 4) and O-acetyl groups which block lysozyme hydrolysis of the glycan portion (2, 9). PG-PC (15

* Corresponding author.
† Present address: Department of Bacteriology, Hirosaki University School of Medicine, Hirosaki, Aomori 036, Japan.
‡ Present address: Rickettsial Diseases Laboratory, Aerobiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701.

TABLE 1. Solubility of phase I *C. burnetii* PG-PC under various conditions

Treatment of PG-PC[a]	Proteins bound to PG-PC[b] (%)
None	100
2% SDS	100
2% SDS + 1% 2-ME + 10% glycerol	99
2% SDS + 1% 2-ME + 10% glycerol + 2 mM EDTA	94

[a] Experimental details are described in the text.
[b] Proteins bound to PG were calculated from the molar ratios of aspartic acid, threonine, serine, valine, histidine, lysine, and arginine contents divided by muramic acid and DAP contents. No treatment was taken as 100%.

mg) was hydrolyzed in 4 ml of 1 N NaOH at room temperature for 20 h and centrifuged at 100,000 × *g* for 1 h. The pellet was washed twice with distilled water and lyophilized. This preparation was designated PG-PC-NaOH(PPT), and the yield was 5.7 mg. The supernatant was dialyzed against distilled water. The nondialyzable fraction was designated PG-PC-NaOH(Sup) and the yield was 7.8 mg. Hydrochloric acid treatment of PG-PC was performed as described previously (2).

Analytical methods. Quantitative and qualitative analyses of amino acids, amino sugars, proteins, neutral sugars, reducing groups, 3-deoxy-D-mannooctulosonic acid (KDO), and fatty acids were performed as described previously (2, 4).

The optical configurations of alanine and glutamic acid was determined in hydrolyzed samples before and after treatments with D-amino acid oxidase (Sigma Chemical, St. Louis, Mo.) and L-glutamic acid decarboxylase (Sigma), respectively (2). The optical configuration of DAP was determined by the method of Bricas et al. (8). N-terminal amino acids were determined by the difference between amino acid contents of a dinitrophenylated sample and an untreated control (24).

Treatment of LCV-CW, SCV-CW, PG-PC, and PG-PC-NaOH(PPT) with lysozyme. Each fraction (0.4 mg) was incubated at 37°C with 30 μg of lysozyme (Worthington Diagnostics, Freehold, N.J.) and 4 μg of NaN_3 in 800 μl of 50 mM Tris buffer (pH 7.2) as described previously (2). Samples were withdrawn and assayed for turbidity (optical density at 500 nm) and reducing groups.

RESULTS

Attempts to dissociate proteins from PG. Various combinations of SDS, 2-ME, glycerol, and EDTA were used in an attempt to remove proteins from PG of *C. burnetii* (Table 1). Although SDS alone or a mixture of SDS, 2-ME, and glycerol did not remove proteins, the addition of EDTA to the mixture facilitated the removal of 6% of the protein. This result suggests that only 6% of the PG-associated proteins may be tightly but noncovalently associated with PG or proteins, whereas 94% of the proteins may be covalently bound to PG.

We were interested in determining the profile of proteins released by such treatments of PG-PC. Therefore, an analysis of released components was carried out by SDS-PAGE after boiling each sample in sample buffer in the presence of 2-ME and dithiothreitol (DTT) (Fig. 1). Several components were resolved in silver-stained SDS-polyacrylamide gels. Some bands were detected in the gels when selected samples were solubilized at 100°C for 5 min in the presence of 2-ME and DTT in the sample buffer. The SCV-CW of *C. burnetii*, gave two major components (apparent molecular weights, 18.3×10^3 and 16.7×10^3) which were detected in SDS-polyacrylamide gels stained by silver (Fig. 1, lane 2) and Coomassie blue R250 (data not shown). Other bands with molecular weights ranging from 30×10^3 to $<13 \times 10^3$ were observed by silver staining. The 16.7×10^3 component was removed from the SCV-CW by hot SDS treatment as shown by the pattern of bands in SDS-treated SCV-CW (Fig. 1, lane 3). Of the proteins released from the PG-PC (Table 1) when treated with the solubilizing mixture containing SDS, 2-ME, glycerol, and EDTA, 6% may correspond to the 18.3×10^3 component (Fig. 1, lanes 3 and 4). When the PG-PC was hydrolyzed with 1 N NaOH, almost all of the proteins and other components were released from the PG into the supernatant, which had new components of low molecular weights as well as the two 16.7×10^3 and 18.3×10^3 components (Fig. 1, lane 6). The low-molecular-weight components may be proteins partially digested with alkali. The precipitate of the PG-PC alkaline hydrolysate from *C. burnetii* showed no bands (Fig. 1, lane 5).

Analysis of PG-PC alkaline hydrolysate. As described previously (4), proteolytic enzyme treatments of PG-PC with trypsin, protease VI, and proteinase K indicated that the PG-associated proteins were resistant to protease digestion since the protein content of PG-PC proteins was not significantly decreased. Since proteolysis did not remove PG-associated proteins, we used 1 N NaOH and 0.1 N HCl digestions of the PG-PC to extract associated and presumably covalently bound proteins. Treatment of PG-PC with

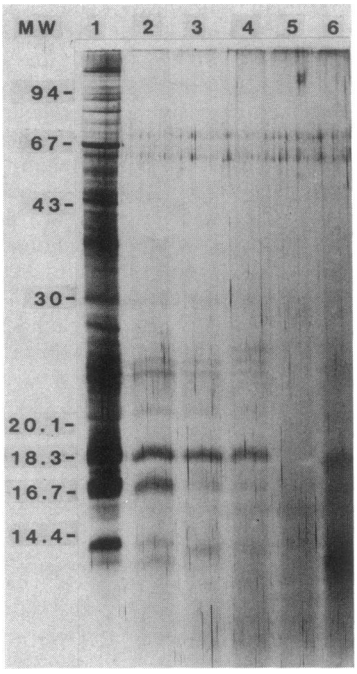

FIG. 1. SDS-PAGE of *C. burnetii* subfractions in the presence of reducing reagents. 2-ME (1%) and DTT (1.5%) were added to the sample buffer. Samples were heat treated in boiling water for 5 min, and 50-μl samples (20 μg) were applied to each slot. after electrophoresis, the slab gel was fixed and silver stained after periodate oxidation (23). Lanes: 1, whole cells of Ohio strain; 2, SCV-CW; 3, hot SDS-treated SCV-CW; 4, PG-PC; 5, PG-PC-NaOH(PPT); 6, PG-PC-NaOH(Sup). Molecular weight (MW) markers (10^3) phosphorylase b (94), albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactoalbumin (14.4).

TABLE 2. Amino acid and amino sugar compositions of alkali-treated PG-PC from small-cell variants of *C. burnetii*

Amino acid or amino sugar[a]	PG-PC[b]		PG-PC-NaOH (PPT)		PG-PC-NaOH (Sup)	
	nmol/mg	Molar ratio	nmol/mg	Molar ratio	nmol/mg	Molar ratio
Aspartic acid	519	1.63	39	0.06	499	14.26
Threonine	349	1.09	17	0.02	245	7.00
Serine	402	1.26	33	0.05	362	10.34
Muramic acid	287	0.90	634	0.91	—[c]	—
Glutamic acid	492	1.54	656	0.95	282	8.06
Proline	198	0.62	—	—	129	3.69
Glycine	609	1.90	173	0.25	558	15.94
Alanine	811	2.53	933	1.35	421	12.03
Cysteine	—	—	2	0.00	—	—
Valine	418	1.31	25	0.04	319	9.11
Methionine	—	—	—	—	—	—
DAP	320	1.00	693	1.00	35	1.0
Isoleucine	112	0.35	—	—	161	4.60
Leucine	344	1.08	34	0.05	278	7.94
Tyrosine	258	0.81	—	—	307	8.77
Phenylalanine	182	0.57	—	—	158	4.51
Glucosamine	300	0.94	597	0.86	—	—
Histidine	87	0.27	7	0.01	78	2.23
Lysine	126	0.39	11	0.02	100	2.86
Arginine	81	0.25	7	0.01	61	1.74

[a] Experimental details are described in the text.
[b] See reference 4.
[c] —, Undetectable.

NaOH removed proteins from the PG without significant loss of PG constituents (Table 2). Major PG components in the PG-PC-NaOH(PPT) were muramic acid, glucosamine, glutamic acid, alanine, and DAP in a molar ratio of 0.9:0.9:1.0:1.4:1.0, respectively (Table 2). Aspartic acid, threonine, serine, glycine, valine, leucine, histidine, lysine, and arginine remained attached to the PG. These residual amino acids may be short peptides still covalently bound to the PG. Significant amounts of glycine (0.25 molar ratio to DAP) were detected.

The PG-PC-NaOH(SUP) contained no detectable muramic acid, a small amount of DAP, and no detectable glucosamine. The release of DAP by NaOH hydrolysis of PG-PC suggested that some DAP subunit peptides were not cross-linked. Moreover, these data suggest that glycyl peptides which are labile to alkali treatment (7) may not be involved in peptide cross-linkages since the PG was not completely dissolved by this treatment. HCl treatment did not remove covalently bound proteins since PG-PC-HCl(PPT) contained roughly the same molar ratios after treatment (data not shown).

Free amino groups in PG-PC-NaOH(PPT). To estimate the free amino groups of DAP and other amino acids that may be involved in cross-linkage, PG-PC-NaOH(PPT) was derivatized with dinitrofluorobenzene (DNFB). Reduction in specific amino acid content of DNFB-treated PG was determined by an amino acid analyzer (Table 3). The data of this analysis indicated that about 61, 20, and 7% of the free amino groups of DAP, alanine, and glycine, respectively, were derivatized by DNFB, whereas the contents of glutamic acid and glucosamine were not reduced. However, the reason for the increased content of muramic acid is unclear (Table 3). These data indicated that, in the PG units, roughly 40% of ε-amino groups of DAP may be cross-linked.

Optical configurations of amino acids of PG. Determinations of the optical configurations of glutamic acid and alanine residues by enzymatic methods showed that the quantity of glutamic acid was unchanged after treating hydrolysates of PG-PC-NaOH(PPT) with L-glutamic acid decarboxylase, whereas the alanine concentration was reduced by about 30% after treatment of hydrolysates with D-amino acid oxidase. These data indicate that the glutamic acid residues were in the D-configuration, and the ratio of L-alanine to D-alanine was approximately 2:1.

The configuration of DAP (8) was determined to be *meso* type from the following analysis. Hydrolysates of PG-PC-NaOH(PPT) and standards of DD-, LL-, and *meso*-DAP were treated with DNFB and compared by silica gel chromatography, using a solvent system consisting of benzyl alcohol-chloroform-methanol-water-concentrated NH_4OH (30:30:30:6:2). Dinitrophenol (DNP) hydrolysates had relative mobility values identical to that of DNP-*meso*-DAP. No component corresponding to the DNP-DD-DAP and DNP-LL-DAP spots were detected (data not shown).

Effect of lysozyme on LCV-CW, SCV-CW, PG-PC, and PG-PC-NaOH(PPT). Each sample was incubated with lysozyme to degrade PG structure. During the treatment, turbidometric readings (500 nm) were recorded periodically

TABLE 3. Major amino acid and amino sugar constituents of PG-PC-NaOH (PPT) before and after dinitrophenylation

Amino acid or amino sugar	Relative content	
	Before dinitrophenylation	dinitrophenylation
Muramic acid	0.97	1.31
D-Glutamic acid[a]	1.00	1.00
Glycine	0.26	0.19
Alanine	1.42	1.22
L-Alanine	0.98	ND[b]
D-Alanine	0.44	ND
meso-DAP	1.06	0.45
Glucosamine	0.91	0.91

[a] Glutamic acid content is referred to as 1.00.
[b] ND, Not done.

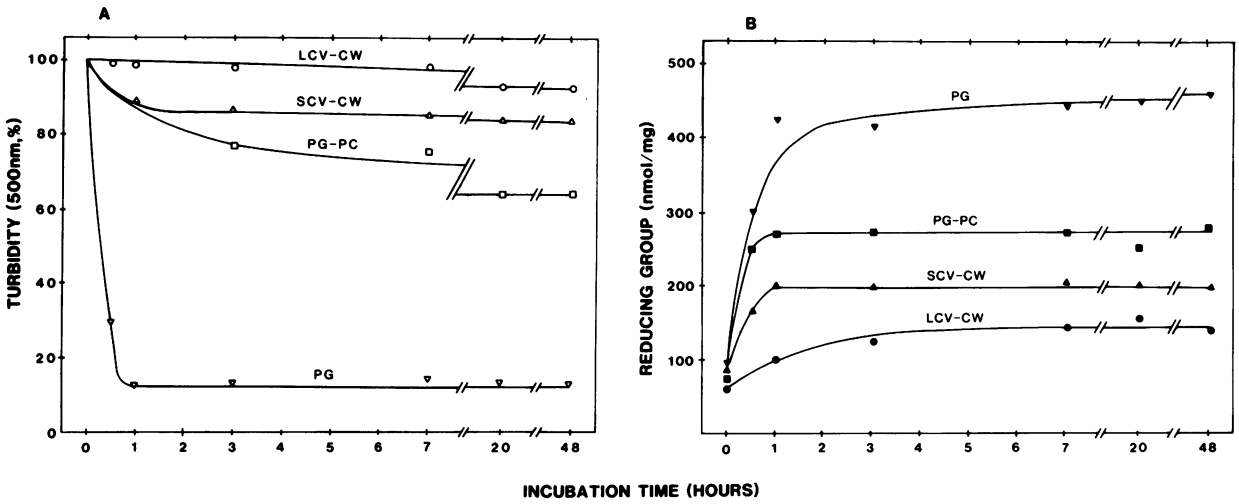

FIG. 2. Lysozyme digestion of *C. burnetii* subfractions. Lysozyme digestions were monitored by measuring (A) decrease in turbidities of LCV-CW (○), SCV-CW (△), PG-PC (□), and PG-PC-NaOH(PPT) (▽) and (B) increase in reducing groups of LCV-CW (●), SCV-CW (▲), PG-PC (■), and PG-PC-NaOH(PPT) (▼).

for 48 h (Fig. 2A and B). Lysozyme treatments of LCV-CW and SCV-CW were not significantly effective in reducing the turbidity; however, the turbidity of PG-PC was reduced slightly by lysozyme treatments (approximately 35%) (Fig. 2A). In contrast, a significant reduction in turbidity of PG-PC-NaOH(PTT) occurred after lysozyme treatment (Fig. 2A). These data suggested that the presence of PG-associated proteins apparently interfered with the action of lysozyme or simply prevented disassociation of the sacculus structure. Since lysozyme treatments of LCV-CW, SCV-CW, and PG-PC were weakly effective when measured by turbidity, we tested the effect of lysozyme by measuring changes in reducing groups. Lysozyme hydrolysis of the glycan portion of PG was effective (Fig. 2B) as measured by increased reducing groups in all samples. The content of muramic acid of PG-PC-NaOH(PPT) was 634 nmol/mg (Table 2), and that of the reducing groups after digestion was roughly 370 nmol/mg (Fig. 2B). Thus, about 60% of the *N*-acetylmuranyl–*N*-acetylglucosamyl disaccharide units were hydrolyzed by lysozyme. The actual percentage may be higher than this, because the color reaction of disaccharide-peptide by the Park-Johnson reaction may be less than that of standard glucose used in this study.

The concentrations of muramic acid in LCV-CW, SCV-CW (4), and PG-PC (Table 2) were 25, 65, and 287 nmol/mg, respectively, whereas those of the reducing groups were roughly 80, 100, and 200 nmol/mg (Fig. 2), respectively. Therefore, approximately all of the disaccharide units in LCV-CW and SCV-CW, and about 70% of the disaccharide units in PG-PC were digested with lysozyme. These data suggest that most of the disaccharide untis of *C. burnetii* PG were hydrolyzed by lysozyme, but the sacculus was not completely degraded to monomeric units.

DISCUSSION

The presence of muramic acid in *C. burnetii* cell walls was first described by Allison and Perkins (1). Myers et al. (17) also reported the presence of DAP from *C. burnetii* cell walls. Schramek et al. (21) attempted to purify the PG of *C. burnetii* but they isolated insufficiently purified PG which contained over 13 non-PG amino acids. Based on the results of our preceding paper, the PG isolated by Schramek et al. (21) may correspond to the PG-PC which was purified from SCV-CW (4). This PG-PC was resistant to proteolysis with some proteases (4). In the present study, PG-associated proteins also were resistant to solubilization with SDS and 2-ME (Table 1). These associated proteins were split into two groups by means of a solubilization technique. (i) The first group was probably noncovalently but tightly associated proteins. Of these proteins, 6% were noncovalently bound to the PG or PG-PC because those proteins were released by treatment with SDS–2-ME–glycerol–EDTA, but not with SDS–2-ME–glycerol. (ii) The second group was covalently linked or tightly bound to the PG since almost all (94%) of the proteins were still associated with the PG after treatment with SDS–2-ME–glycerol and EDTA. The proteins tightly associated with the PG were removed by chemical hydrolysis with 1 N NaOH treatment. This harsh treatment released about 90% of the proteins from the PG-PC-NaOH(PPT). However, short peptides were apparently still covalently bound to the PG since non-PG amino acids, namely, aspartic acid, threonine, glycine, cysteine, serine, valine, leucine, histidine, lysine, and arginine, were associated with the PG. Significant amounts of glycine (0.25 molar ratio to DAP) were associated with the PG; therefore, this amino acid may be a component of the linkage region between the PG and the proteins.

Although we did not determine the exact structure of the PG because of the presence of contaminating non-PG components, the tentative structure of PG may be the A1γ type (20) based on the major components of PG-PC-NaOH(PPT). Chemical analysis of PG-PC-NaOH(PPT) revealed no unusual components but rather the same components as those previously described for other gram-negative bacteria (20). PG consisted of muramic acid, glucosamine, glutamic acid, alanine, and *meso*-DAP in a molar ratio of 0.9:0.9:1.0:1.4:1.0. About 60% of the *meso*-DAP and 14% of alanine were freely accessible for derivatization by DNFB. Thus, 40% of the amino group of DAP may be cross-linked with the carboxyl group of D-alanine.

Previous studies by Perkins and Allison (19) showed that cell walls of *C. burnetii* extracted with fat solvents and trichloroacetic acid and treated with proteolytic enzymes were sensitive to lysozyme treatment. However, studies carried out by Burton et al. (10) and Schramek et al. (21) suggested that lysozyme was not effective on *C. burnetii*

cells and PG. Burton et al. (10) reported that *C. burnetii* cells exposed to EDTA-lysozyme were not morphologically different from untreated cells when examined by electron microscopy, whereas Schramek et al. (21) showed that lysozyme treatment of *C. burnetii* PG-PC was only 10 to 15% effective when measured by turbidity. Our studies indicated that the glycan portion of *C. burnetii* cell wall fractions (LCV-CW and SCV-CW), PG-PC, and PG-PC-NaOH(PPT) were sensitive to lysozyme digestion as measured by increase in reducing groups (Fig. 2). However, the cell wall fractions and PG-PC were not completely disassociated by lysozyme treatment (i.e., slight decrease in turbidity) because of the presence of the PG-associated proteins. After these proteins were removed with alkali, the PG-PC-NaOH(PPT) was almost completely solubilized by lysozyme as measured by turbidity.

Previous studies have shown that resistance of the glycan portion of PG to lysozyme digestion may arise by at least five mechanisms: (i) the presence of O-acetyl groups in glycan (9), (ii) the attachment of polymers such as teichoic acids and polysaccharides to muramic acid-6-phosphate (12), (iii) the occurrence of free amino groups in the peptide portion (18), (iv) a high degree of peptide cross-linking (22), and (v) the occurrence of *N*-unsubstituted glucosamine residues (6). In addition, the rigidity of sacculus structure of *Legionella pneumophila* cell walls and PG-PC after lysozyme hydrolysis of the disaccharides was prevented by a high degree of peptide cross-linking (2). In this study, we have shown that the glycan portion of *C. burnetii* PG is sensitive to lysozyme hydrolysis. However, dissociation of the sacculus structure of *C. burnetii* PG-PC may be prevented by the attachment of proteins to the PG. This mechanism may account for the apparent insensitivity of *C. burnetii* whole cells, cell walls, and PG-PC to lysozyme and protease treatments, thus providing a rigid sacculus structure which withstands the microbicidal conditions of the phagolysosome and environment.

ACKNOWLEDGMENT

The assistance provided by the Medical Illustrations Department (USAMRIID) is gratefully appreciated.

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