

Analysis of the Peptidoglycan of *Rickettsia prowazekii*

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In the present study, peptidoglycan from *Rickettsia prowazekii*, an obligate intracellular bacterium, was purified. The rickettsial peptidoglycan is like that of gram-negative bacteria; that is, it is sodium dodecyl sulfate insoluble, lysozyme sensitive, and composed of glutamic acid, alanine, and diaminopimelic acid in a molar ratio of 1.0:2.3:1.0. The small amount of lysine found in the peptidoglycan preparation suggests that a peptidoglycan-linked lipoprotein(s) may be present in the rickettsiae. D-Cycloserine, a D-alanine analog which inhibits the biosynthesis of bacterial cell walls, prevented rickettsial growth in mouse L929 cells at a high concentration and altered the morphology of the rickettsiae at a low concentration. These effects were prevented by the addition of D-alanine. This suggests that *R. prowazekii* contains D-alanine in the peptidoglycan and has D-Ala-D-Ala ligase and alanine racemase activities.

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate intracellular bacterium with typical gram-negative bacterial morphology (31, 32). In thin sections of *R. prowazekii*, a cytoplasmic membrane and an outer membrane defining a prominent periplasmic space are routinely observed (2, 3, 15). In most bacteria, a defined structural element in the cell envelope, peptidoglycan, maintains the integrity and the shape of the bacterium (27). It is not established whether *R. prowazekii*, or any other member of the genus, has peptidoglycan. As small obligate intracellular parasites, growing in eucaryotic cytoplasm and migrating from host cell to host cell in extracellular biological fluids, rickettsiae may not be challenged by the osmotic stresses faced by free-living bacteria. It has been reported that peptidoglycan appears to be either absent or highly deficient in *Rickettsia tsutsugamushi* (1). Indirect evidence indicates that *R. prowazekii* contains peptidoglycan: muramic acid and diaminopimelic acid (DAP) have been found in cell lysates of rickettsiae (10, 18), and penicillin inhibits growth and induces the formation of spheroplasts in cultures of *R. prowazekii* (33). However, some bacteria contain DAP as a precursor of lysine but have lysine, not DAP, in the peptidoglycan (4, 24). Moreover, the amino acid composition in the rickettsial cell wall preparations used in previous studies (9, 10, 18) was not much different from that in the whole-cell extract. It is unlikely that 15 or more amino acids represent the composition of the rickettsial peptidoglycan. In the present study, we demonstrated that *R. prowazekii* has sodium dodecyl sulfate (SDS)-insoluble, lysozyme-sensitive peptidoglycan and that the purified peptidoglycan has an amino acid profile, including DAP, similar to that of *Escherichia coli* peptidoglycan. To the best of our knowledge, this is the first isolation and characterization of the amino acid composition of the peptidoglycan of either an obligate intracellular parasite or a member of the alpha-purple group of eubacteria.

Isolation of peptidoglycan from *R. prowazekii*. The methods of Braun and Sieglin (6) and Glauner (12) were used to isolate peptidoglycan from Renografin-purified *R. prowazekii* (7, 14) and *E. coli* (as a control), except that the amounts of bacteria used in the published methods (6, 12) were scaled down.

Briefly, rickettsiae (approximately 5 mg of total protein) or *E. coli* (approximately 3 mg of total protein) was boiled in 4% SDS, digested with pronase (200 µg/ml) (Boehringer Mannheim Corporation, Indianapolis, Ind.) after being washed with distilled water, boiled again in 4% SDS, and washed again with distilled water (12). Portions of the pellets were digested with 100 µg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml and examined by electron microscopy after negative staining (20). As shown in Fig. 1A, *R. prowazekii* was rod shaped and bounded by a cell wall. After SDS and pronase treatments, rickettsia-shaped sacculi were obtained in the ultracentrifugation pellets (Fig. 1B). However, after lysozyme treatment, these sacculi disappeared, and only irregular debris remained in the pellets (Fig. 1C). No significant amino acid peaks were obtained when this debris was analyzed by high-performance liquid chromatography (HPLC) (data not shown). Lysozyme is a glycosidase which hydrolyzes the β-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial peptidoglycan (27). These data demonstrated that lysozyme-sensitive repeating disaccharide structures are located in the rickettsial SDS-insoluble sacculi, which are peptidoglycan.

Amino acid composition of the rickettsial peptidoglycan. The amino acid composition of the peptidoglycan was determined by HPLC (23) after hydrolysis of the peptidoglycan in 6

TABLE 1. Amino acid composition of peptidoglycan of *R. prowazekii* compared with that of *E. coli*

Component	Amt in:			
	<i>E. coli</i>		<i>R. prowazekii</i>	
	µmol/g of protein ^a	Relative amt ^b	µmol/g of protein	Relative amt
Glutamic acid	52 ± 2.5 (50) ^b	1.2 (1)	21 ± 4.3	1.0
Alanine	94 ± 4.4 (100)	2.2 (2)	50 ± 4.1	2.3
DAP	42 ± 0.3 (50)	1.0 (1)	22 ± 1.7	1.0
Lysine	16 ± 1.1 (5)	0.4 (0.1)	0.7 ± 0.0	0.03

^a Each value represents the mean ± standard deviation for three experiments. The values in parentheses were compiled and calculated for *E. coli* B/r in balanced growth at 37°C in aerobic glucose minimal medium (mass doubling time, 40 min) (19).

^b Normalized to the average amount of DAP.

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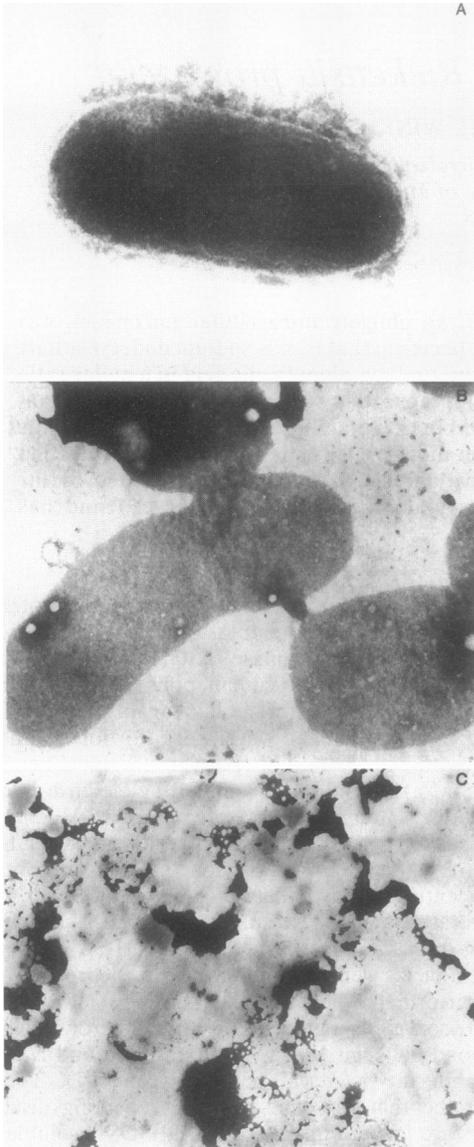


FIG. 1. Morphology of *R. prowazekii* after different treatments, as observed with an electron microscope. (A) Untreated cells. (B) SDS-treated cells. (C) SDS- and lysozyme-treated cells.

N HCl (Pierce Chemical Company, Rockford, Ill.) (6, 12) and derivatization with dansyl chloride (8, 28). The amino acid profile of the peptidoglycan of *R. prowazekii* was very similar to that of the peptidoglycan of *E. coli* (data not shown) and contained only glutamic acid, alanine, DAP, and lysine. The molar ratio of glutamic acid, alanine, DAP, and lysine was 1.0:2.3:1.0:0.03 in the rickettsial peptidoglycan and 1.2:2.2:1.0:0.4 in the peptidoglycan of *E. coli* (Table 1). These ratios are very similar to that previously reported for *E. coli* (1.0:2:1.0:0.1) (19). Small amounts of lysine could be found in the rickettsial peptidoglycan, which suggests that a peptidoglycan-linked lipoprotein(s) may be present in the rickettsiae. As is known for *E. coli*, a molecule of lipoprotein is attached to about 1 of every 10 repeating units of the peptidoglycan, and the last lysine residue of this protein remains linked to peptidoglycan after SDS and pronase treatments (5, 6). However, when normalized to the total amount of protein in the

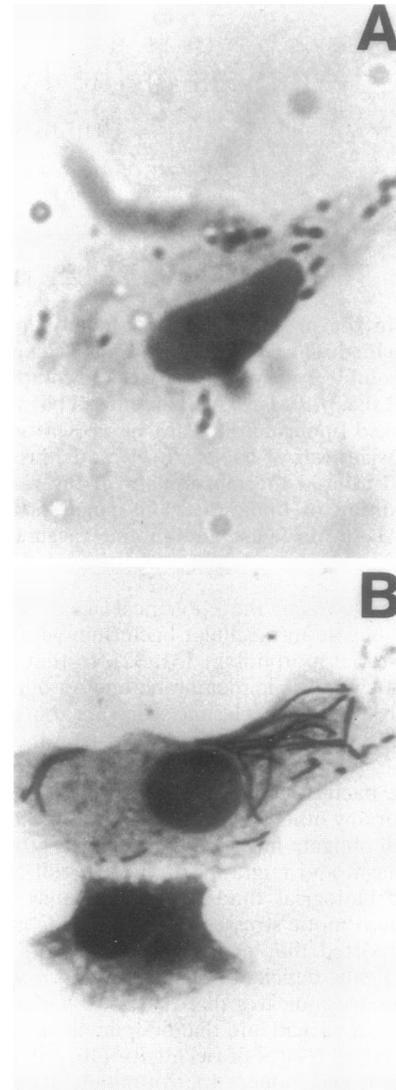


FIG. 2. Morphology of *R. prowazekii* grown in mouse L929 cells in MEM with and without D-cycloserine, as observed with a light microscope. (A) *R. prowazekii* grown in MEM with 10% serum. (B) *R. prowazekii* grown in MEM with 10% serum and D-cycloserine (50 µg/ml).

cell extract, the amount of the rickettsial peptidoglycan was only half of that of *E. coli* (Table 1). This could be due to contamination by adherent host proteins in the rickettsial preparations, a potential problem when working with intracellular organisms. Another possibility is that *R. prowazekii* has only one layer of peptidoglycan instead of two as found in *E. coli* (13, 16, 17, 26).

Effect of D-cycloserine on the growth of *R. prowazekii* in mouse L929 cells. D-Cycloserine is a D-alanine analog which affects the biosynthesis of bacterial peptidoglycan by inhibiting D-Ala-D-Ala ligase and alanine racemase (21, 22, 25). Mouse L929 cells (Flow Laboratories, Inc., McLean, Va.) were infected with the rickettsiae as described previously (29). After incubation overnight, the media were changed to Eagle minimal essential medium (MEM) (Mediatech, Washington, D.C.)

TABLE 2. Effect of D-cycloserine on growth of *R. prowazekii* in mouse L929 cells

Addition(s) to MEM ^a	Growth of <i>R. prowazekii</i> ^b			
	% R	RI	NR	% of control ^c
None	100 ± 0	58 ± 1	5,666 ± 101	100
D-Cycloserine	44 ± 9	5 ± 3	83 ± 6	1
D-Alanine	97 ± 3	59 ± 1	5,671 ± 164	100
D-Cycloserine plus D-alanine	95 ± 2	56 ± 2	5,253 ± 61	95

^a The indicated compounds were added to MEM with 10% serum as described in the text. The concentrations of D-cycloserine and D-alanine were 100 and 800 µg/ml, respectively.

^b The growth of the rickettsiae was expressed as the percentage of cells infected with rickettsiae (% R), the number of rickettsiae per infected cells (RI), and the total number of rickettsiae (NR) in 100 cells counted. The initial infection gave 77 ± 3% of the cells infected with 5 ± 1 rickettsiae per infected cell. Each value represents the mean ± standard deviation for two experiments.

^c An index of rickettsial growth based on the average total number of rickettsiae (NR) per 100 cells relative to that for the control.

plus 10% newborn calf serum with or without D-alanine (800 µg/ml) or D-cycloserine (50 to 100 µg/ml) and 1 µg of emetine (Sigma Chemical Co.), an inhibitor of eucaryotic protein synthesis, per ml. After 24 h, duplicate coverslips were stained by a modification of the method of Gimenez (11), and the growth and morphology of the rickettsiae were monitored microscopically. *R. prowazekii* failed to grow in mouse L929 cells in the presence of 100 µg of D-cycloserine per ml (Table 2). At a lower concentration of D-cycloserine (50 µg/ml), the morphology of the rickettsiae was altered; i.e., the rickettsiae were enlarged and/or elongated and failed to septate (Fig. 2). However, the effect of D-cycloserine could be prevented by the addition of D-alanine (Table 2), the normal substrate which competes with D-cycloserine for both the transport system and the D-Ala-D-Ala ligase (30). These data strongly suggested that *R. prowazekii* contains D-alanine in the peptidoglycan and has D-Ala-D-Ala ligase and alanine racemase activities.

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