INFECTION AND IMMUNITY, Sept. 1986, p. 678–684
0019-9567/86/090678-07$02.00/0

Vol. 53, No. 3

Demonstration of a Peptidoglycan-Linked Lipoprotein and Characterization of Its Trypsin Fragment in the Outer Membrane of *Brucella* spp.

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Received 26 February 1986/Accepted 22 May 1986

The sodium dodecyl sulfate (SDS) extraction-trypsin digestion protocol used by Braun and Sieglin (V. Braun and U. Sieglin, Eur. J. Biochem. 13:336–346, 1970) to show the peptidoglycan-linked lipoprotein of *Escherichia coli* was applied to both *Brucella abortus* and *E. coli*. Whereas a single polypeptide of 8,000 molecular weight was obtained from *E. coli*, several proteins of apparent molecular weight lower than 35,000 were demonstrated by SDS-polyacrylamide gel electrophoresis in *B. abortus*. These results did not change when the trypsin digestion conditions were modified. On the other hand, when the SDS extractions were performed under conditions more stringent than those used for other gram-negative bacteria, only a polypeptide fragment of apparent molecular weight of 8,000 was obtained from *B. abortus*. This polypeptide was similar to the trypsin fragment of the *E. coli* lipoprotein with respect to its behavior in SDS-polyacrylamide gels, isoelectric point in urea, molecular weight, and presence of both ester- and amide-linked fatty acids. Moreover, the amino acid analysis showed an overall similarity with respect to the amino acid composition of *E. coli* lipoprotein. A polypeptide of the same molecular weight, isoelectric point, and amino acid composition was also obtained from *Brucella ovis* by the same method. These results demonstrated that *B. abortus* and *B. ovis* cell envelopes contain a lipoprotein and strongly support the hypothesis that it is the only major protein covalently linked to the peptidoglycan.

The *Brucella* cell envelope (CE) is a three-layered structure in which an inner or cytoplasmic membrane, a periplasmic space, and an outer membrane (OM) can be differentiated (13). This OM contains lipopolysaccharide (LPS) (13) and, as examined by the Sarkosyl-Zwittergent method (36, 41, 45), several major proteins which by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) cluster into three different groups (45). Little is known of the role or properties of group 1 proteins (apparent molecular weight, 88,000 to 94,000), but it has been shown that proteins in group 2 (40,000 to 35,000 molecular weight) are matrix proteins (37) with porin activity (10) and that group 3 proteins (30,000 to 25,000 molecular weight) are likely to be the counterparts of the *Escherichia coli* OmpA protein (45). Thus, although significant differences in physicochemical properties have been found (36), this OM is similar to those of other gram-negative bacteria (30) with respect to the presence of LPS, porins, and OmpA-like proteins as major components. Despite this similarity, a lipoprotein similar to the one originally described by Braun and Rehn (3) in *E. coli* has not been found in *Brucella* spp. (45). Such a major OM protein, which exists as both free and peptidoglycan-linked forms (23, 24), has been found in several gram-negative bacteria (1, 2, 30, 33, 34) and, at least in *E. coli*, seems to play an important structural role in the stability of the OM (30, 43).

In a previous work (36), we have shown that the OM of *Brucella abortus* contains proteins accessible to extrinsic labeling with ^{125}I-lactoperoxidase which are not present in Sarkosyl-Zwittergent extracts of CEs, including several of molecular weight lower than 16,000. This observation prompted us to search in *Brucella* spp. for a low-molecular-weight protein similar to the *E. coli* lipoprotein found by Braun and Rehn (3). We report here that a protein linked to the peptidoglycan of *B. abortus* and *Brucella ovis* could be obtained by trypsin digestion of the CE fraction insoluble in SDS. A partial characterization of this polypeptide by using the trypsin fragment of the lipoprotein of *E. coli* as control is also presented.

MATERIALS AND METHODS

Bacterial strains. The avirulent rough (R) *B. abortus* 45/20, *Brucella melitensis* 115, and *B. ovis* REO198 used in this work have been described by other authors (9, 26). For cell fractionation or LPS extraction, the bacteria were grown in 3-liter flasks containing 800 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) on a rotary shaker at 37°C. *E. coli* B MH1283 was grown in the same medium and conditions.

Cell fractionation. Exponentially growing *B. abortus* 45/20, *B. ovis* REO198, and *E. coli* MH1283 cells were harvested by centrifugation (15 min at 7,000 × *g* and 4°C), washed once with saline, and suspended in a minimal amount of 10 mM Tris hydrochloride (pH 7.5) supplemented with DNase (100 μg/ml; Miles Laboratories, Inc., Elkhart, Ind.) and RNase (100 μg/ml, type III-A; Sigma Chemical Co., Saint Louis, Mo.). Cells were disrupted by ballistic disintegration in an MSK-Braun cell homogenizer (B. Braun Mesulgen AG, Leinfelden, Federal Republic of Germany), and after the glass beads were decanted, the CE fraction was obtained by ultracentrifugation as described previously (36).

Preparation of protein-peptidoglycan sacculi. The SDS-insoluble protein-peptidoglycan sacculi were prepared exactly as described by Braun and Sieglin (5). Alternatively, this protocol was modified as follows. CEs were first thoroughly suspended in water, and this suspension was added dropwise with continuous magnetic stirring to the boiling SDS buffer (4% SDS [Serva, Heidelberg, Federal Republic

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of Germany] in 10 mM Tris hydrochloride [pH 7.5]) up to a final concentration of 10 mg of CE protein per ml. The extraction was carried out for 20 min at 100°C with reflux. After cooling to room temperature, the insoluble residue was sedimented by centrifugation (40,000 × *g*, 30 min, 15°C), resuspended again in distilled H_2O, and reextracted two more times under the same conditions. The final SDS-resistant material was washed extensively with acetone-water (6:1, vol/vol) to remove most of the detergent bound during the extraction steps; these washings were essential to obtain good lipoprotein yields, presumably because bound SDS interfered with the enzymatic digestion described below.

Trypsin and lysozyme digestions. The SDS-resistant material was suspended in 10 mM Tris hydrochloride (pH 8.3) by brief sonication, trypsin was added (1 mg/10 mg of protein; Sigma Chemical Co.), and the mixture was incubated for 3 h at 37°C. Proteolysis was stopped either with an excess of soybean trypsin inhibitor (Sigma Chemical Co.) or by cooling the mixture to 4°C. After removal of the trypsin-resistant fraction by centrifugation (40,000 × *g* for 30 min at 4°C), the protein present in the supernatant fluid was precipitated either by acidification (pH 3.5) with formic acid or by the addition of 5 volumes of acetone (−20°C overnight). Finally, the precipitate was collected by centrifugation, suspended in a minimal amount of distilled H_2O, and stored at −20°C until further use.

Alternatively, the acetone-water-washed, SDS-resistant residue was digested with lysozyme (1 mg/mg of protein in residue; Sigma Chemical Co.). After overnight incubation at 37°C, the mixture was centrifuged as above, and the supernatant fluid was precipitated with acetone as indicated above. Both the precipitate of the supernatant fluid and the sedimented insoluble residue were kept for analysis.

LPS preparations. *B. melitensis* 115 R-LPS was extracted from acetone-dried cells by the petroleum ether-chloroform-phenol method (16); since these extracts contained less than 1% protein, they were used without further purification. For enzyme-linked immunosorbent assays (see below), R-LPS was coupled to bovine serum albumin (BSA) as described by Galanos et al. (17).

Gel filtration chromatography. Gel filtration was performed in Bio-Gel P-60 (200- to 400-mesh) columns (1.6 by 54 cm). The gel was equilibrated in 10 mM Tris hydrochloride (pH 8)–2% SDS and calibrated with dextran blue, horse cytochrome *c* (12,000 molecular weight), and insulin (6,000 molecular weight) as the standards. Chromatography was performed at a flow rate of 4 ml/h, and 1-ml fractions were collected.

Analytical methods. Protein was determined colorimetrically with the Folin reagent as described by Dulley and Grieve (14), using BSA as a standard. For amino acid analysis, samples were hydrolyzed under vacuum in 6 N HCl–2% phenol for 24 h at 100°C and analyzed with ninhydrin in a Durrum D-500 (Dionex) amino acid analyzer.

Gas-liquid chromatography was performed in a Sigma-300 dual flame ionization detector apparatus (Perkin-Elmer Ltd., Beaconsfield, England) equipped with a stainless-steel Supelcoport (100/120-mesh), 3% SP-2,100 column (Supelco Inc., Bellefonte, Pa.) and a Sigma-15 chromatograph data station (Perkin-Elmer). A 150 to 230°C temperature gradient was used in the analysis. Ester- and amide-linked fatty acids were released from the samples by treatment with alkaline hydroxylamine and by saponification of the hydroxylamine-insoluble residue by the methods of Snyder and Stephens (42) as modified by Rietschel et al. (39). Methyl esters were prepared with BF_3 and methanol as described by Morrison and Smith (38) and tentatively identified by comparison of their retention times with those of known standards (Qualmix M and Bacterial Fatty Acid Methyl Esters Mixture; Supelco).

SDS-PAGE was performed by the method of Laemmli (28). When acrylamide gradients were used, they were performed in 7 M urea by the method of Hashimoto et al. (21). Molecular weight standards were phosphorylase *b* (94,000), BSA (66,600), *E. coli* B OmpF (38,000) obtained by the method of Rosenbusch (40), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000), egg white lysozyme (14,300), and polymixin B (8,000). After electrophoresis, polyacrylamide gels were stained for proteins with Coomassie blue (15) or by the alkaline-silver method of Merrill et al. (32).

Isoelectric focusing was carried out in 7 M urea–16% Pharmalite 10-3 (Pharmacia, Uppsala, Sweden)–5% polyacrylamide gels at 2,000 V for 1.5 h with running tap water as the coolant. Electrode solutions were 0.1 M H_2SO_4 and 0.1 M NaOH, respectively. After focusing, the gels were fixed first with 5% sulfosalicylic acid in 10% trichloroacetic acid for 60 min and then with methanol-acetic acid-distilled H_2O (3:1:6, vol/vol/vol) for 30 min. Staining was performed with 0.2% Coomassie blue R-250 in methanol-acetic acid-H_2O, a mixture which was also used as the destaining solution. Isoelectric point standards were bovine carbonic anhydrase B (5.8), β-lactoglobulin A (5.2), soybean trypsin inhibitor (4.5), and amyloglucosidase (3.5).

Immunological methods. Antisera to purified *B. abortus* 45/20 lipoprotein were raised in rabbits. The animals were immunized once intracutaneously (100 μg of protein in Freund complete adjuvant) and 10 times intramuscularly (weekly doses of 50 μg in Freund incomplete adjuvant) and bled 5 days after the last injection. Antibody to R-LPS was obtained from rabbits after four weekly intramuscular doses of 1 mg in Freund incomplete adjuvant.

Enzyme-linked immunosorbent assays were performed on polystyrene plates. Lipoprotein was attached to the plates by overnight incubation of a 5-μg/ml solution in 60 mM carbonate buffer (pH 9.6) at 37°C. BSA-coupled R-LPS at a concentration of 10 μg/ml was attached similarly. The test was performed as described elsewhere (29) with peroxidase-conjugated goat anti-rabbit immunoglobulin G (heavy and light chain specific) of commercial origin (Nordic Laboratories, Tilburg, Holland).

For Western blot analysis (6), proteins and R-LPS were transferred (200 mA, 18 h) onto nitrocellulose sheets (type HA, 0.45-μm pore size; Millipore Corp., Bedford, Mass.), and the sheets were incubated for 1 h at room temperature with the corresponding antiserum diluted in 0.15% Tween 20–20 mM phosphate-buffered saline (pH 7.3). After the unbound immunoglobulin was removed with several washes of the same buffer, peroxidase-conjugated anti-rabbit immunoglobulin G serum was added, and incubation was carried out for 1 h at room temperature. 4-Chloro-1-naphthol was used as the developing substrate (0.5 mg/ml in H_2O_2-Tris-saline-methanol [22]). As a control, both polyacrylamide gels and nitrocellulose sheets were stained for proteins with Coomassie blue and either amido black or India ink (19), respectively.

RESULTS

As a first approach to studying the presence of proteins covalently linked to the peptidoglycan, CEs of *B. abortus*

45/20 were extracted with SDS at 100°C as described by Braun and Sieglin (5). SDS-PAGE analysis of the trypsin digestion products of the fraction resistant to such extraction demonstrated several proteins of apparent molecular weights ranging from 35,000 to the lower limit of the gel (Fig. 1, lane 2). In contrast, the preparations obtained from *E. coli* CEs by the same protocol showed only a single major polypeptide of apparent molecular weight close to 8,000 (Fig. 1, lane 1), as well as traces of trypsin.

The results obtained with *B. abortus* were essentially the same when different trypsin concentrations and times of incubation were used (Fig. 1, lanes 3 and 4). On the other hand, modification of the conditions of extraction with SDS had a strong influence on the protein profile of the fractions released by trypsin digestion. When the extraction with SDS was performed under the stringent conditions described in Materials and Methods, instead of the numerous protein bands observed before, a single band of apparent molecular weight close to 8,000 was detected by SDS-PAGE in both *B. abortus* and *E. coli* preparations (Fig. 2A, lanes 3 and 4). Shorter times of extraction than those indicated in Materials and Methods also removed the high-molecular-weight proteins, but instead of the 8,000-molecular-weight band, a major 14,000-molecular-weight band and a cluster of bands in the 10,000- to 8,000-molecular-weight range were observed (Fig. 2A, lanes 1 and 2). The banding pattern of these preparations was, however, dependent on the electrophoretic conditions. When the same preparations were analyzed in 10 to 18% acrylamide gradient–7 M urea gels, a single major band was demonstrated instead of the 14,000- and 10,000- to 8,000-molecular-weight bands observed before (Fig. 2B). This result suggested the presence of a single major protein in the preparations, an interpretation also supported by the analysis described below.

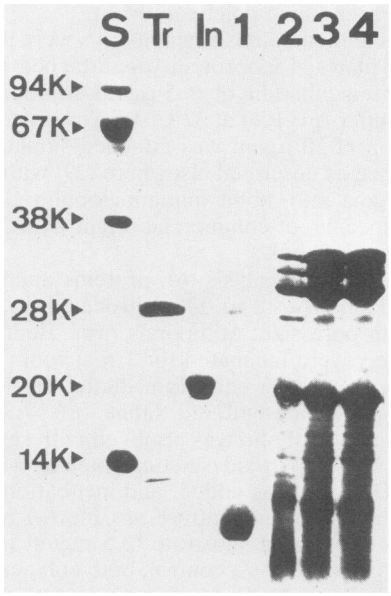

FIG. 1. SDS-PAGE analysis of the proteins obtained by the SDS extraction-trypsin digestion protocol of Braun and Sieglin (5) from *E. coli* B MH1283 (lane 1, 5 μg of trypsin per mg of protein, 2 h of incubation) or *B. abortus* 45/20 (lane 2, 5 μg of trypsin per mg of protein, 4 h; lane 3, 2.5 μg of trypsin per mg of protein, 4 h; lane 4, 2.5 μg of trypsin per mg of protein, 2 h). S, Standards; Tr, trypsin; In, soybean trypsin inhibitor. Numbers on left show molecular weight standards; K, 10^3.

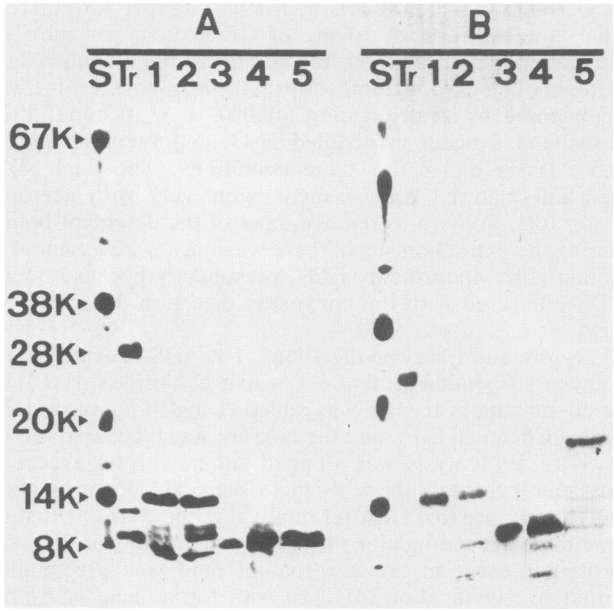

FIG. 2. Analysis by standard (A) or 7 M urea-acrylamide gradient (B) SDS-PAGE of the trypsin digests of CEs extracted once (10 min) with SDS at 100°C (lane 1, *E. coli* B MH1283; lane 2, *B. abortus* 45/20) or three times (20 min each time) with SDS at 100°C (lane 3, *E. coli* B MH1283; lane 4, *B. abortus* 45/20; lane 5, *B. ovis* REO198). S, Standards; Tr, trypsin. Numbers on left show molecular weights; K, 10^3.

Isoelectric focusing in urea showed a single acidic protein in those *B. abortus* and *E. coli* preparations that by standard SDS-PAGE contained both the 14,000- and the 10,000- to 8,000-molecular-weight group of bands (Fig. 3A and B). In the *E. coli* preparations that contained just the 8,000-molecular-weight band, this analytical method showed an acidic band of the same isoelectric point as the one corresponding to the 14,000- and 10,000- to 8,000-molecular-weight preparations (Fig. 3A and B). In some of the *B. abortus* preparations that contained the 8,000-molecular-weight band, isoelectric focusing revealed two very close bands (Fig. 3C). Of note, although the focused proteins were easily observed by their precipitation in the trichloroacetic acid-sulfosalicylic fixing solution (Fig. 3B), they often stained poorly with Coomassie blue (Fig. 3A).

The presence of proteins covalently linked to the peptidoglycan of *B. abortus* was also studied with lysozyme instead of trypsin. Although the presence of lysozyme prevented the demonstration by SDS-PAGE of a possible 14,000-molecular-weight protein, the supernatants of *E. coli* and the insoluble residues of both *B. abortus* and *E. coli* contained a polypeptide of molecular weight lower than 14,000, which was demonstrated by silver staining of SDS-PAGE gels (Fig. 4). The results of these experiments also ruled out the possibility that the polypeptides observed after trypsin digestion of the SDS-resistant fraction were due to proteolysis of proteins of high molecular weight.

Gel filtration of the polypeptide released from the SDS-resistant fraction by trypsin resolved only one major peak eluting between the cytochrome *c* and insulin markers and from whose K_{av} (0.27) a molecular weight of 10,300 was estimated. For the *E. coli* lipoprotein preparations, this same analysis gave a K_{av} of 0.29 and a molecular weight of 10,500.

The polypeptide purified by gel filtration was used to hyperimmunize rabbits, and the serum obtained was ana-

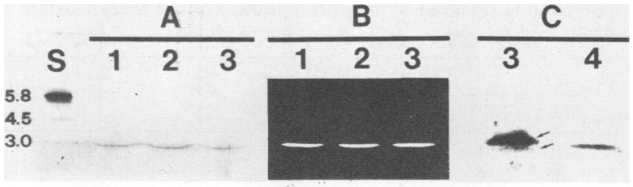

FIG. 3. Isoelectric focusing of the preparations shown in Fig. 2. (A) Coomassie blue-stained gel of preparation from *E. coli* B MH1283 CE extracted once (10 min) with SDS at 100°C (lane 1); preparation from *E. coli* B MH1283 CE extracted three times, 20 min each time, with SDS at 100°C (lane 2); and preparation from *B. abortus* 45/20 CE extracted once (10 min) with SDS at 100°C (lane 3). S, Standards. (B) Protein bands precipitated during fixation of the same gel shown in panel A. (C) Coomassie blue-stained gel of preparation from *B. abortus* 45/20 CE extracted three times, 20 min each time, with SDS at 100°C (lane 3) and preparation from *B. ovis* CE extracted three times, 20 min each time, with SDS at 100°C (lane 4). Numbers on the left are the isoelectric points.

lyzed by immunoenzymatic methods. Although this serum contained high titers of antibody to the immunizing protein preparation, it failed to react with BSA–R-LPS in either enzyme-linked immunosorbent assays or Western blots (data not shown). Moreover, the anti-R-LPS serum did not react with the protein (data not shown). Both results demonstrated that the protein preparations obtained after gel filtration were free of LPS, and therefore they were used for chemical analysis.

With respect to the amino acid composition reported for the lipoprotein of *E. coli* (2), the polypeptide from *B. abortus* showed both similarities and differences (Table 1). Particularly, glycine, phenylalanine, and histidine, which are not present in the *E. coli* lipoprotein, were found in the protein from *B. abortus*. Despite these differences, the similarity between both proteins was remarkable with respect to the estimated minimal number of amino acids (61 to 63 in *B. abortus* versus 58 in *E. coli*), minimal molecular weight (7,095 to 7,375 versus 7,286, repsectively), and proportions of polar uncharged amino acids (28.6% versus 28.1%, respectively) and nonpolar amino acids (41.3% versus 35.1%, respectively).

The purified protein was also tested for the presence of ester- and amide-linked fatty acids. Although in preliminary experiments the analysis showed high proportions (up to 80%) of methyl laurate among the methyl esters of the fatty acids bound by hydroxylamine-sensitive linkages, controls run with SDS showed that the presence of this methyl ester was due in all likelihood to contamination of the protein by tightly bound detergent. The interpretation of these controls is in keeping with recent studies in which no lauric acid has been found in *Brucella* spp. (7, 8, 25), and, therefore, the values of the hydroxylamine-sensitive fatty acids were corrected accordingly. The results are presented in Table 2, and they show the presence of fatty acids, both ester (hydroxylamine sensitive) and amide (hydroxylamine resistant) linked. The major fatty acids found were myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), and oleic ($C_{18:1}$) acids. No hydroxylated fatty acids were detected, a result consistent with those presented above showing no contaminant LPS in the protein preparations used to obtain the immunoserum. Similarly, although the major fatty acids of the protein were some of those reported for *Brucella* phospholipids (44), lactobacillic acid ($C_{19:0\ cyc}$) which is characteristic of *Brucella* phospholipid was only present in very small amounts.

Part of the experiments and analysis performed with *B. abortus* 45/20 were repeated with *B. ovis* REO198. The results demonstrated that this strain also contained a low-molecular-weight protein bound to the peptidoglycan (Fig. 2A, lane 5), although its mobility in acrylamide gradient-urea gels was different from that of its *B. abortus* counterpart (Fig. 2B, lane 5). In addition, both the isoelectric point in urea and the overall amino acid composition were similar to those of the protein from *B. abortus* (Fig. 3C; Table 1).

DISCUSSION

The results presented in this report demonstrate that when the protocol used for members of the family *Enterobacteriaceae* (2) and *Pseudomonas* species (33, 34) to show the covalently linked form of the lipoprotein was applied to *B. abortus*, several proteins remained in the SDS-resistant residue. However, when more stringent SDS extractions were performed, only a single protein remained attached to the peptidoglycan, as shown by both SDS-PAGE in urea and isoelectric focusing. This result is in contrast with those of Dubray and Bezard (11) and Dubray and Charriaut (12) who reported that digestion with lysozyme of a CE fraction resistant to extraction with SDS at 100°C brought about the release of several polypeptides of molecular weights ranging from 37,000 to 15,000. These authors interpreted their results as supporting a model in which the major polypeptides of the *B. abortus* OM would be covalently linked to the underlying peptidoglycan layer (12). It is clear that the evidence presented here does not support such a model. It must be noted that Verstreate et al. (45) have reported that extraction of *B. abortus* living or Formalin-killed cells with repeated changes of SDS at 100°C during 4 h did not completely remove the OM major proteins of *B. abortus* from the lysozyme-sensitive residue. However, as pointed out by Verstreate et al. (45), these observations do not unequivocally support the covalent linkage of the OM major proteins with the peptidoglycan because similar results were obtained for *E. coli* when Formalin-killed cells were used. Indeed, it is well established

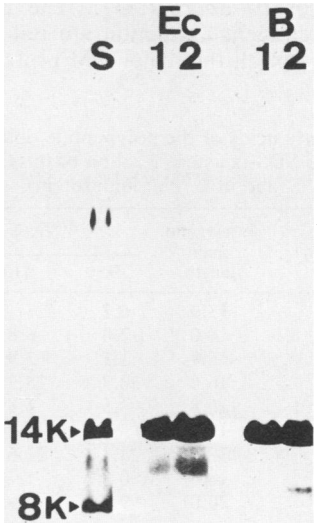

FIG. 4. SDS-PAGE analysis of the proteins released by lysozyme digestion of the SDS-resistant fraction of the CE of *E. coli* B MH1283 (Ec) and *B. abortus* 45/20 (B). Lane 1, Proteins released into the supernatant fluid; lane 2, insoluble proteins. The standards (S) were lysozyme (14,000 molecular weight [14K]) and polymyxin B (8,000 molecular weight [8K]). The gel was stained with ammoniacal silver.

TABLE 1. Amino acid composition of the *E. coli* lipoprotein and the polypeptide released by trypsin digestion of the SDS-resistant fraction of the CE of *B. abortus* and *B. ovis*

Amino acid	*B. abortus*		*B. ovis*		*E. coli*[a] (NI[c])
	Mean ± SE[b]	NI[c]	Mean ± SE[b]	NI[c]	
Asx	7.18 ± 0.17	7	6.20 ± 0.16	6	14
Thr	2.96 ± 0.26	3	48 ± 0.06	2–3	2
Ser	2.85 ± 0.38	3	3.62 ± 0.36	4	6
Glx	5.27 ± 0.16	5	6.02 ± 0.05	6	5
Pro	1.84 ± 0.10	2	ND[d]		
Gly	9.04 ± 0.43	9	8.38 ± 0.38	8	
Ala	5.10 ± 0.23	5	5.16 ± 0.27	5	9
Val	4.77 ± 0.42	5	5.15 ± 0.06	5	4
Cys	0.58 ± 0.17	1	ND[d]		1
Met	1.46 ± 0.36	1–2	1.09 ± 0.50	1	2
Ile	3.16 ± 0.11	3	1.03 ± 0.13	1	1
Leu	3.51 ± 0.14	3–4	3.19 ± 0.29	3	4
Tyr	2.80 ± 0.11	3	0.81 ± 0.05	1	1
Phe	2.09 ± 0.08	2	3.05 ± 0.09	3	
Lys	2.15 ± 0.25	2	1.88 ± 0.01	2	5
His	1.00	1	1.00	1	
Arg	0.97 ± 0.07	1	0.64 ± 0.10	1	4
NH_4	3.40 ± 0.35	3	3.79 ± 0.17	4	

[a] From reference 2.
[b] Figures are the mean of the number of residues ± the standard error calculated from the data of four independent analyses on the basis of one histidine residue per mol of protein.
[c] NI, Nearest integer.
[d] ND, Not determined.

that the lipoprotein of Braun and Rehn (3) is the only major protein covalently linked to the peptidoglycan in exponentially growing *E. coli* cells (30).

The high SDS resistance that was found for *B. abortus* is in agreement with the results of a previous comparative study in which it was demonstrated that *Brucella* CEs were more resistant to nonionic and dipolar ionic detergents and EDTA than the CE of *E. coli* (36). Similarly, we have reported that, in contrast to *E. coli*, it is necessary to repeat the 50°C SDS extraction step of Rosenbusch's protocol (40) several times to remove the nonmatrix proteins from the peptidoglycan of *B. abortus* (37). The reasons for this resistance to detergent extraction are not clear, but since covalent linkage of all the major OM proteins to the peptidoglycan must be ruled out, they could relate to the peculiar fatty acid composition of both the LPS and phospholipids of *Brucella* spp. (35, 44).

TABLE 2. Fatty acids of the polypeptide obtained by trypsin digestion of the SDS-resistant fraction of the CE of *B. abortus* and of *E. coli* lipoprotein[a]

Fatty acid	Equivalent chain length	*B. abortus*		*E. coli*[b]	
		HS[c]	HR[d]	Ester	Amide
Unknown	13.9	6.1			
$C_{14:0}$	14.0	12.0	6.8	1.5	2.4
$C_{16:1}$	15.7	1.1	2.9	10.6	10.9
$C_{16:0}$	16.0	20.7	28.3	44.5	65.0
$C_{17:0}$ cyc	16.8	3.5	1.8	11.6	1.7
$C_{18:1}$	17.7	3.2	12.1	23.7	10.8
$C_{18:0}$	18.0	24.1	22.6		1.7
$C_{19:0}$ cyc	18.6	0.8	1.7	7.6	
$C_{20:0}$	20.0	9.9	4.9		
$C_{14:OH}$					4.0
Other (no. of peaks)		17.6 (9)	18.9 (8)	1.4	3.6

[a] Figures represent the percentage of the corresponding species of fatty acid. The values of the *B. abortus* polypeptide were corrected for the interference owing to lauryl sulfate as indicated in the text.
[b] From reference 20.
[c] Hydroxylamine-sensitive fatty acids.
[d] Hydroxylamine-resistant fatty acids.

The use of both CE from cells that had not been inactivated and an exhaustive SDS extraction protocol eliminated the artifacts described above. However, this protocol brought about the binding of important amounts of SDS to the detergent-resistant fraction, as demonstrated by the fatty acid analysis. Removal of all the SDS possible was found to be essential for the lytic enzymes to act. Also, it is important to note that when lysozyme was used the low-molecular-weight protein released from the peptidoglycan of *B. abortus* remained in the insoluble residue. All these methodological problems could explain why a low-molecular-weight protein has not been observed in lysozyme digests of SDS-extracted CEs of *B. abortus* (11, 12, 45).

Under some conditions of extraction with SDS several bands (14,000 to 18,000 molecular weight) appeared in Laemmli polyacrylamide gels in both *E. coli* and *B. abortus* preparations. However, in 7 M urea–10 to 18% acrylamide gradient gels the same preparations showed only a major protein band suggesting that in fact they contained a single polypeptide. This interpretation is consistent with the results of the isoelectric focusing analysis, which also showed a single protein band. Braun et al. (4) have reported that the precise banding pattern of the lipoprotein depends on the amount of SDS available for binding, presumably because of the strong tendency of this amphiphilic protein to self-association. Also, Gmeiner et al. (18) have shown that the banding pattern of *Proteus mirabilis* lipoprotein is affected by both the SDS concentration and the presence of urea in the polyacrylamide gels. These observations are consistent with our own results because, in addition to the effect of urea and acrylamide gradients, multiple bands were not observed in standard gels with protein preparations that had undergone an intense exposure to SDS as a result of the exhaustive detergent extraction. The same reasons could explain the different pattern presented in urea-acrylamide gradient

gels by *E. coli* and *Brucella* preparations that had been extracted with SDS for different times.

It is clear that the polypeptide obtained by trypsin digestion of the exhaustively extracted *Brucella* CE must represent a fragment and not the whole protein. However, as it has been shown for the *E. coli* lipoprotein, such a fragment is very likely to contain most of the protein. This conclusion is supported by the similarity of the polypeptide fragments obtained from both bacteria with respect to overall amino acid composition, isoelectric point in urea, apparent molecular weight in SDS-PAGE, and molecular weight estimated by gel filtration. In addition, like the *E. coli* lipoprotein fragment, the polypeptide obtained from *B. abortus* contained fatty acids, both ester and amide linked. The proportion that the lipid moiety represents in both lipoproteins is probably similar because the difference between the molecular weight of the whole fragment (estimated by gel filtration) and the molecular weight of its protein moiety (estimated from the amino acid analysis) was similar in both cases. Finally, the fatty acid composition of the lipoprotein of Braun and Rehn (3) resembles that of the *E. coli* phospholipid (2), and the same was found to be true for the fatty acids of the lipoprotein and phospholipids of *B. abortus*. It has to be stressed that the controls performed ruled out the possibility that the results of the fatty acid analysis could be due to contaminant LPS. Since LPS is a tenacious contaminant of *B. abortus* OM major proteins (37, 45), its absence from the preparations analyzed is remarkable and probably relates to the exhaustive SDS extractions carried out.

Although not all the experiments and analyses performed with the lipoproteins of *E. coli* and *B. abortus* were repeated with *B. ovis*, the data of the SDS-PAGE, isoelectric focusing, and amino acid analyses should be enough to demonstrate the presence in this species of a lipoprotein linked to the peptidoglycan. As could be expected, although both the molecular weight and isoelectric point were similar to those of the *E. coli* lipoprotein, the amino acid composition of the protein from *B. ovis* was closer to that of *B. abortus*.

There are a few reports on the precise composition of *Brucella* peptidoglycan (27, 31), but the data available suggest that it is not substantially different from that of other gram-negative bacteria. Thus, the data presented also suggest that the lipoprotein-peptidoglycan linkage is not very different from that of *E. coli*, at least with respect to the relative location within the protein of the peptide bond cleaved by trypsin. Studies on the immunological cross-reactivity between the lipoproteins of *Brucella* spp. and *E. coli*, which could shed some light on additional similarities, are in progress.

ACKNOWLEDGMENTS

We are grateful to D. T. Berman and A. J. Iriarte for the amino acid analysis and to R. Díaz for many helpful suggestions and discussions.

This investigation was supported in part by the Comision Asesora de Investigación Científica y Tecnológica of Spain (grants 0173/81 and 2910/83) and by fellowship support for M.J.G.-M. from the Fondo de Investigaciones Sanitarias de la Seguridad Social.

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