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PROTEIN NEIGHBORHOODS IN THE OUTER MEMBRANE OF *SALMONELLA TYPHIMURIUM*

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Summary

The organization of proteins in the outer membrane of *Salmonella typhimurium* was analyzed by cross-linking with cleavable reagents and symmetrical two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. The major outer membrane proteins could be cross-linked to form multimeric complexes. The pore-forming 44 000, 36 000 and 34 000 dalton proteins were cross-linked to form dimers and trimers. Lipoprotein was cross-linked to 33 000 and 17 500 dalton proteins. In addition the 33 000, 24 000, 17 500 dalton proteins and the free form of lipoprotein were cross-linked to the peptidoglycan layer of the cell wall. The cross-linked complexes found were similar to those of analogous proteins in the outer membrane of *Escherichia coli*, thus suggesting a similar organization of outer membrane proteins in these species.

Introduction

The outer membrane of Gram-negative bacteria seems to be a rather rigid structure with strong protein interactions. In *Escherichia coli* the major proteins: I, II*, III [1] and lipoprotein [2] are interacting tightly with each other [3—5]. Some of the proteins also interact with the peptidoglycan layer: bacteriophage λ receptor protein [6], protein I (which exists as two subspecies Ia and Ib [7]) and at least part of the free lipoprotein, can be isolated as peptidoglycan complexes [8—12]. We [10,13—16] and Reithmeier and Bragg [17] have studied the specificity of these protein interactions by cross-linking techniques. The pore-forming proteins, λ receptor [18] and protein [19—22], are cross-linked to multimers [10,13,15,17] in which the basic unit appears to

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be a trimer [10,15]. In addition the bacteriophage PA-2 determined porin, protein 2 [23—25], seems to have a similar trimeric arrangement [26]. In addition to multimers, the other major proteins could be cross-linked to form heterocomplexes [14,17]. Lipoprotein was found cross-linked to protein II* [14,17] and to 17 500 dalton protein (probably identical to protein III) [14].

Protein II*, free lipoprotein [14,17,27] and the 17 500 dalton protein [14] could also be cross-linked to peptidoglycan. Thus the major proteins seem to interact with the peptidoglycan layer of the cell wall.

The outer membrane of *Salmonella typhimurium* contains a set of major proteins which seem to be analogous to the *E. coli* proteins [28]. The 44 000 dalton protein [12] which is induced by maltose is analogous to λ receptor in *E. coli*. It has a trimeric arrangement [15] and it forms pores through the outer membrane (Palva, E.T., unpublished results). It is also associated with peptidoglycan [12,15] as are the major porins [29,30], the 36 000 and 34 000 dalton proteins [31] of *S. typhimurium* [9]. A lipoprotein is present in *S. typhimurium* analogous to that of *E. coli* [32], and it exists in both free form and covalently linked to peptidoglycan [33]. As will be discussed in Results proteins similar to 17 500 and 15 500 dalton [14] proteins are also present in *S. typhimurium*.

In this study the protein interactions in the outer membrane of *S. typhimurium* have been analyzed by cross-linking techniques. Evidence is presented for interactions similar to those found in *E. coli*.

Materials and Methods

Chemicals. The cleavable cross-linking reagents tartryl-diazide (0.6 nm bridge length) and tartryl-di-(glycylazide) (1.3 nm bridge length) [34] were a generous gift from Hans Fasold. [^{35}S]Methionine (specific activity 800—1000 Ci/mmol) came from the Radiochemical Center (Amersham, U.K.).

Strains, media and growth conditions. E. coli K12 B14 [35] is a wild-type strain in respect to outer membrane proteins. *S. typhimurium* LT2 strains SH6749 (wild type in respect to outer membrane proteins), SH6751 (*ompB* derivative of SH6749 lacking the 36 000 dalton protein), SH6761 (*ompD* derivative of SH6749 lacking the 34 000 dalton protein), SH6896 (*ompD* derivative of SH6751 lacking both 36 000 and 34 000 dalton proteins) (Mäkelä, P.H., personal communication) and SL1909 (*ompA* mutant lacking the 33 000 dalton protein [37]) were kindly donated by P.H. Mäkelä.

Cells were grown in Luria broth [37] with or without the addition of 0.4% maltose, or in M9 minimal medium [37] with maltose or glycerol (0.4%) as carbon source and the required amino acids. Growing of cells and labeling of proteins with [^{35}S]methionine was as described previously [14].

Isolation of cell envelopes and cell walls. Cell envelopes were isolated as described in Ref. 12 and the cell wall fraction (outer membrane and peptidoglycan) as described in Ref. 10.

Isolation of peptidoglycan layer. Peptidoglycan layer was isolated from cells disrupted by mild ultrasonic treatment (two 5-s periods in a salt/ice bath) [14].

Isolation of peptidoglycan-associated proteins. Protein-peptidoglycan com-

plexes were isolated as described by Rosenbusch [8] but using the cell envelope preparation as starting material. Proteins non-covalently associated with peptidoglycan were released by 0.5 M NaCl treatment [38] and purified on a sucrose gradient in the presence of sodium dodecyl sulfate as described [10].

Cross-linking. Cross-linking of whole cells, isolated cell walls, protein-peptidoglycan complexes or isolated proteins was with the reagents at concentrations indicated, essentially as described previously [10,13,14].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Discontinuous 10 or 14% acrylamide slab gels (0.5 mm thick) were with the modified Laemmli [39] buffer system as described [13]. The symmetrical two-dimensional gel system [34] used to analyze cross-linked proteins was as described previously [10,13]. For analysis of the mobility of the cross-linked complexes of the 36 000 and 34 000 dalton proteins 3—10% acrylamide slab gels with the continuous buffer system described by Davies and Stark [40] were used. The staining and autoradiography of gels have been described [10].

Results

Comparison of the protein composition of the cell wall of E. coli and S. typhimurium

The cell wall fraction (outer membrane and peptidoglycan) was isolated from *E. coli* and *S. typhimurium* and analyzed on 14% acrylamide dodecyl sulfate gels (Fig. 1). The protein patterns obtained are remarkably similar in these two species, counterparts for all the major outer membrane proteins in *E. coli* seem to present in *S. typhimurium*, including analogs to the 24 000, 17 500 and 15 500 dalton proteins, of *E. coli*. The *S. typhimurium* 24 000 dalton protein is apparently a major band when labeled with [^{35}S]methionine, while in *E. coli* this is a minor species. A general feature of the outer membrane protein patterns in these species is that many *S. typhimurium* proteins (44 000, 36 000, 34 000, 33 000 dalton proteins and lipoprotein) tend to be slightly larger than corresponding *E. coli* proteins. Whether this is due to the analogous polypeptide chains being actually longer in *S. typhimurium* or being differently modified leading to different mobilities on dodecyl sulfate gels is not known.

The identify of the bands corresponding to 36 000, 34 000 and 33 000 dalton proteins was verified by using appropriate mutants and that of 44 000 by induction with maltose (Fig. 1B). The protein band evident in the double mutant SH6896 between the positions of 36 000 and 34 000 dalton proteins is the 35 000 dalton protein [31], which is increased in some mutants lacking 36 000 and 34 000 dalton proteins [41,42].

Cross-linking of the cell wall

The isolated cell wall of *S. typhimurium* was treated with various concentrations of tartryl-di-(glycylazide) and analyzed on two-dimensional 14% acrylamide dodecyl sulfate gels (Fig. 2). The cross-linked complexes separated in the first dimension were cleaved by 15 mM $NaIO_4$ before electrophoresis in the second dimension [10,13]. Fig. 2A shows the protein pattern of the control

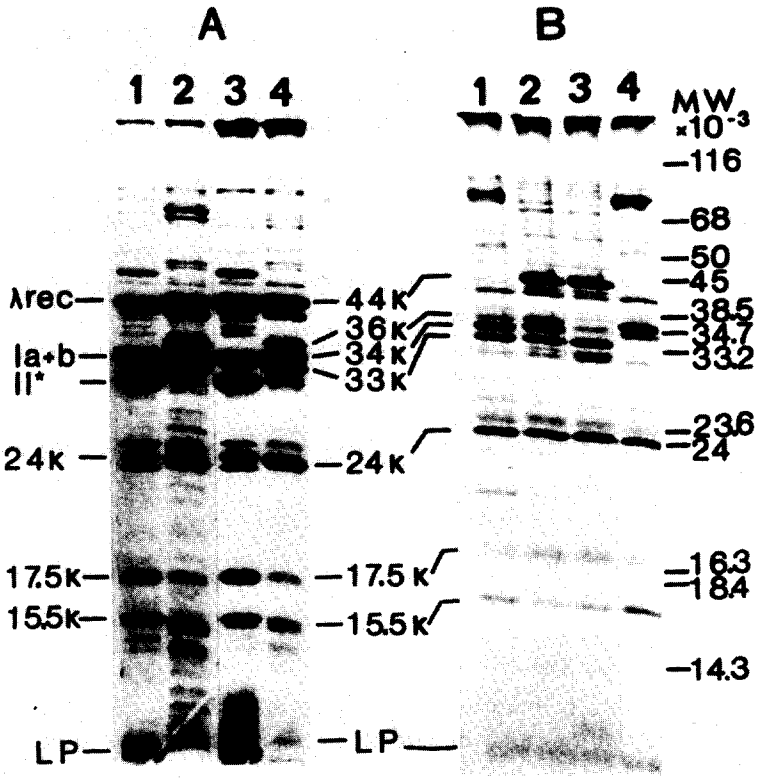

Fig. 1. Cell wall proteins of *S. typhimurium* and *E. coli* analyzed by sodium dodecyl sulfate polyacrylamide (14%) gel electrophoresis. A cell wall fraction from [^{35}S]methionine-labeled cells grown in M9 minimal medium was isolated. (A) A comparison of *E. coli* and *S. typhimurium* proteins. Slots 1 and 3, *E. coli* K12 B14 (wild type); slots 2 and 4 *S. typhimurium* LT2, SH6749 (wild type). Slots 1 and 2, stained gel; slots 3 and 4, an autoradiogram of the same gel. LP, lipoprotein. (B) *S. typhimurium* mutants used. Slots 1 and 2, strain SH6749 (wild type); slot 3, strain SH6896 (a double mutant lacking the 36 000 and 34 000 dalton proteins); slot 4, strain SL1909 (a mutant lacking the 33 000 dalton protein). The cells analyzed in slots 1 and 4 were grown using glycerol as the carbon source; those in slots 2 and 3 using maltose. The arrows on the right indicate the migration of standard proteins: β-galactosidase (M_r 116 000); bovine serum albumin (68 000); immunoglobulin H chain (50 000); ovalbumin (45 000); maltose-binding protein (38 500); pepsin (34 700); arabinose binding protein (33 200); 50 S ribosomal protein L1 (23 600); trypsinogen (24 000); β-lactoglobulin (18 400); 50 S ribosomal protein L13 (16 300); egg-white lysozyme (14 300).

which was not cross-linked. The proteins indicated in the one-dimensional gel (Fig. 1) can be identified (arrows in Fig. 2A). They seem to fall on the diagonal except a part of the 36 000—34 000 dalton proteins, which move slower in the second dimension and appear above diagonal. This aberrant mobility may be due to periodate modification.

When the cell wall fraction was cross-linked prior to electrophoresis various off-diagonal spots representing cross-linked and cleaved proteins were obtained (Fig. 2B—D). The cleaved proteins are identified by their horizontal alignment with spots on the diagonal. The different protein components of heterocomplexes of two or more proteins can be tentatively identified by the vertical alignment of the off-diagonal spots. The major proteins were readily cross-linked with the lowest concentration of tartryl-di-(glycylazide) used (0.5 mM)

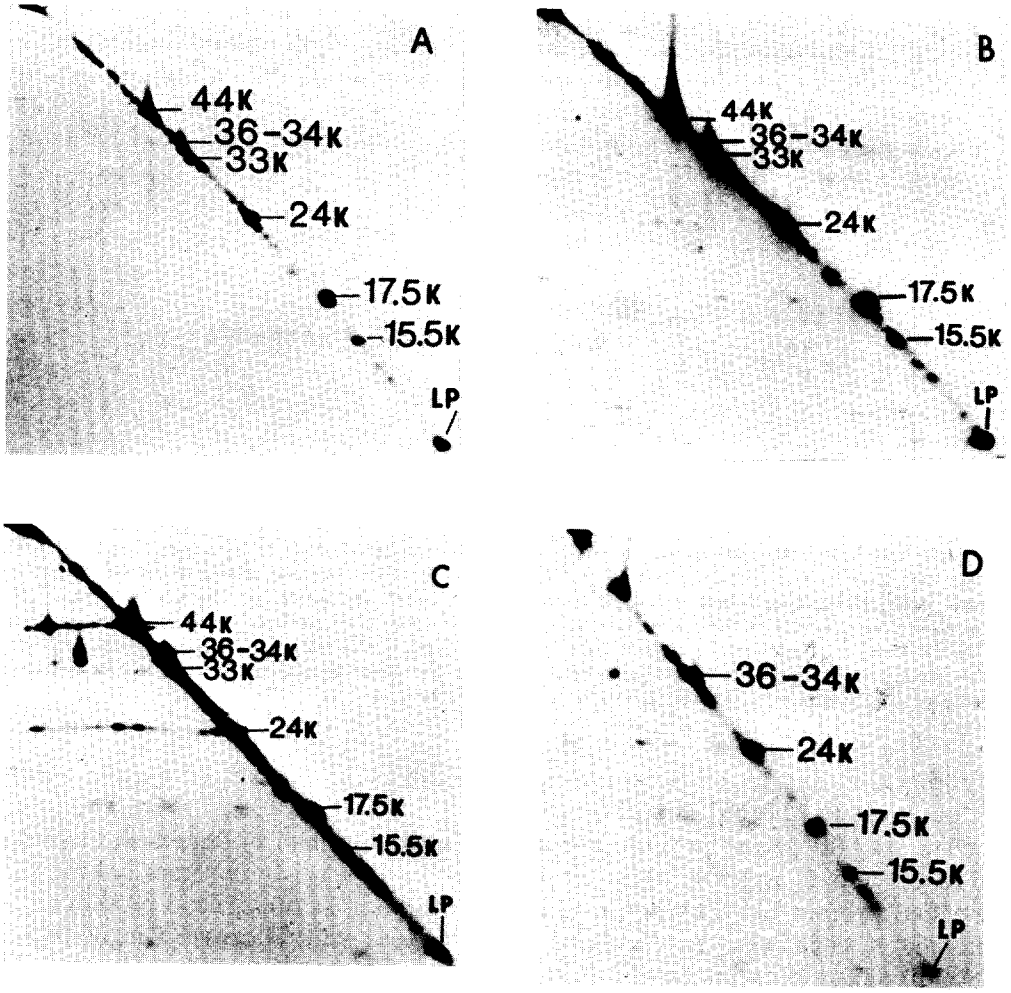

Fig. 2. Autoradiograms of two-dimensional sodium dodecyl sulfate polyacrylamide (14%) gel patterns from cell walls of *S. typhimurium*. The isolated cell walls were treated with tartryl-di-(glycylazide) for 30 min at room temperature before electrophoresis as follows. (A) Untreated control of strain SH6749 (wild type). (B) Strain SH6749 treated with 0.5 mM reagent. (C) Strain SH6749 treated with 5 mM reagent. (D) Strain SL1909 (lacking the 33 000 dalton protein) treated with 5 mM reagent. The cross-links were cleaved with 15 mM $NaIO_4$ for 15 min after electrophoresis in the first dimension. Cells in A—C were grown in M9 maltose, those in D in M9 glycerol. LP, lipoprotein.

(Fig. 2B), increasing the concentration led to the appearance of additional higher molecular weight complexes (Fig. 2C). 5 mM tartryl-di-(glycylazide) seemed to be optimal for cross-linking. Increasing the concentration further up to 20 mM although giving higher yields of complexes, did not result in appearance of any new off-diagonal spots and simultaneously resulted in decreased resolution of the spots (not shown).

Fig. 3 shows a schematic diagram of the two-dimensional gel pattern obtained after cross-linking the cell wall with 5 mM tartryl-di-(glycylazide). The number of components in a cross-linked complex can be estimated from the apparent molecular weights, although certain caution is necessary as indicated

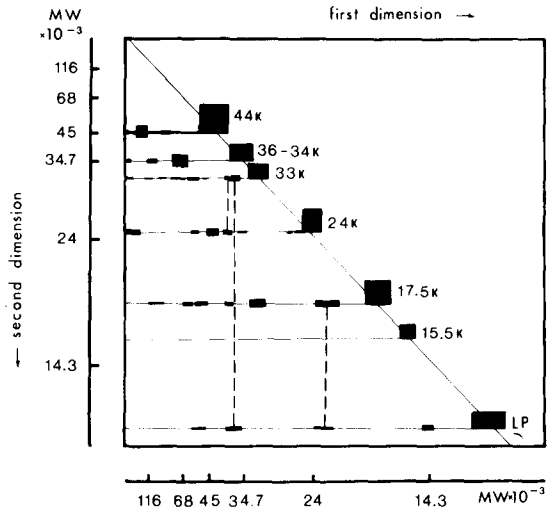

Fig. 3. A schematic diagram of the cross-linking pattern obtained with 5 mM tartryl-di-(glycylazide) from cell walls of the wild-type strain SH6749 analyzed by two-dimensional gels (as in Fig. 2c). Only those off-diagonal spots which could be reproducible detected are indicated. The width of each spot represents the range of molecular weights covered and the height is an estimate of the amount of material seen in the autoradiograms. For clarity only the upper halves of the spots on the diagonal are depicted. The vertical dotted lines connect those spots which are probable components of the same cross-linked complex. The standard proteins are as in Fig. 1. LP, lipoprotein.

by the aberrant mobilities of some cross-linked complexes of certain membrane proteins [10,15] (see also below).

The 44 000 dalton protein seems to form predominantly dimers and trimers (Fig. 2B and C and Fig. 3) as we have shown previously by cross-linking the purified protein [15]. No spots other than those of 44 000 dalton protein were missing if cells were not induced with maltose (not shown). The 36 000—34 000 dalton proteins cross-linked also to form dimers and trimers (Fig. 2B—D and Fig. 3) (see also below). Due to aberrant mobility often observed in the second dimension gels of the 36 000 and 34 000 dalton proteins these could not be separated in the two-dimensional gels. The 33 000 dalton protein was cross-linked to form numerous oligomeric complexes, the lowest of these probably representing a complex between 33 000 dalton protein and lipoprotein. The lipoprotein spot vertically aligned with the 33 000 dalton protein spot was missing in the 33 000 dalton protein-lacking mutant (Fig. 2D). One of the spots of 24 000 dalton protein was also missing in this mutant, thus it might represent a complex between 33 000 and 24 000 dalton proteins (Fig. 2D). The 24 000 dalton protein in addition formed numerous multimeric complexes but the exact composition of these is not clear. The 17 500 dalton protein was cross-linked to various oligomers. The one with the lowest molecular weight is vertically aligned with a spot from the lipoprotein, thus these two proteins seem to be cross-linked. The next multimer is probably a dimer of the 17 500 dalton protein, while the composition of the higher multimers cannot be unambiguously deduced from these results. As in *E. coli* [14] the 15 500 dalton protein was not cross-linked under the conditions used. The lipoprotein in addition to being cross-linked to 33 000 and 17 500 dalton proteins forms at least a dimer and a

higher multimer, probably a hexa-octamer (Figs. 2 and 3). Thus most of the major outer membrane proteins can be cross-linked to form multimeric species, and the 33 000 and 17 500 dalton proteins also to lipoprotein.

Similar results to those shown in Figs. 2 and 3 were obtained with the shorter reagent tartryl-diazide, although higher concentrations of reagent were necessary. The cross-linking with tartryl-di-(glycylazide) was also carried out with whole cells prior to cell wall isolation with similar results (not shown) indicating that the interactions seen are not due to artefacts in cell wall preparation. The digestion of the peptidoglycan with lysozyme prior to cross-linking did not result in changes in the cross-linking pattern, nor did the cross-linking of the cell wall in an ice bath (not shown), which suggests that the interactions are quite stable.

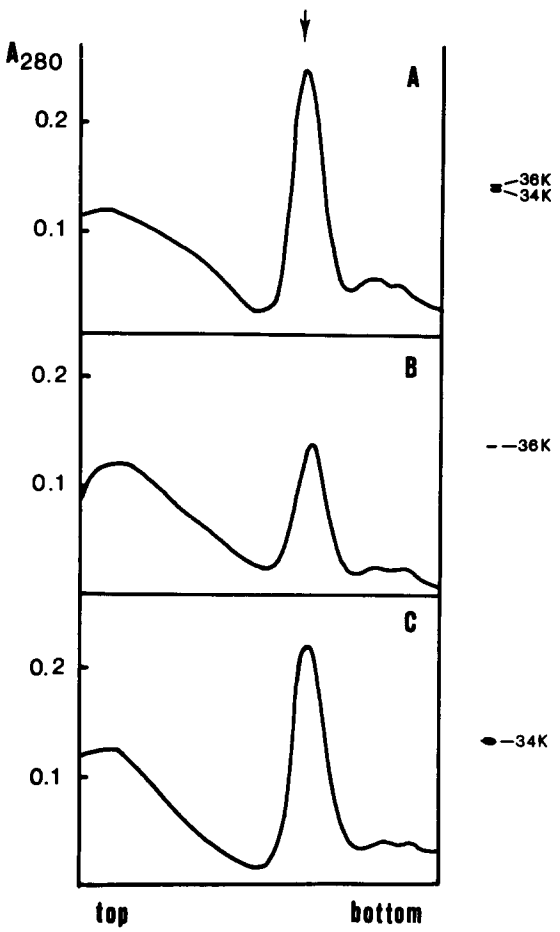

Fig. 4. Sedimentation of the 36 000 and 34 000 dalton proteins in a sucrose gradient. Cells were labeled with [^{35}S]methionine, protein-peptidoglycan complexes isolated, and the peptidoglycan associated proteins released with salt treatment. The released proteins were applied to sucrose gradients containing 0.1% dodecyl sulfate and the gradients centrifuged in Sorvall ODT-2 ultracentrifuge at 130 000 × *g* for 16 h. The gel patterns on the right of each gradient profile show the peak material analyzed on sodium dodecyl sulfate polyacrylamide (14%) gels. (A) Strain SH6749 (wild type). (B) Strain SH6761 (*ompD* mutant lacking the 34 000 dalton protein). (C) Strain SH6751 (*ompB* mutant lacking the 36 000 dalton protein). The arrow indicates the sedimentation of protein I trimers under identical conditions.

Arrangement of the 36 000 and 34 000 dalton proteins

Since the 36 000 and 34 000 dalton proteins were not well enough resolved in two-dimensional electrophoresis they were purified from wild type and mutant which had either one of each of these species. These proteins are easily purified by a scheme similar to that used for *E. coli* protein I [10] (see Materials and Methods). The only impurity seen in dodecyl sulfate gels after the gradient centrifugation is the 44 000 dalton protein which is present in minor amounts, even when not induced, and partially cosediments with the 36 000 and 34 000 dalton proteins. Fig. 4 shows the gradient profiles obtained from the salt-released proteins [39], and the 14% acrylamide dodecyl sulfate gel patterns of the peak material of the gradient. These proteins sediment at a rate similar to that of *E. coli* protein I trimers [10] (arrow in Fig. 4A), thus indicating a similar multimeric arrangement.

The purified proteins were cross-linked with tartryl-di-(glycylazide) and analyzed on 10% acrylamide dodecyl sulfate gels (Fig. 5). At low concentration of the reagent, dimers of both proteins were observed (Fig. 5, slots 4—6). The dimers of both 36 000 and 34 000 dalton proteins had similar mobilities.

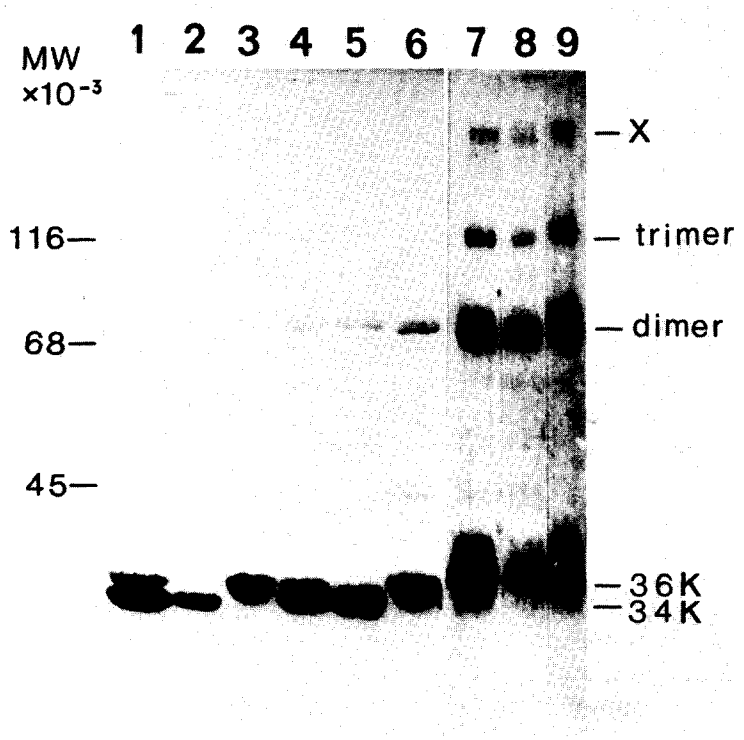

Fig. 5. Cross-linking of the 36 000 and 34 000 dalton proteins. The purified proteins from strains SH6749 (wild type; slots 1, 4 and 7), SH6751 (lacking the 36 000 dalton protein; slots 2, 5 and 8) and SH6761 (lacking the 34 000 dalton protein; slots 3, 6 and 9) were treated as follows before electrophoresis on sodium dodecyl sulfate polyacrylamide (10%) gels. The material in slots 1—3, untreated control; slots 4—6, treated with 1 mM tartryl-di-(glycylazide) for 30 min at room temperature; slots 7—9 treated with 10 mM tartryl-di-(glycylazide). The arrows on the left indicate the migration of the standard proteins as in Fig. 1.

Therefore it was impossible to deduce whether, in the wild type, the 36 000 and 34 000 dalton proteins were cross-linked only to themselves or if even hetero-complexes of 36 000 and 34 000 dalton proteins exist. With higher reagent concentrations both 36 000 and 34 000 dalton proteins were cross-linked to form trimers (Fig. 5, slots 7—9) and higher molecular weight complexes (X in Fig. 5). The higher molecular weight material is probably a form of trimers, which has an aberrant mobility in 10% gels. When the mobility of the cross-linked complexes was analyzed on gels of different acrylamide concentrations (from 3 to 10%), the monomers, dimers and trimers of both 36 000 and 34 000 dalton proteins had normal mobilities well within the range of standard proteins. However, the mobility of the higher molecular weight complex X was found to be the same as that of the trimers in 3 and 4% gels but aberrantly low in higher acrylamide concentrations (Fig. 6). Similar results have been previously obtained for *E. coli* protein I [10]. Thus these porins, 34 000 and 36 000 dalton proteins, appear to have similar trimeric arrangement to that of the pore-forming protein I in *E. coli* [10].

Cross-linking of outer membrane proteins to peptidoglycan

In *E. coli* major outer membrane proteins seem to be associated with peptido-

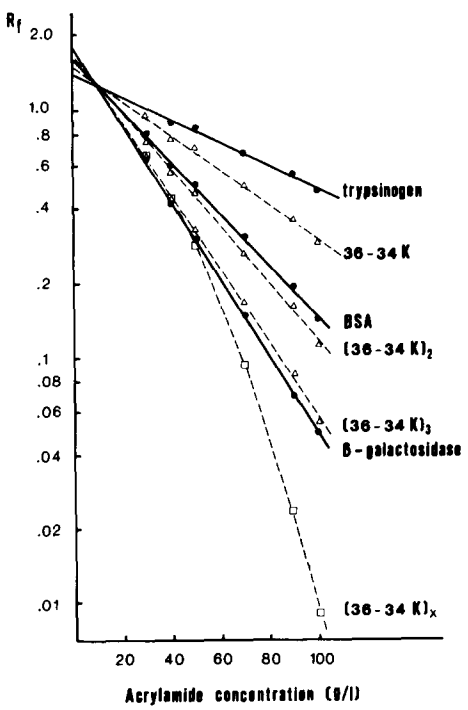

Fig. 6. Ferguson plot [43,44] of the cross-linked multimers of the 36 000 and 34 000 dalton proteins. The cross-linked proteins were subjected to electrophoresis in sodium dodecyl sulfate gels of different acrylamide concentrations (from 3 to 10%), using a continuous buffer system. The logarithm of the mobility of protein band relative to that of the tracking dye (bromophenol blue) was plotted against the acrylamide concentration. •, standard proteins; △, monomers, dimers and trimers of the 36 000 and 34 000 dalton proteins; □ complex X of the 36 000 and 34 000 dalton proteins. BSA, bovine serum albumin.

glycan: λ receptor and protein I can be isolated as protein-peptidoglycan complexes [8—12] and proteins II*, 17 500 dalton protein and the free form of lipoprotein can be cross-linked to peptidoglycan [14,17,27,45]. In *S. typhimurium* the 44 000, 36 000 and 34 000 dalton proteins can be isolated as peptidoglycan complexes [9,12,15].

The cross-linking of *S. typhimurium* proteins to peptidoglycan was studied by treating whole cells or isolated cell envelopes with tartryl-di-(glycylazide) and isolating the peptidoglycan layer. This peptidoglycan isolation involved an extraction with boiling dodecyl sulfate. Thus only proteins covalently attached stay with the peptidoglycan. The proteins cross-linked to peptidoglycan were analyzed after cleavage of the cross-links on 14% acrylamide dodecyl sulfate gels (Fig. 7). No proteins were found in control samples not cross-linked prior to peptidoglycan isolation. In the cross-linked preparations

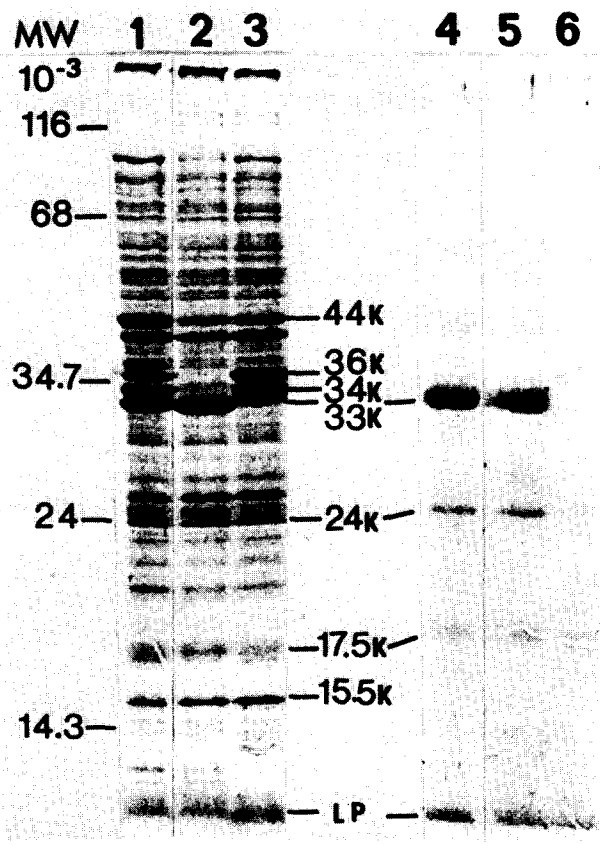

Fig. 7. Cross-linking of cell wall proteins to peptidoglycan. Isolated cell envelopes of M9 maltose-grown cells were cross-linked with 10 mM tartryl-di-(glycylazide) for 30 min at room temperature and the peptidoglycan fraction isolated. The cross-links were cleaved with 15 mM $NaIO_4$ for 10 min prior to the analysis on sodium dodecyl sulfate polyacrylamide (14%) gels. Slots 1—3: uncross-linked cell envelopes of strains SH6749 (wild type, slot 1), SH6896 (lacking the 36 000 and 34 000 dalton proteins, slot 2) and SL1909 (lacking the 33 000 dalton protein, slot 3). Slots 4—6: isolated and cleaved peptidoglycan fractions of cross-linked cell envelopes of strains SH6749 (slot 4), SH6896 (slot 5) and SL1909 (slot 6). LP, lipoprotein.

the 33 000, 24 000, 17 500 dalton proteins and free form of lipoprotein could be detected after cleavage (Fig. 7, slots 4–6). The 44 000, 36 000 or 34 000 dalton proteins were not cross-linked to peptidoglycan. Only in the 33 000 dalton protein lacking mutant small amounts of the 36 000 dalton protein were sometimes detected in cross-linked peptidoglycan preparations (not shown). The cross-linking of the 24 000 dalton protein was probably through the 33 000 dalton protein, since this band was missing in the mutant lacking the 33 000 dalton protein (Fig. 7, slot 6).

Discussion

The results presented above establish the presence of tight protein interactions in the *S. typhimurium* outer membrane. Most of the major outer membrane proteins were found in cross-linked complexes. The specificity of these interactions could be studied by using cleavable cross-linking reagents and symmetrical two-dimensional gel electrophoresis. Also a tentative composition of some of the cross-linked complexes could be deduced from the mobilities in gels, although this analysis is hampered by the clearly aberrant mobility of some of the cross-linked complexes [10,15]. The interactions observed by using cross-linking techniques should be considered as measures of the minimum interactions present in vivo, since the lack of cross-linking of two proteins does not necessarily mean the lack of an interaction. It could merely mean that the reactive groups are not in the right position. An example of this is that protein I in *E. coli* is not cross-linked to lipoprotein [14,17], but an interaction is known to exist between these proteins [45]. The pore-forming 44 000, 36 000 and 34 000 dalton proteins were cross-linked to form dimers and trimers, suggesting that they are arranged as trimers in the cell wall. The 36 000 and 34 000 dalton proteins were also cross-linked to form an aberrantly moving complex X (Fig. 6) similar to a complex seen previously when cross-linking *E. coli* protein I [12]. This is probably a form of trimer, which has a different conformation [12], rather than being caused by cross-linking of non-protein moieties. No preferential cross-linking of lipopolysaccharide to form complex X was observed. Actually very little lipopolysaccharide (less than 1%) could be cross-linked to the 36 000 and 34 000 dalton proteins (Palva, E.T., unpublished results), although lipopolysaccharide probably interacts with these proteins in vivo as shown with the analogous *E. coli* proteins Ia and Ib [47,48].

Further evidence of the trimeric arrangement of the 36 000 and 34 000 dalton proteins comes from the sedimentation data (Fig. 4) which shows that the purified proteins sediment as multimeric species, at the same rate as protein I trimers of *E. coli* [10]. Purified 36 000 and 34 000 dalton proteins could be cross-linked to form up to trimers. Also the 35 000 dalton minor protein has a similar sedimentation rate as the 36 000 and 34 000 dalton proteins (not shown) suggesting a similar arrangement. Recently it has been also shown by sedimentation analyses of *E. coli* protein I and *S. typhimurium* 36 000 and 34 000 dalton proteins (Nakae, T., personal communication) and by gel filtration of *E. coli* protein Ib [46] that the unheated proteins exist as trimers in dodecyl sulfate solutions. Thus it appears that all the peptidoglycan-associated porins studied so far in *E. coli* (protein I [10], λ receptor [15] and protein 2

[26]) and in *S. typhimurium* (the 44 000 dalton protein [15] and the 36 000–34 000 dalton proteins) have similar trimeric arrangements. This might be a general feature of outer membrane porins.

An interaction was established between the 33 000 dalton protein and lipoprotein. The 33 000 dalton protein and lipoprotein interaction was further verified by using a 33 000 dalton protein-lacking mutant. The corresponding lipoprotein spot was also missing in the mutant (Fig. 2D). The mutant study also suggested an interaction between the 33 000 and 24 000 dalton proteins. All these proteins formed multimeric complexes in addition to heterocomplexes. Dimers were seen of both lipoprotein and the 17 500 dalton protein. The exact composition of the higher multimers of these proteins is harder to establish, due to the possibility of aberrant mobility of these complexes as a result of high levels of internal cross-linking and/or cross-linking to non-protein moieties such as phospholipids and lipopolysaccharides, as discussed previously [10,14]. However, in the case of proteins I, II* and lipoprotein in *E. coli* it has been shown that many of cross-links formed do not involve phospholipids or lipopolysaccharide [45].

The results presented (Fig. 7) also suggest an interaction of 33 000, 24 000 and 17 500 dalton proteins and the free lipoprotein with the peptidoglycan. The interaction of the 24 000 dalton protein was probably through the 33 000 dalton protein since the 24 000 dalton protein was missing in the 33 000 dalton protein-lacking mutant. The directness of the interaction of the other proteins with peptidoglycan could not be established unambiguously due to the lack of appropriate mutants in *S. typhimurium*. At least the 33 000 dalton protein is not needed for the association of the 17 500 dalton protein and the lipoprotein with peptidoglycan. These results and the results obtained previously for the peptidoglycan association of the 44 000 [12,15], 36 000 and 34 000 dalton proteins [9] suggest that most of the major outer membrane proteins are associated with the peptidoglycan layer of the cell wall.

These interactions observed in the *S. typhimurium* outer membrane proteins are remarkably similar to those seen previously with *E. coli*. The results also suggest an analogy of the 24 000, 17 500 and 15 500 dalton proteins in these species; these proteins have similar molecular weights and can form similar cross-linked complexes. Thus all major outer membrane proteins seem to have counterparts in both species, and form similar cross-linked complexes. This suggests that the organization of the cell wall is very similar in these bacteria with strong protein-protein and protein-peptidoglycan interactions.

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