INFECTION AND IMMUNITY, Mar. 1994, p. 974–979
0019-9567/94/$04.00+0
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Vol. 62, No. 3

Spatial Arrangement of Gene Products of the P1 Operon in the Membrane of *Mycoplasma pneumoniae*

G. LAYH-SCHMITT* AND R. HERRMANN

ZMBH, Mikrobiologie, Universität Heidelberg, 69120 Heidelberg, Federal Republic of Germany

Received 24 September 1993/Returned for modification 26 October 1993/Accepted 13 December 1993

The spatial arrangement of three P1 operon-encoded proteins—attachment protein P1 (ORF5 gene product) and the ORF6-derived proteins of 40 and 90 kDa—in the membrane of *Mycoplasma pneumoniae* M129 was investigated by nearest-neighbor analysis. For these studies, the homobivalent, thiol-cleavable, and non-membrane-permeating cross-linking reagent 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was used. The cross-linked proteins were isolated by immunoprecipitation with antibodies directed against fusion proteins of selected regions of the 40-kDa, the 90-kDa, or the P1 protein. The individual components of the immunoprecipitated protein complexes were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography, and immunoblot analysis. This study showed that the P1 protein, the ORF6 gene product, and an unidentified 30-kDa protein were linked to each other in the intact bacterial membrane by the reagent DTSSP, indicating that these proteins are located at a maximal distance of 12 Å (1 Å = 0.1 nm) on the tip structure of *M. pneumoniae*.

Mycoplasma pneumoniae is a wall-less, extracellular pathogen of the human respiratory tract (12, 28). Since this organism lacks a cell wall or other cell appendages, the macromolecules which are directly involved in adherence of the bacterium to the host cell must be located in the membrane. The interaction between surface components of this bacterium and the host is a prerequisite for virulence and pathogenicity, because there is a strong correlation between the cytadherence and virulence of *M. pneumoniae* (2, 13, 17, 18). It is believed that the adhesion of *M. pneumoniae* is mediated by the P1 protein, an integral membrane protein partially exposed on the surface and located on the tip-like attachment organelle of *M. pneumoniae* (3, 6, 11). The tip structure is stabilized by internal high-molecular-weight proteins (HMW1 to HMW5) which form a cytoskeleton-like structure, as shown by electron microscopy studies (16, 26, 32). A second adhesin-related protein, of 30 kDa, was described by Baseman et al. (4) and Dallo et al. (5). The P1 gene is part of the P1 operon, consisting of three open reading frames, which are ordered tandemly ORF4-ORF5 (P1 gene)-ORF6 (14, 15, 34). ORF6 has the coding capacity for a protein of 130 kDa. However, Sperker et al. (30) identified two proteins, of 90 and 40 kDa, as the gene product of this open reading frame. These two proteins might be the result of cotranslational cleavage, because a precursor protein of 130 kDa was never found. These proteins are not expressed by avirulent mutant *M. pneumoniae* M129-B176 (30). Hu et al. (13) found that monoclonal antibodies recognize 35- and 85-kDa proteins in wild-type *M. pneumoniae* M129 but failed to detect these proteins in avirulent mutant M129-B176. Consistent with these data were the findings of Franzoso et al. (7) for reconvalescent-phase sera of *M. pneumoniae*-infected chimpanzees. Hansen et al. (8) used two-dimensional polyacrylamide gel electrophoresis (PAGE) to compare protein profiles of virulent (broth passage B10) and avirulent (broth passage B181) *M. pneumoniae* M129. The avirulent strain lacked three proteins, A, B, and C, of 72, 85, and 37 kDa, respectively. The 85-kDa protein was accessible to trypsin and iodination, like the 90-kDa protein of the ORF6 gene product, as described previously (22, 23). All these results indicate that the two proteins with molecular masses of 85 and 35 kDa (37 kDa) are identical to the ORF6-derived 40- and 90-kDa proteins (23, 30). Moreover, Yayoshi et al. (37) suggested a correlation between the development of antibodies to an 85-kDa protein of *M. pneumoniae* in vaccinated animals and the effects of protection by live vaccines. We believe that this 85-kDa protein is identical to the ORF6-encoded 90-kDa protein. To confirm that the 90-kDa protein is an ORF6 gene product, 11 amino acids of the N-terminal region were sequenced and found to correspond to the amino acids derived from the DNA sequence between positions 455 and 465 of ORF6 (23). Thus, the proposed cotranslational cleavage must occur N terminal to amino acid position 455. Recently, we showed that the two proteins of 40 and 90 kDa (ORF6 gene product) are membrane proteins with surface-exposed regions (22, 23). Franzoso et al. confirmed these results by localizing the ORF6 gene product to the cell membrane (7).

Mutants that express the P1 protein or the adhesin-related 30-kDa protein but lack either the ORF6 gene product or cytoskeletal proteins HMW1 to HMW5 are avirulent (13, 17, 18, 30). This fact indicates that the attachment of *M. pneumoniae* to host cells is a multifunctional process involving a number of proteins. The localization in the cell and the biochemical and functional features of membrane proteins such as the P1 protein, the 30-kDa protein, and the ORF6 gene product have already been studied (3–7, 11, 22, 23, 30). However, the organization of these proteins within the intact *M. pneumoniae* membrane has not been studied so far. Nearest-neighbor analysis with chemical cross-linking reagents is one method for probing the spatial arrangement of membrane proteins (25, 31). Such studies were carried out to characterize the spatial distances of surface-exposed regions of the ORF6 gene product and the P1 protein by use of the thiol-cleavable, non-membrane-permeating cross-linking reagent 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP).

* Corresponding author. Mailing address: ZMBH, Mikrobiologie, Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Federal Republic of Germany. Phone: 49-6221-566828. Fax: 49-6221-565893.

MATERIALS AND METHODS

Growth of *M. pneumoniae*. *M. pneumoniae* M129 (ATCC 29342) (broth passage B21) and avirulent strain M129-B176 (ATCC 29343) were grown in modified Hayflick medium at 37°C for 48 h (9). Strain M129 was cultured in 137-cm^2 Roux flasks containing 100 ml of medium. The adherent mycoplasmal colonies were washed three times with phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate [pH 7.4]); the cells were scraped off into PBS and processed for the cross-linking procedures. Avirulent strain M129-B176 was grown in 500 ml of Hayflick medium in 1,000-ml glass bottles with gentle shaking for 48 h at 37°C. The cells were harvested by centrifugation at 8,000 × *g*, washed three times with PBS, and prepared for cross-linking experiments.

In vivo ^{35}S labelling of *M. pneumoniae*. For in vivo L-[^{35}S]methionine-cysteine labelling, *M. pneumoniae* M129 was grown in 10 Roux bottles, each containing 100 ml of Hayflick medium. Strain M129-B176 was cultured in two-liter glass bottles, each containing 500 ml of Hayflick medium. To 100 ml of medium, 300 μCi of L-[^{35}S]methionine-cysteine (L-^{35}S-protein labelling mix; specific activity, 11 mCi/ml; NEN) was added. The cells were cultured, washed, and harvested as described above.

Cloning, expression, and purification of FPs. Selected DNA regions of ORF6 of the P1 operon were cloned in expression vectors, and fusion proteins (FPs) 130 k-1 and 130 k-5 were expressed in *Escherichia coli* 537 as described previously (30, 36). The FPs were enriched as described by Küpper et al. and Strebel et al. (19, 20, 33) and then electroeluted from a preparative 10% polyacrylamide gel as described earlier (23). A *Sau*3A DNA fragment of the P1 gene (nucleotide positions 5023 to 5335 according to Inamine et al. [14]) was cloned in expression vector pDS 15. This plasmid links the *M. pneumoniae* DNA to the modified mouse dihydrofolate reductase (DHFR) gene. This DHFR gene encodes at the N terminus six histidine residues, which permit rapid and efficient one-step purification of the FPs by metal chelate affinity chromatography by taking advantage of the affinity of histidine for divalent nickel ions, as described by Hochuli et al. (10) and Proft and Herrmann (27). For cloning of a selected DNA region of the gene encoding the adhesin-related 30-kDa protein, the 9.2-kb *Eco*RI fragment of cosmid pcosMPH8 (36) was cleaved with restriction endonuclease *Hae*II (Boehringer Mannheim GmbH) into several subfragments. The *Hae*II fragment including nucleotide positions 68 to 543 (according to Dallo et al. [5]) was further digested with restriction enzyme *Hha*I (Boehringer). Protruding ends of the DNA fragments were removed with S1 nuclease (Boehringer). One of the two resulting fragments (nucleotide positions 306 to 543) was cloned in expression vector pDS 13 and fused in frame to the modified mouse DHFR gene. After ligation, competent cells of *E. coli* M15 were transformed. To identify recombinant plasmids, *E. coli* colonies were transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Federal Republic of Germany [FRG]) and hybridized with an appropriate ^{32}P-5′-end-labelled oligonucleotide. The radioactive labelling was carried out as described by Maxam and Gilbert (24). Plasmids from positive clones were isolated and sequenced by the method of Sanger et al. (29) with a Sequenase sequencing kit from US Biochemical Corp. according to the manufacturer's instructions. The expression of the FPs was induced with 2 mM isopropyl-β-thiogalactopyranoside (IPTG) (Serva, Heidelberg, FRG). The FPs were purified with Ni^{2+} NTA-Sepharose 6B (Dianova, Hamburg, FRG) columns as described by Hochuli et al. (10) and Proft and Herrmann (27). Originally, we intended to clone the complete gene for the adhesin-related 30-kDa protein. Although we were able to select transformed *E. coli* colonies containing recombinant plasmids with the gene for the 30-kDa protein, we were unable to express the whole protein in *E. coli* M15 (UGA suppressor strain).

Preparation of antisera. Antisera against FPs from regions of the 40- and 90-kDa proteins (ORF6 gene product), the P1 protein, and the 30-kDa protein were prepared by use of rabbits as described previously (23).

Cross-linking with DTSSP. DTSSP is a non-membrane-permeating cross-linker containing two imidoester groups, which can react with primary amine moieties and with epsilon amine groups on lysine or N-terminal amines, which results in covalent bonds. The distance between these groups is approximately 12 Å (1 Å = 0.1 nm). An internal disulfide bond can be cleaved by reduction with β-mercaptoethanol. The cross-linking procedure was basically done by the method of Newhall et al. (25). For all cross-linking experiments, *M. pneumoniae* M129 and M129-B176 were grown and radiolabelled as described above. Freshly harvested cells were washed once with TEA buffer (50 mM triethanolamine [Roth, Karlsruhe, FRG], 5 mM $MgCl_2$, 100 mM KCl [pH 8.5]) and resuspended in the same buffer to yield 50 mg of cells (wet weight) per ml. To this suspension, the desired concentration of DTSSP (Pierce, Rockford, Ill.) was added. To determine the appropriate concentration of DTSSP, increasing amounts of the cross-linking reagent (0.3, 0.6, 0.75, 1.25, 2.5, 3, 5, and 7.5 mM) were added to 50 mg of cells per ml. After incubation at 23°C for 30 min with gentle mixing, the reaction was quenched for 15 min by the addition of 50 mM Tris-HCl (pH 7.5) (final concentration). The pellets were washed twice with PBS containing 50 mM Tris-HCl (pH 7.5). After centrifugation at 8,000 × *g*, the cell pellets were frozen at −70°C or immediately processed for sodium dodecyl sulfate (SDS)-PAGE, immunoblotting, and immunoprecipitation. To ensure that the cells remained intact during cross-linking, the protein or DNA content of the supernatants of the pelleted bacteria was measured at 260 and 280 nm.

Immunoprecipitation of cross-linked protein complexes. Immunoprecipitation was performed by a modification of the methods described by Andersen and Blobel (1) and Layh-Schmitt and Herrmann (23). Pellets of DTSSP-treated cells (50 mg [wet weight]) were lysed in 200 μl of lysis buffer (2% SDS, 25 mM NaCl, 25 mM Tris-HCl [pH 7.2], 10 U of aprotinin [Sigma, Munich, FRG] per ml), heated to 100°C for 5 min, and diluted in 800 μl of dilution buffer (2.5% Triton X-100 [Pierce], 190 mM NaCl, 60 mM Tris-HCl, 6 mM EDTA [pH 7.4], 10 U of aprotinin per ml). To 1-ml lysates, samples of 100 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) to which antibodies against the 90-kDa, the 40-kDa, or the P1 protein had been covalently bound as described earlier (23) were added. Immunoprecipitation was done overnight at 4°C with thorough mixing. The protein A matrix with the bound antigen-antibody complexes was washed four times with 1,000 μl of buffer I (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA) and two times with buffer II (buffer I without detergents). The antigens were released from the antibody matrix by the addition of 30 μl of 2× Laemmli buffer without β-mercaptoethanol (21) and incubation of the suspension for 5 min at 37°C. After removal of the solid matrix by centrifugation, portions of the supernatants containing the antigens were reduced with 50 mM β-mercaptoethanol and compared with untreated samples by SDS-PAGE, autoradiography, and immunoblot analysis.

SDS-PAGE and autoradiography. The protein profiles of lysates of DTSSP-treated or untreated *M. pneumoniae* cells as well as reduced and unreduced immunoprecipitates of cross-

linked protein complexes were separated by SDS-PAGE with 12% gels as described by Laemmli (21). The high-molecular-weight protein complexes obtained after cross-linking with DTSSP were further analyzed in SDS–5% polyacrylamide gels. For autoradiography, the gels were incubated in Amplify (Amersham) at room temperature for 30 min with gentle shaking. The gels were then washed for 1 h in distilled water, dried, and exposed for 5 to 8 days to Kodak X-Omat AR diagnostic films (Kodak, Rochester, N.Y.).

Immunoblot analysis. Western blotting (immunoblotting) was done by the method of Towbin et al. (35). Proteins were separated by SDS-PAGE as described above and electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell) at 5 mA/cm^2 for 20 min by use of a semidry blot apparatus (Biometra, Göttingen, FRG). The transfer buffer consisted of 25 mM Tris–150 mM glycine–10% methanol (pH 8.3). After the nitrocellulose sheets were blocked with 5% skim milk in PBS, they were incubated with a 1:2,000 dilution of antisera (to the 40-kDa, 90-kDa, and P1 proteins) in buffer 1 (150 mM NaCl, 100 mM Tris-HCl [pH 7.5], 0.05% Nonidet P-40 [Sigma]) for 1 h at 37°C. Thereafter, the blots were rinsed twice with buffer 1 for 10 min each time and incubated with alkaline phosphatase-conjugated rabbit immunoglobulin G (heavy and light chains) (Dianova) diluted 1:7,500 in buffer 1 for 1 h at 37°C. The blots were washed as described above and developed by the addition of 70 μg of nitroblue tetrazolium chloride (Biomol, Hamburg, FRG) per ml and 35 μg of 5-bromo-4-chloro-3-indolylphosphate sodium (Biomol) per ml in a buffer consisting of 100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris-HCl (pH 9.5).

RESULTS

Identification of *M. pneumoniae* proteins with anti-FP antisera. To obtain specific antisera for the detection of the adhesin-related 30-kDa protein, the 40-kDa protein, the 90-kDa protein, and the P1 protein, we immunized rabbits with FPs which were constructed, expressed, and purified as described in Materials and Methods. Using *M. pneumoniae* lysates for immunoblot analysis, we showed that rabbit antisera against the FPs 130 k-1 (N-terminal part of ORF6) and 130 k-5 (C-terminal part of ORF6) recognized a 40-kDa protein and a 90-kDa protein, respectively (30). Rabbit antisera raised against the DHFR FP of the adhesin-related 30-kDa protein (amino acid positions 102 to 181) detected a 30-kDa protein, and antisera raised against the P1 FP were reactive with a 170-kDa protein.

Cross-linking with DTSSP. To determine optimal reaction conditions, freshly harvested *M. pneumoniae* cells (50 mg [wet weight] per ml) were treated with various concentrations of DTSSP. Immunoblot analysis revealed that all three monomeric protein bands, the 40- and 90-kDa proteins and the P1 protein, disappeared and that high-molecular-weight protein complexes appeared when concentrations of between 3 and 7.5 mM DTSSP were used for cross-linking experiments. Concentrations of DTSSP of between 0.3 and 2 mM were not sufficient for effective cross-linking of the 40-kDa, the 90-kDa, and the P1 proteins. For all further experiments, 4 mM DTSSP per 50 mg of cells (wet weight) per ml was used (Fig. 1). The cells remained intact during incubation with the cross-linking reagent DTSSP, since neither protein nor DNA was detected in the supernatants of DTSSP-treated cell pellets. Since all three proteins, the ORF6-derived proteins and the P1 protein, were cross-linked in intact cells and shifted in high-molecular-weight complexes, efforts were made to analyze the individual components of those protein complexes.

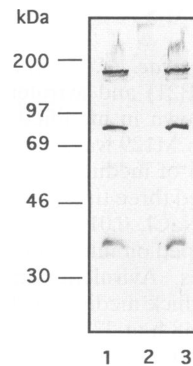

FIG. 1. Immunoblot analysis of *M. pneumoniae* lysates with antisera against the 40-kDa, the 90-kDa, and the P1 proteins. Lanes: 1, lysate of untreated *M. pneumoniae*; 2, lysate of cells cross-linked with 4 mM DTSSP; 3, reduced lysate of cells reacted with 4 mM DTSSP.

SDS-PAGE and autoradiography of immunoprecipitated cross-linked proteins. The cross-linked protein complexes of *M. pneumoniae* M129 were isolated from lysates of DTSSP-treated cells by immunoprecipitation with protein A-Sepharose-coupled antibodies against the 40-kDa, the 90-kDa, and the P1 proteins. The three immunoprecipitated complexes were dissociated into their protein components by cleavage of the disulfide bonds of the cross-linking reagent with β-mercaptoethanol. Thereafter, the protein components were resolved in SDS–12% polyacrylamide gels. Coomassie blue or silver staining of the gels revealed that the cross-linked protein complexes were composed of four proteins with molecular masses of 170, 90, 40, and 30 kDa. When in vivo ^{35}S-radiolabelled *M. pneumoniae* M129 cells were used for DTSSP treatment, three proteins with molecular masses of 170, 90, and 30 kDa were detected as components of all three immunoprecipitated protein complexes (Fig. 2). The 40-kDa protein could not be unambiguously detected, most likely because it contains only two methionines, which might not be sufficient for efficient in vivo radiolabelling, while the 90-kDa and P1 proteins contain five and seven methionines, respectively. In *M. pneumoniae* M129-B176, which lacks the ORF6 gene product, the 170-kDa protein (P1 protein) is cross-linked to a 30-kDa protein (Fig. 3). Additional experiments are required to prove whether the 30-kDa proteins found in the cross-linked protein complexes of the immunoprecipitates of *M. pneumoniae* M129 and M129-B176 are identical.

Immunoblot analysis of immunoprecipitated cross-linked proteins. The cross-linked protein complexes were immunoprecipitated with antisera against the P1, 40-kDa, or 90-kDa protein. The disulfide bonds of the DTSSP-cross-linked, immunoprecipitated protein complexes were reduced and analyzed by SDS-PAGE and immunoblot analysis. In all three immunoprecipitated protein complexes, cross-linked proteins with molecular masses of 40 and 90 kDa could be identified as the ORF6 gene product by immunoblot analysis. Antiserum against the P1 protein reacted with the 170-kDa protein in the protein complexes of all three immunoprecipitates (Fig. 4A to C). However, we were unable to identify the nature of the 30-kDa protein, which is one component of the cross-linked proteins. This protein was not reactive with antiserum against an FP containing a region of the adhesin-related 30-kDa protein (amino acids 102 to 181) (Fig. 5). Furthermore,

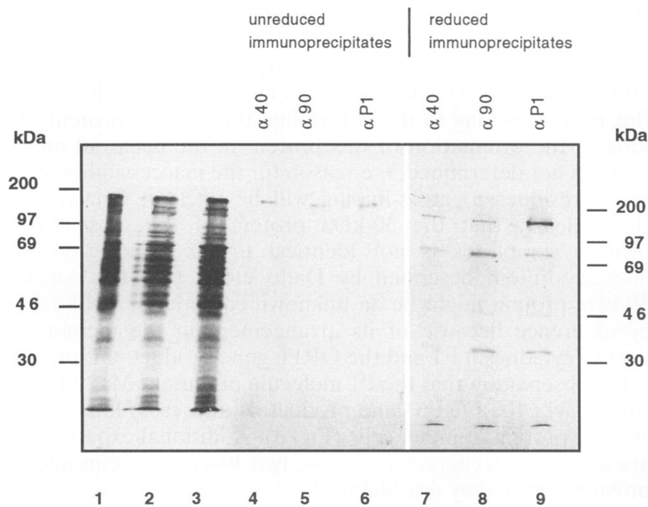

FIG. 2. Autoradiography of an SDS-polyacrylamide gel in which proteins of in vivo ^{35}S-radiolabelled *M. pneumoniae* M129 were separated. Lanes: 1, protein profile of DTSSP-cross-linked bacteria; 2, protein profile of reduced lysate of DTSSP-cross-linked bacteria; 3, protein profile of reduced lysate of untreated bacteria; 4 to 9, unreduced (lanes 4, 5, and 6) and reduced (lanes 7, 8, and 9) immunoprecipitates from lysates of DTSSP-cross-linked cells tested with antibodies against the 40-kDa protein (lanes 4 and 7), the 90-kDa protein (lanes 5 and 8), and the P1 protein (lanes 6 and 9) coupled to protein A-Sepharose.

immunoblot analysis of lysates of cross-linked *Mycoplasma* cells revealed that the adhesin-related 30-kDa protein is not cross-linked by DTSSP to other proteins or to itself because there is no detectable shift of its molecular mass (Fig. 5). The immunoprecipitated cross-linked protein complex which was isolated by use of antiserum against the P1 protein and separated in an SDS–5% polyacrylamide gel could be detected as a diffuse band of about 300 kDa by immunoblot analysis.

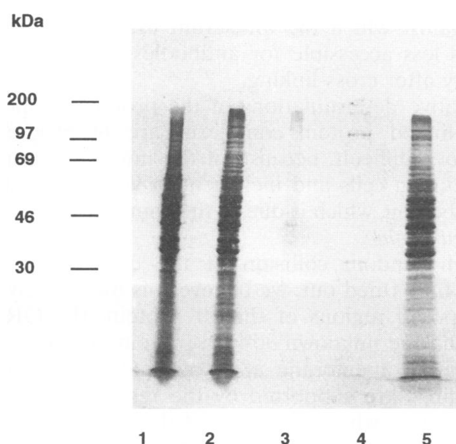

FIG. 3. Autoradiography of an SDS-polyacrylamide gel in which proteins of in vivo ^{35}S-radiolabelled *M. pneumoniae* M129-B176 were separated. Lanes: 1, protein profile of DTSSP-cross-linked bacteria; 2, protein profile of lysate of untreated bacteria; 3 and 4, unreduced (lane 3) and reduced (lane 4) immunoprecipitates from lysates of DTSSP-cross-linked cells tested with antibodies against the P1 protein coupled to protein A-Sepharose; 5, protein profile of reduced lysate of DTSSP-cross-linked bacteria.

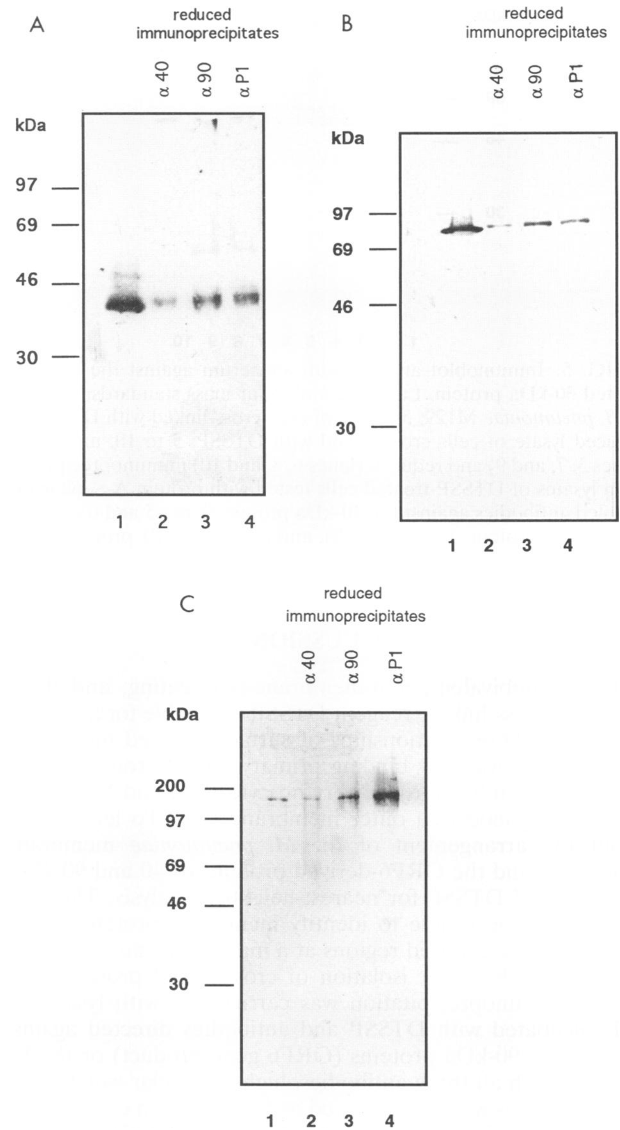

FIG. 4. (A) Immunoblot analysis with antiserum against the 40-kDa protein. Lanes: 1, lysate of *M. pneumoniae*; 2 to 4, reduced immunoprecipitates from lysates of DTSSP-cross-linked cells tested with protein A-Sepharose-coupled antibodies against the 40-kDa protein (lane 2), against the 90-kDa protein (lane 3), and against the P1 protein (lane 4). (B) Immunoblot analysis with antiserum against the 90-kDa protein. Lanes: 1, lysate of *M. pneumoniae*; 2 to 4, reduced immunoprecipitates from lysates of DTSSP-cross-linked cells tested with protein A-Sepharose-coupled antibodies against the 40-kDa protein (lane 2), against the 90-kDa protein (lane 3), and against the P1 protein (lane 4). (C) Immunoblot analysis with antiserum against the P1 protein. Lanes: 1, lysate of *M. pneumoniae*; 2 to 4, reduced immunoprecipitates from lysates of DTSSP-cross-linked cells tested with protein A-Sepharose-coupled antibodies against the 40-kDa protein (lane 2), against the 90-kDa protein (lane 3), and against the P1 protein (lane 4).

This protein complex was reactive not only with antiserum against the P1 protein but also with antisera against the 90- and 40-kDa proteins (ORF6 gene product) (Fig. 6), indicating that after cross-linking, one protein complex which contains at least those three proteins is formed.

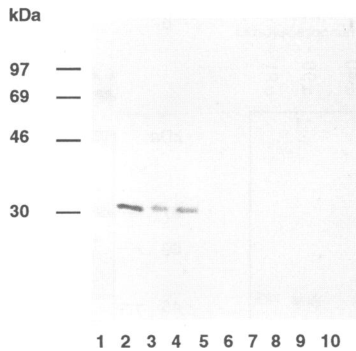

FIG. 5. Immunoblot analysis with antiserum against the adhesin-related 30-kDa protein. Lanes: 1, molecular mass standards; 2, lysate of *M. pneumoniae* M129; 3, lysate of cells cross-linked with DTSSP; 4, reduced lysate of cells cross-linked with DTSSP; 5 to 10, unreduced (lanes 5, 7, and 9) and reduced (lanes 6, 8, and 10) immunoprecipitates from lysates of DTSSP-treated cells tested with protein A-Sepharose-coupled antibodies against the 40-kDa protein (lanes 5 and 6), against the 90-kDa protein (lanes 7 and 8), and against the P1 protein (lanes 9 and 10).

DISCUSSION

The homobivalent, non-membrane-permeating, and thiol-cleavable cross-linking reagent DTSSP is suitable for analyzing nearest-neighbor relationships of surface-exposed membrane proteins by covalently binding primary amine groups of proteins, as shown by Staros for erythrocytes (31) and Newhall et al. for the gonococcal outer membrane (25). To learn more about the arrangement of the *M. pneumoniae* membrane protein P1 and the ORF6-derived proteins of 40 and 90 kDa, we also used DTSSP for nearest-neighbor analysis. Thus, we should have been able to identify membrane proteins which exhibit surface-exposed regions at a maximal distance of 12 Å from each other. For isolation of cross-linked protein complexes, immunoprecipitation was carried out with lysates of cells incubated with DTSSP and antibodies directed against the 40- and 90-kDa proteins (ORF6 gene product) or the P1 protein. With all three antibodies, high-molecular-weight protein complexes were precipitated and consisted in each case of the same set of proteins: 170-, 90-, 40-, and 30-kDa proteins (Fig. 2). By immunoblot analysis, we identified the 170-kDa protein as cytadherence protein P1 and the 90- and 40-kDa proteins as the ORF6 gene product (Fig. 4A to C). Immunoblot analysis of lysates of cross-linked bacteria and a whole *M. pneumoniae* extract revealed that the adhesin-related 30-kDa protein is neither cross-linked with DTSSP (although it contains nine lysine residues to which DTSSP could bind) nor detected in the complexes with antiserum raised against the 30-kDa FP (Fig. 5). Interestingly, eight of the nine lysines in this protein belong to the N-terminal third of the protein. As long as the orientation of this protein in the bacterial membrane is not determined, the reason for the inaccessibility of its lysine residues to cross-linking will be unclear. Our results show clearly that the 30-kDa protein of the cross-linked protein complexes is not identical to the adhesin-related 30-kDa protein described by Dallo et al. (5). The former 30-kDa protein might be an unknown component involved in cytadherence because of its arrangement in the membrane next to cytadhesin P1 and the ORF6 gene product. Of interest is the observation that the P1 molecule of mutant M129-B176, which lacks the ORF6 gene product, is also cross-linked to a 30-kDa protein in intact cells (Fig. 3). Additional experiments are necessary to characterize those two 30-kDa proteins and to prove whether they are identical.

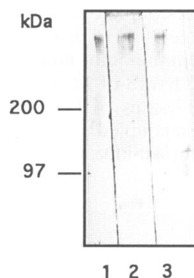

FIG. 6. Immunoblot analysis of the cross-linked protein complex isolated by immunoprecipitation with antiserum against the P1 protein. The cross-linked proteins were reactive with antisera against the P1 protein (lane 2) and against the 90- and 40-kDa proteins (lane 3). Lane 1, molecular mass standards (Rainbow marker).

So far, we do not know how the individual proteins (the P1 protein, the ORF6 gene product, and the 30-kDa protein) are cross-linked to each other. The proteins of the cross-linked complexes might have been linked directly to each other or indirectly via one or two individual nearest neighbors. As already mentioned, the components of each immunoprecipitated cross-linked protein complex were identical, no matter which antisera were used for immunoprecipitation. However, the amounts of the individual proteins in the cross-linked protein complexes varied, depending on the antisera used for immunoprecipitation. Higher amounts of the P1 protein were immunoprecipitated with antisera against P1 than with antisera against the 90- and 40-kDa proteins; this was also the case when an aliquot of the same lysate of DTSSP-treated cells was used. This result indicates that the P1 molecule is cross-linked not only to the ORF6 gene product and a 30-kDa protein but also to itself. Immunoprecipitation of monomeric P1 protein can be ruled out, because after cross-linking, monomeric P1 was undetectable by immunoblot analysis (Fig. 1). The anti-40-kDa protein antiserum did not prove as effective as the antiserum directed against the 90-kDa or P1 protein when used for immunoprecipitation of cross-linked protein complexes. The reason for this might be that the region of the 40-kDa protein against which the antiserum used in this study was directed is less accessible for antibodies or exhibits reduced antigenicity after cross-linking.

Quantitative determinations of the protein components in the cross-linked protein complexes are in progress. Such studies prove difficult, because of the minute amounts of all four proteins in cells and inefficient in vivo [^{35}S]methionine-cysteine labelling, which is due to the complex culture medium for *M. pneumoniae.*

Although random collision of the cross-linked proteins cannot be fully ruled out, we believe it is more likely that the surface-exposed regions of the P1 protein, the ORF6 gene product, and the unknown 30-kDa protein are located on the *M. pneumoniae* membrane at a maximal distance of 12 Å. These findings are supported by the results of immunogold labelling studies, which showed that the P1 protein (3, 6, 11) and the 90-kDa protein (7) are located on the tip structure of *M. pneumoniae.*

There is evidence that the P1 protein requires both the 90-kDa protein and the 40-kDa protein for proper functioning, since a mutant (*M. pneumoniae* M129-B176) lacking the ORF6 gene product is avirulent and shows reduced adherence to host cells (2, 22). The importance of the location of the P1 protein on the tip structure of the mycoplasmal cell for cytadherence is

further indicated by the fact that the P1 protein of avirulent, nonadhering mutants lacking cytoskeletal proteins (HMW1 to HMW4) or proteins A, B, and C (75, 85, and 37 kDa, respectively) is not concentrated on the tip of the *M. pneumoniae* cell but is equally distributed in the cell membrane (8, 18). Thus, in this respect, the determination of P1 protein distribution in the mutant lacking the ORF6 gene product (M129-B176) or in mutants lacking individual cytoskeleton-forming proteins is of interest, and such studies are in progress.

ACKNOWLEDGMENTS

We thank E. Pirkl, ZMBH, Mikrobiologie, Heidelberg, Federal Republic of Germany, for excellent technical assistance with the cloning procedures and L. Runkel, ZMBH, for critical reading of the manuscript.

This work was supported by the BMFT (BCT O381/5).

REFERENCES

1. **Andersen, D. J., and G. Blobel.** 1979. Biogenesis and assembly of membrane proteins. Methods Enzymol. **96:**111–120.
2. **Barile, M. F., D. F. K. Chandler, H. Yoshida, M. W. Grabowski, R. Harasawa, and S. Razin.** 1988. Parameters of *Mycoplasma pneumoniae* infection in Syrian hamsters. Infect. Immun. **56:**2442–2449.
3. **Baseman, J. B., R. M. Cole, D. C. Krause, and D. K. Leith.** 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J. Bacteriol. **151:**1514–1522.
4. **Baseman, J. B., J. Morrison-Plummer, D. Provillard, B. Puleo-Scheppke, V. V. Tryon, and S. C. Holt.** 1987. Identification of a 32-kilodalton protein of *Mycoplasma pneumoniae* associated with hemadsorption. Isr. J. Med. Sci. **23:**474–479.
5. **Dallo, S. F., A. Chavoya, and J. B. Baseman.** 1990. Characterization of the gene for a 30-kilodalton adhesin-related protein of *Mycoplasma pneumoniae*. Infect. Immun. **58:**4163–4165.
6. **Feldner, J., U. Göbel, and W. Bredt.** 1982. *Mycoplasma pneumoniae* adhesin localized to tip structure by monoclonal antibody. Nature (London) **298:**765–767.
7. **Franzoso, G., P.-C. Hu, G. A. Meloni, and M. F. Barile.** 1993. The immunodominant 90-kilodalton protein is localized on the terminal tip structure of *Mycoplasma pneumoniae*. Infect. Immun. **61:**1523–1530.
8. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1979. Two-dimensional gel electrophoretic comparison of proteins from virulent and avirulent strains of *Mycoplasma pneumoniae*. Infect. Immun. **24:**468–475.
9. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1)**:**285–303.
10. **Hochuli, E., W. Bammwarth, H. Döbli, R. Gentz, and D. Stüber.** 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. Bio/Technology **11:**1321–1325.
11. **Hu, P.-C., R. M. Cole, V. S. Huang, T. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216:**313–315.
12. **Hu, P.-C., A. M. Collier, and J. Baseman.** 1977. Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. J. Exp. Med. **145:**1328–1343.
13. **Hu, P.-C., A. M. Collier, and W. A. Clyde, Jr.** 1984. Serological comparison of virulent and avirulent *Mycoplasma pneumoniae* monoclonal antibodies. Isr. J. Med. Sci. **20:**870–873.
14. **Inamine, J. M., T. P. Denny, S. Loechel, U. Schaper, C.-H. Huang, K. F. Bott, and P.-C. Hu.** 1988. Nucleotide sequence of the P1 attachment protein gene of *Mycoplasma pneumoniae*. Gene **64:**217–219.
15. **Inamine, J. M., S. Loechel, and P.-C. Hu.** 1988. Analysis of the nucleotide sequence of P1 operon of *Mycoplasma pneumoniae*. Gene **73:**175–183.
16. **Krause, D. C., and K. K. Lee.** 1991. Juxtaposition of the genes encoding *Mycoplasma pneumoniae* cytadherence-accessory proteins HMW1 and HMW3. Gene **107:**83–89.
17. **Krause, D. C., D. K. Leith, and J. B. Baseman.** 1983. Reacquisition of specific proteins confers virulence in *Mycoplasma pneumoniae*. Infect. Immun. **39:**830–839.
18. **Krause, D. C., D. K. Leith, R. M. Wilson, and J. B. Baseman.** 1982. Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect. Immun. **35:**809–817.
19. **Küpper, H., J. Delamater, B. Otto, and H. Schaller.** 1982. Expression of major foot and mouth disease antigen in *E. coli*, p. 222–226. *In* Y. I. Reda and T. Neppu (ed.), Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms. Kodansha Ltd., Tokyo.
20. **Küpper, H., W. Keller, C. Kurz, S. Forss, H. Schaller, R. Franze, K. Strohmeier, G. Marquardt, V. G. Zaslavskiy, and P. H. Hofschneider.** 1981. Cloning of cDNA of major antigen of foot and mouth disease virus and expression in *E. coli*. Nature (London) **289:**555–559.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Layh-Schmitt, G.** 1993. The ORF6 gene product of the P1 operon. Zentralbl. Bakteriol. Int. J. Med. Microbiol. **278**(Suppl.)**:**287–295.
23. **Layh-Schmitt, G., and R. Herrmann.** 1992. Localization and biochemical characterization of the ORF6 gene product of the *Mycoplasma pneumoniae* P1 operon. Infect. Immun. **60:**2904–2913.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
25. **Newhall, W. J., V. Newhall, W. D. Sawyer, and R. A. Haak.** 1980. Cross-linking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **28:**785–793.
26. **Ogle, K. F., K. K. Lee, and D. C. Krause.** 1992. Nucleotide sequence analysis reveals novel features of the phase-variable cytadherence accessory protein HMW3 of *Mycoplasma pneumoniae*. Infect. Immun. **60:**1633–1641.
27. **Proft, T., and R. Herrmann.** 1992. Identification and characterization of gene products of *Mycoplasma pneumoniae*. IOM Lett. **2:**266.
28. **Razin, S.** 1981. *Mycoplasmas*: the smallest pathogenic procaryotes. Isr. J. Med. Sci. **17:**510–515.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Sperker, B., P.-C. Hu, and R. Herrmann.** 1991. Identification of gene products of the P1 operon of *Mycoplasma pneumoniae*. Mol. Microbiol. **5:**299–306.
31. **Staros, J. V.** 1982. *N*-Hydroxysulfosuccinimide active esters: bis (*N*-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. Biochemistry **21:**3950–3955.
32. **Stevens, M. K., and D. Krause.** 1991. Localization of the *Mycoplasma pneumoniae* cytadherence accessory proteins HMW1 and HMW4 in the cytoskeleton-like triton shell. Infect. Immun. **173:**1041–1050.
33. **Strebel, K., E. Beck, K. Strohmaier, and H. Schaller.** 1986. Characterization of foot-and-mouth disease virus gene products with antisera against bacterially synthesized fusion proteins. J. Virol. **57:**983–991.
34. **Su, C. J., V. V. Tryon, and J. B. Baseman.** 1987. Cloning and sequence analysis of cytadhesin P1 gene from *Mycoplasma pneumoniae*. Infect. Immun. **55:**3023–3029.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
36. **Wenzel, R., and R. Herrmann.** 1988. Physical mapping of the *Mycoplasma pneumoniae* genome. Nucleic Acids Res. **16:**23–36.
37. **Yayoshi, M., T. Sasaki, E. Hayatsu, M. Araake, and M. Yoshioka.** 1990. Relationship between an 85 kDa protein and the protective effects of *Mycoplasma pneumoniae*. IOM Lett. **1:**468.