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# Localization and Biochemical Characterization of the ORF6 Gene Product of the *Mycoplasma pneumoniae* P1 Operon

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**ORF6 represents one of the two open reading frames flanking the P1 attachment protein gene of *Mycoplasma pneumoniae* in the order ORF4-P1-ORF6 (J. M. Inamine, T. P. Denny, S. Loechel, U. Schaper, C.-H. Huang, K. F. Bott, and P.-C. Hu, Gene 64:217–219, 1988; J. M. Inamine, S. Loechel, and P.-C. Hu, Gene 73:175–183, 1988; C. J. Su, V. V. Tryon, and J. B. Baseman, Infect. Immun. 55:3023–3029, 1987), indicating an operonlike organization. As described previously, we identified two proteins with molecular masses of 40 and 90 kDa (B. Sperker, P.-C. Hu, and R. Herrmann, Mol. Microbiol. 5:299–306, 1991) which might represent two cotranslational cleavage fragments of the ORF6 gene product. To determine the site of the putative cotranslational cleavage, the first 10 amino acids of the N terminus of the isolated 90-kDa protein were sequenced. The data are consistent with the DNA-deduced amino acid sequence between amino acid positions 455 and 465 (RAGNSSETDAL). Thus, the cleavage site was identified at amino acid position 455 (R). In this study, the two proteins were localized and biochemically characterized. Both proteins are part of the insoluble fraction of *M. pneumoniae* as shown by immunoblots of supernatants and pellets of mechanically disrupted cells subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Surface proteolysis followed by SDS-PAGE and Western blot (immunoblot) analysis, covalent labelling of surface-exposed proteins with [$^{125}$I]iodide and subsequent immunoprecipitation of both radiolabelled proteins, immunofluorescence studies with formalized and living *M. pneumoniae*, and immunoadsorption experiments provided strong evidence that the 40- and 90-kDa proteins are membrane-associated proteins expressing surface-exposed regions.**

*Mycoplasma pneumoniae* is an extracellular pathogen of the human respiratory tract (13, 25). The adhesion of *M. pneumoniae* to higher-order cells is mediated by the P1 protein, a 170-kDa membrane protein clustering at a tiplike organelle (3, 9, 12). The DNA sequence of the P1 gene was determined by Su et al. (31) and Inamine et al. (16). A second adhesin-related *M. pneumoniae* protein of 30 kDa which also clusters at the tiplike structure was described by Baseman et al. (4) and Dallo et al. (6). Since mutants lacking proteins other than those two proposed attachment proteins are avirulent and show reduced adherence to host cells, there must exist proteins which are involved in cytadsorption besides protein P1 and the 30-kDa protein (4, 19, 20). Krause and Baseman (18) reported binding of a number of detergent-solubilized proteins to glutaraldehyde-fixed host cells, which supports the idea that several different proteins are responsible for adherence. Inamine et al. (16) analyzed the DNA sequences of two open reading frames flanking the P1 gene, designated ORF4 and ORF6, with coding capacities for proteins of 28 and 130 kDa, respectively. On the basis of these results, the P1 operon was proposed to consist of the P1 gene flanked by two open reading frames in the order ORF4-P1-ORF6. As functionally interacting genes are often localized in an operon, we searched for the gene product of ORF6, because it might be an important protein with accessory function in cytadsorption. Two proteins of 90 and 40 kDa instead of an expected protein of 130 kDa were identified as gene product of ORF6 by Sperker et al. (29). In this study, we expanded upon those results by using biochemical and immunological methods to examine the identities of the two ORF6-gene-derived proteins as well as their cellular locations.

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## MATERIALS AND METHODS

**Growth of *M. pneumoniae*.** *M. pneumoniae* M129-B18 (ATCC 29342) was grown in modified Hayflick medium (11) at 37°C for 48 h. One-hundred-milliliter cultures were grown attached to glass by using 137-cm$^2$ Roux flasks washed before harvesting with three changes of PBS (0.14 M NaCl, 10 mM sodium phosphate, pH 7.4), scraped, resuspended in PBS, and processed for labelling with $^{125}$I, trypsin treatment, and immunofluorescence or immunoadsorption experiments. For protein purification, the suspended cells were centrifuged at 10,000 × *g* for 5 min at 4°C and the pellets were stored at −70°C.

**Protein determination in dilute detergent solution.** The protein concentrations of cell lysates used for purification and of cell suspensions were determined by the bicinchoninic acid procedure of Pierce (28). Cell suspensions were solubilized in 1% sodium dodecyl sulfate (SDS) before the protein assay was performed.

**Expression and purification of FP.** On the basis of antigenicity plots (5) of the DNA-deduced amino acid sequence, selected ORF6 regions of the DNA were cloned in MS2 expression vectors and fusion proteins (FP) were expressed in *Escherichia coli*. The subcloning procedure for *M. pneumoniae* DNA from the cosmid pcos MPE7, described by Wenzel and Herrmann (34), was performed by B. Sperker (29). FP were expressed in *E. coli* 537 after plasmid transformation into competent *E. coli* 537 cells as described previously (29). The expression of FP was performed by the method of Strebel et al. (30). The protein pattern was analyzed on SDS–12% polyacrylamide gels.

FP were purified according to a modified procedure described by Küpper et al. (21, 22) by using sequential Triton X-100 and urea extractions followed by preparative SDS–10% poly-acrylamide gel electrophoresis (PAGE). The excised FP were electroeluted overnight at 4°C and 200 V with a Biotrap apparatus (Biometra) by using Laemmli electrophoretic buffer containing only 0.01% SDS. The eluate contained approximately 1 mg of protein per ml.

**Preparation of antisera against FP.** Rabbits were immunized subcutaneously with 50 μg of protein suspended in Freund's complete adjuvant. They were given three booster immunizations with the same amount of protein suspended in Freund's incomplete adjuvant after 3 to 4 weeks. Preimmune sera were taken from all rabbits as controls.

**SDS-PAGE and Western blotting (immunoblotting).** *E. coli* and *M. pneumoniae* proteins were subjected to SDS-PAGE as described by Laemmli (23). For Western blot analysis according to the method of Towbin et al. (32), proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Schleicher & Schuell) in a semidry blot apparatus (Biometra) with the transfer buffer 25 mM Tris–150 mM glycine–10% methanol, pH 8.3. The nitrocellulose sheet was blocked for 1 h at 37°C with 5% skimmed dry milk in PBS. Anti-FP antisera were diluted 1:2,000 in a buffer containing 150 mM NaCl, 100 mM Tris hydrochloride (pH 7.5), and 0.05% Nonidet P-40 (buffer 1) and added to the nitrocellulose. After incubation for 1 h at 37°C, the blot was washed twice in buffer 1 for 10 min at 37°C. The reaction with the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G [IgG] [H+L] [Dianova]) diluted 1:7,500 in buffer 1 was performed at 37°C for 1 h. After the nitrocellulose sheets were intensively rinsed in buffer 1, color development was observed by adding a buffer consisting of 100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris hydrochloride (pH 9.5) and containing 70 μg of Nitroblue Tetrazolium chloride (Biomol) per ml and 35 μg of 5-bromo-4-chloro-3-indolylphosphate sodium (Biomol) per ml. The reaction was stopped with 20 mM Tris hydrochloride, pH 8.0, containing 5 mM EDTA.

**Preparation of antibody protein A matrix.** The IgG portion of rabbit anti-FP 130 K-5 and 130 K-1 antisera was purified by protein A-Sepharose according to the method of Goding (10) and covalently bound to protein A-Sepharose (Pharmacia) with dimethyl pimelimidate (Pierce) by using a modified method described by Schneider et al. (27). The binding of the affinity-purified IgG to protein A-Sepharose was performed by incubating 2 ml of IgG (6 mg/ml of PBS) with 2 ml of preswollen protein A-Sepharose (0.6 mg [dry weight]) for 1 h at room temperature with gentle shaking. Thereafter, the gel was washed three times with 10 volumes of 0.2 M borate buffer (pH 9.0). The antibodies were cross-linked to protein A with 10 volumes of 20 mM dimethyl pimelimidate in 0.2 M borate buffer (pH 9.0). The coupling reaction was performed at room temperature for 30 min. After the gel was washed twice with 10 volumes of 0.2 M ethanolamine (pH 8.0), excess reactive groups were blocked by incubating the gel with 10 volumes of 0.2 M ethanolamine (pH 8.0) for 2 h at room temperature. Finally, the gel was washed three times with PBS and stored in PBS containing 0.05% sodium azide at 4°C.

**Purification of the 90-kDa protein by immunoaffinity chromatography.** Immunoaffinity chromatography was performed by modified methods described by Andersen and Blobel (1) and Schmitt et al. (26). Cell lysates were prepared by solubilizing a frozen bacterial pellet of a 2-liter culture in 4 ml of a buffer containing 2% SDS, 25 mM NaCl, 25 mM Tris hydrochloride (pH 7.2), and 10 U of Trasylol (Sigma) per ml (lysis buffer). After being heated to 100°C for 5 min, the lysate was diluted with 4 volumes of buffer containing 2.5% Triton X-100, 190 mM NaCl, 60 mM Tris hydrochloride (pH 7.4), 6 mM EDTA, and 10 U of Trasylol per ml (dilution buffer). The cell lysate was centrifuged at 10,000 × *g* for 30 min, and the clear supernatant was gently agitated with 2 ml of the protein A-antibody matrix prepared as described above for 12 h at 4°C. Thereafter, the gel was removed by centrifugation for 5 min at 500 × *g*. The gel was suspended in washing buffer I (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris hydrochloride [pH 7.5], 5 mM EDTA), packed in a column (Econo-Pack 10; Bio-Rad), and washed four times in 10 volumes of washing buffer I and twice in 10 volumes of washing buffer I without detergents (washing buffer II). Then the protein A-antibody matrix with the adsorbed antigen was incubated for 5 min at room temperature with 2 ml of 3.5 M NaSCN containing 0.1% Zwittergent 3-14 (Calbiochem), and thereafter the antigen was eluted from the gel. The eluate was dialyzed against distilled water and lyophilized in a Speed-Vac centrifuge. The lyophilized protein was solubilized in 75 μl of $H_2O$ and 75 μl of 2× Laemmli sample buffer.

**Electroblotting and sequencing of N-terminal amino acids.** Sequencing of N-terminal amino acids was basically performed as described by Eckerskorn et al. (7). Briefly, samples prepared as described above were loaded on an SDS–10% polyacrylamide gel (1 by 100 by 80 mm) and separated according to the method of Laemmli (23). The proteins were electroblotted in a semidry blotting apparatus (Biometra) onto glass fiber sheets (Gelbond; Biometra) by using 50 mM borate buffer (pH 9.0) and 5 mA/$cm^2$/20 min. After staining of the electroblotted protein with amido black (0.1% amido black, 10% acidic acid, 45% methanol), the protein bands were excised. Amino acid sequencing of the N terminus of the protein was performed on the glass fiber membrane by the method of Edmann with an automatic sequencer.

**Mechanical disruption of *M. pneumoniae*.** Bacteria were broken by treatment with a microdismembrator (Braun) for 15 min at a shaking frequency of 50/s. The lysate was centrifuged at 10,000 × *g* for 30 min. The supernatant and the pellet were subjected to SDS-PAGE followed by Western blotting in order to evaluate the localization of the 90- and 40-kDa proteins.

**Trypsin treatment of *M. pneumoniae*.** To assess surface-exposed regions of the 90- or 40-kDa protein, mild surface proteolysis was performed with intact cells. Fresh *Mycoplasma* cultures grown attached to glass were washed and harvested as described above. The cells were suspended in PBS and adjusted to a protein concentration of 2.5 mg/ml. Two-milliliter samples of this suspension were incubated with 100 or 200 μg of trypsin for different times at 37°C. As controls, untreated bacteria or trypsin-treated Nonidet P-40 (1%) lysates were used. Trypsin cleavage was stopped after 5, 10, 15, or 20 min by transferring 300-μl samples of the reaction mixtures to tubes containing 10 U of Trasylol. The cells were pelleted, washed once with PBS, and lysed in 200 μl of $H_2O$ and 200 μl of 2× Laemmli sample buffer. Three hundred microliters of Nonidet P-40 lysates were diluted with 100 μl of 4× Laemmli sample buffer. All samples were subjected to SDS–12% PAGE, and Western blot analysis was performed as described above with 10 μl of samples per lane.

**[$^{125}$I]iodide labelling of *M. pneumoniae*.** Enzymatic iodination of *M. pneumoniae* cells was performed by the procedure

of Marchalonis et al. (24) in order to covalently radiolabel surface-exposed proteins. Four 100-ml cultures grown in Roux flasks (137cm$^2$) were harvested as described above and resuspended in 1.3 ml of PBS. Aliquots of 300 μl were used for the labelling procedure. One aliquot was lysed in 1% Nonidet P-40 before iodination. To 300 μl of cells or Nonidet P-40 lysate 5 μl of KJ ($5 \times 10^{-5}$ M; Merck) in PBS, 10 μl of lactoperoxidase (0.2 mg/ml of PBS; Sigma), and 50 μCi of [$^{125}$I]iodide (Amersham) were added. The reaction was started with 3 μl of $H_2O_2$ ($9 \times 10^{-4}$ M). After 5 min, another 3 μl of $H_2O_2$ was added. Iodination of cell suspensions was stopped by dilution with 1 ml of chilled Hayflick medium containing 20% horse serum. The bacteria were pelleted, washed once with PBS, and processed either for SDS-PAGE and autoradiography or for immunoprecipitation.

**Immunoprecipitation.** Immunoprecipitations were performed as described by Andersen et al. (1). Pellets of radiolabelled cells were lysed in the same way as described for immunoaffinity chromatography. The pellet of a 100-ml *Mycoplasma* culture was lysed in 200 μl of lysis buffer and 800 μl of dilution buffer as described above. One hundred microliters of the antibody-protein A matrix was added to 1 ml of these lysates and incubated with thorough mixing for 14 h at 4°C. The antibody-protein A matrix was pelleted and washed four times in 1 ml of washing buffer I and twice in 1 ml of washing buffer II. The matrix was resuspended in 30 μl of 2× Laemmli sample buffer and heated to 100°C for 5 min. After removal of the solid matrix by centrifugation, the supernatant containing the antigen was subjected to SDS–12% PAGE.

**Immunofluorescence tests.** To evaluate membrane association of both proteins, immunofluorescence studies were performed with formaldehyde-fixed and living cells. For immunofluorescence tests, *M. pneumoniae* was grown for 48 h on eight-chamber slides (Falcon). The bacteria grown attached to the slides were washed three times with PBS and fixed with 2% formaldehyde in PBS for 10 min at room temperature. After fixation, the slides were washed twice with PBS for 10 min. The air-dried preparations were incubated with 100 μl of anti-FP 130 K-1 or 130 K-5 antisera diluted 1:20 in PBS containing 1% bovine serum albumin (BSA) (Biomol) per chamber. As controls, preimmune sera (1:20 diluted in PBS containing 1% BSA) and PBS were used. The incubation was performed for 1 h at 37°C. The slides were washed twice for 10 min in PBS and incubated with fluorescein (DTAF)-conjugated anti-rabbit IgG (H+L) (Dianova) diluted 1:50 in PBS. The incubation and washing procedures were performed as described for the first antibody. The air-dried preparations were embedded in a drop of phosphate-buffered (0.15 M, pH 9.0) glycerol (80%). The slides were examined with a Zeiss epifluorescence microscope. For immunofluorescence staining of live cells, *M. pneumoniae* was grown in Roux flasks and harvested as described above. Each of four washed cell pellets of 50 mg (wet weight) was resuspended in 900 μl of PBS and 100 μl of preimmune sera, and anti-130 K-1 and anti-130 K-5 were added, respectively. The cells were incubated for 1 h at room temperature with gentle shaking. After the cells were washed three times in PBS, the pellets were resuspended in DTAF-conjugated anti-rabbit IgG (H+L) (Dianova) (diluted 1:50 in PBS). For incubation and washing, the same conditions were used as described for the first antibody. Aliquots of the suspensions were observed on slides with a fluorescence microscope as described above.

**Immunoadsorption experiments.** To determine whether the antisera produced against FP 130 K-1 and 130 K-5 recognize surface-exposed epitopes of the two ORF6-derived proteins, immunoadsorption experiments were performed by applying the method of Engleberg et al. (8).

*M. pneumoniae* was grown and harvested as described above. A bacterial cell pellet of 50 mg (wet weight) was resuspended in 1 ml of PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. After 10 μl each of anti-130 K-1 and anti-130 K-5 antisera was added, the mixture was incubated overnight at 4°C with gentle shaking. The bacteria were washed three times in PBS. The adsorbed antibodies were eluted by incubating the cells in 1 ml of 0.2 M NaCl–0.2 M glycine-HCl, pH 2.8, for 30 min at room temperature. The eluate was neutralized by adding 100 μl of 2 M Tris hydrochloride, pH 8.8. To evaluate the immunological reactivity of the eluate, Western blot analysis was performed as described above (see Fig. 7).

## RESULTS

**Analysis and purification of FP expressed in *E. coli*.** The two gene fusions 130 K-1 (N-terminal part of ORF6) and 130 K-5 (C-terminal part of ORF6) were expressed in *E. coli* as FP consistent in size with the expected molecular masses previously shown by Sperker et al. (29). In Fig. 1, the physical map of the P1 operon as described by Inamine et al. (16) and the positions of the cloned DNA fragments are shown. The FP enriched by Triton X-100 and urea extractions were subjected to preparative SDS-PAGE and electroeluted. In order to gain enough antisera for immunological studies and affinity purification of the ORF6 gene product, each FP was used to immunize two rabbits.

**Identification of *Mycoplasma* proteins with anti-FP antisera.** Western blot analysis with *M. pneumoniae* lysates (Fig. 2) revealed that the rabbit anti-FP 130 K-1 and 130 K-5 antisera recognized a 40- and a 90-kDa protein, respectively, instead of an expected 130-kDa protein. These findings fully support the results of Sperker et al. (29) with anti-FP antisera raised in mice. Both proteins are associated with the insoluble fraction of mechanically disrupted *Mycoplasma* cells as shown by SDS-PAGE followed by immunoblot analysis. The 90-kDa protein migrates as a sharp band, whereas the signal representing the 40-kDa protein might be composed of two bands differing at most in about 2 kDa. These bands so far could not be separated at the preparative level.

**Purification of the 90-kDa protein and N-terminal sequencing.** Since the putative cotranslational cleavage site of the ORF6 gene product must be consistent with the N terminus of the 90-kDa protein, this protein was purified by immunoaffinity chromatography and subjected to sequencing of N-terminal amino acids as described above. Twenty milligrams of protein (1 mg/ml) of *M. pneumoniae* lysates was totally depleted of the 90-kDa protein by 2 ml of protein A-antibody Sepharose (6 mg of IgG per ml of matrix) as evaluated by Western blot analysis (data not shown). As estimated by Coomassie blue staining of SDS-polyacrylamide gels by using known amounts of BSA for comparison, the yield of the 90-kDa protein after one-step affinity chromatography is about 20 μg with an initial *Mycoplasma* lysate of 20 mg of protein. The 90-kDa protein was identified by its positive reactivity in Western blot analysis with anti-FP 130 K-5 antiserum. The affinity-purified protein was free of other *Mycoplasma* proteins as examined by SDS-PAGE followed by Coomassie staining (Fig. 3). Because of negligible bleeding of the protein A-antibody matrix, minute amounts of antibodies were coeluted with the 90-kDa protein. The immunoaffinity-purified protein was subjected to SDS-

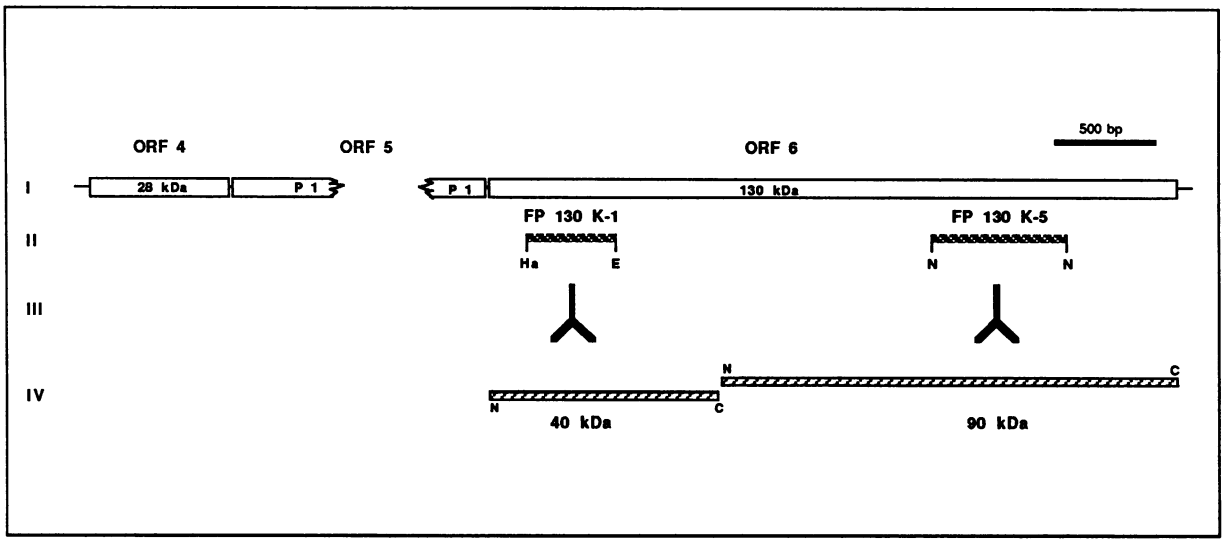


FIG. 1. Identification of the ORF6 gene products by using anti-FP antisera. I, Physical map of the P1 operon; II, FP expressed in *E. coli* after cloning of selected DNA fragments of ORF6 in MS2 expression vectors; III, anti-FP antisera; IV, gene product of ORF6 detected by anti-FP antisera.

PAGE, electroblotted on a glass fiber membrane, and stained with amido black, and the band of the 90-kDa protein was excised for N-terminal sequencing. The amino-terminal end of the 90-kDa protein of two separate preparations (10 μg of protein) was sequenced and determined to be RAGNSSEDAL. The N-terminal amino acid of the 90-kDa protein is number 455 (R) of the amino acid sequence deduced from the DNA sequence of ORF6.

**Proteolysis of surface proteins.** Because of a putative signal sequence and a possible membrane-spanning region identified from the DNA-deduced amino acid sequence, Inamine et al. (16) suggested the predicted 130-kDa protein to be a membrane protein. As described above, after mechanical disruption of *Mycoplasma* cells, the 40- and 90-kDa proteins indeed pelleted together with the membrane fraction. In order to prove the membrane-bound and possibly surface-exposed nature of the ORF6 gene product, proteolysis of intact cells was performed. Intact *M. pneumoniae* cells were incubated with one of two concentrations of trypsin, 100 or 200 μg/ml, for different times. SDS-PAGE and Western blot analysis of bacteria treated with 100 μg of trypsin per ml (shown in Fig. 4A to C) revealed a time-dependent cleavage of the 90- and 40-kDa proteins into smaller fragments. The reactivity of the cleavage fragments with antisera was re-

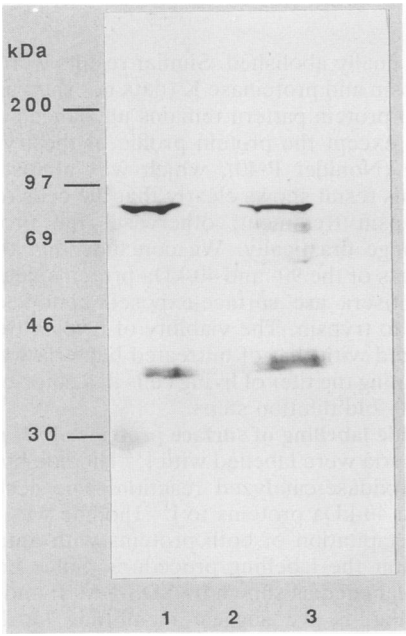


FIG. 2. Identification of ORF6 gene product by Western blot analysis of total cell lysates (lane 1), the soluble fraction (lane 2), and the insoluble fraction (lane 3) of mechanically disrupted *M. pneumoniae* with anti-FP 130 K-1 and anti-FP 130 K-5 antisera, which detect 40- and 90-kDa proteins, respectively.

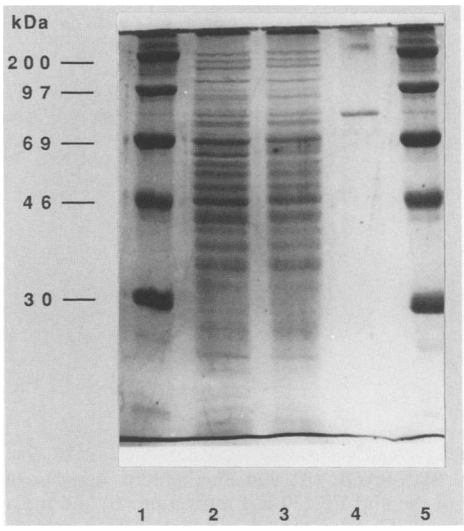


FIG. 3. SDS–12% PAGE of the initial *Mycoplasma* cell lysate (lane 2) used for immunoaffinity chromatography, cell lysate absorbed with anti-FP 130 K-5 antiserum (lane 3), and the eluted 90-kDa protein (lane 4). Lanes 1 and 5 show the molecular weight standard.

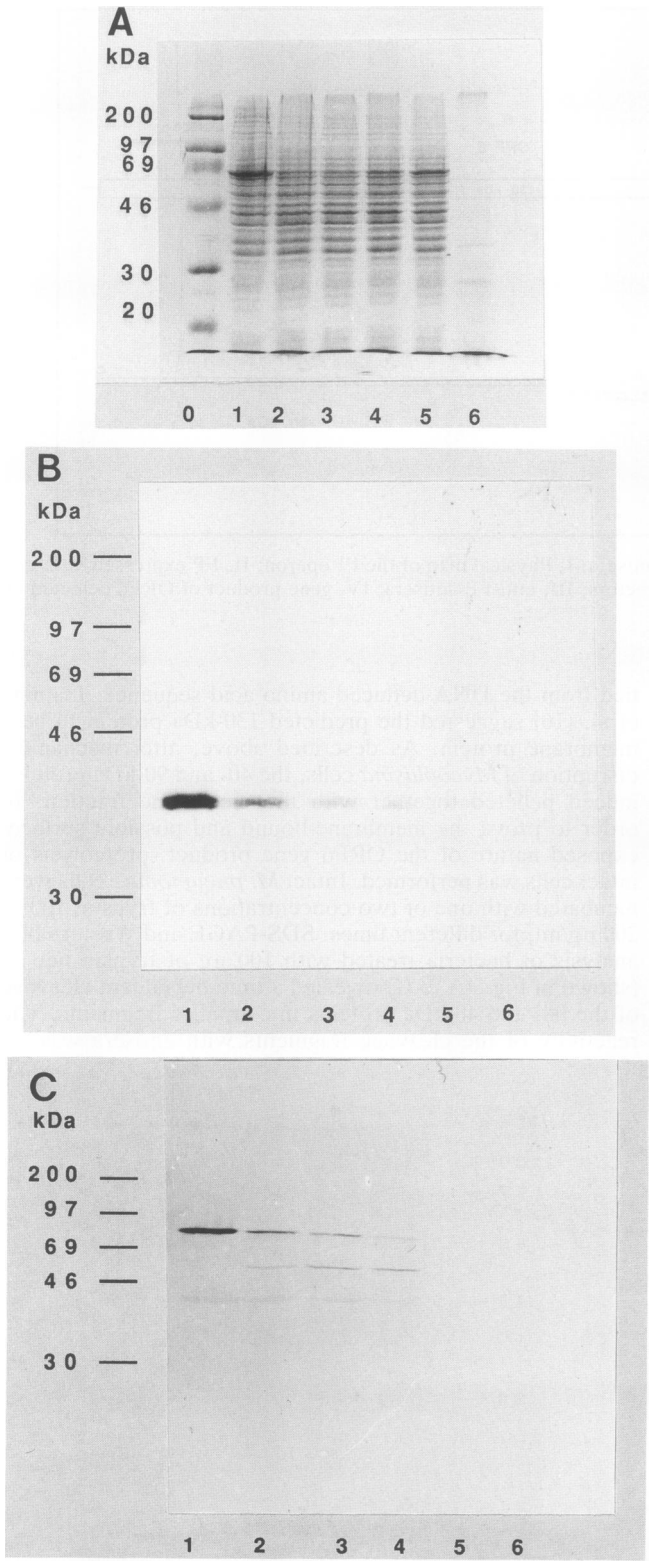


FIG. 4. SDS-PAGE (A) and immunoblot analysis showing the reactivity of the anti-FP 130 K-1 antiserum (B) and the anti-FP 130 K-5 antiserum (C) after time-dependent surface proteolysis of *M. pneumoniae*. Shown are lysate of untreated cells (lane 1); lysates of cells treated with trypsin (100 μg/ml) for 5 (lane 2), 10 (lane 3), 15 (lane 4), or 20 min (lane 5) at 37°C; and cell lysate (Nonidet P-40) treated with trypsin for 10 min at 37°C (lane 6). Numbers and lane 0 (A) indicate molecular masses.

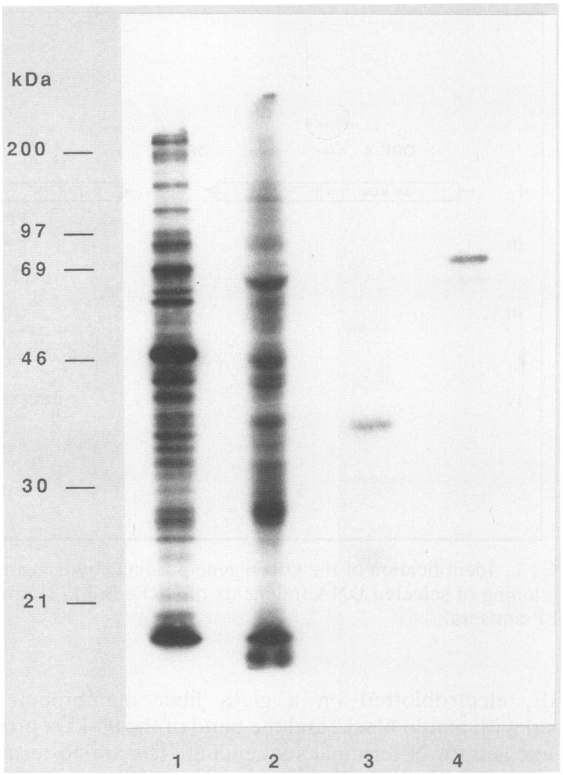


FIG. 5. Evaluation of the surface exposure of the ORF6 gene product by its accessibility to [$^{125}$I]iodide. Autoradiogram of the SDS-PAGE profiles of radioiodinated *M. pneumoniae* cell lysate (Nonidet P-40) (lane 1) and radioiodinated intact *M. pneumoniae* (lane 2); lanes 3 and 4 contain the immunoprecipitated 40-kDa (lane 3) and 90-kDa (lane 4) proteins with lysates of [$^{125}$I]iodide-labelled intact *M. pneumoniae* cells and anti-FP 130 K-1 or 130 K-5 antiserum, respectively.

duced and finally abolished. Similar results were found with chymotrypsin and proteinase K (data not shown). However, the general protein pattern remains unchanged after trypsin treatment, except the protein profile of the trypsin-treated cell lysate (Nonidet P-40), which was almost totally degraded. This result shows clearly that the cells remain intact during trypsin treatment; otherwise, the protein profile would change drastically. We conclude that the antigenic determinants of the 90- and 40-kDa proteins detected by the anti-FP antisera are surface-exposed epitopes which are accessible to trypsin. The viability of trypsin-treated bacteria compared with that of untreated bacteria was examined by determining the titer of living cells in a color-changing test by using 10-fold dilution steps.

**[$^{125}$I]iodide labelling of surface proteins of *M. pneumoniae*.** Intact bacteria were labelled with [$^{125}$I]iodide by performing a lactoperoxidase-catalyzed reaction. The accessibility of the 90- and 40-kDa proteins to [$^{125}$I]iodide was revealed by immunoprecipitation of both proteins with anti-FP protein antisera after the labelling procedure. Since both proteins were radiolabelled as shown by SDS-PAGE and autoradiography in Fig. 5, we suggest membrane localization and surface exposure of both proteins. The 40-kDa protein was labelled with [$^{125}$I]iodide considerably more weakly than was the 90-kDa protein. In order to achieve the same strength of radioactive label for both proteins, we needed about three times more cells for immunoprecipitation of the 40-kDa

protein than for the 90-kDa protein. This result is consistent with the fact that the 90-kDa protein contains 27 tyrosines compared with 10 tyrosines of the 40-kDa protein. By surface labelling of intact cells, about 18 distinct protein bands could be elucidated after SDS-PAGE and autoradiography, suggesting that *M. pneumoniae* exhibits at least 18 membrane-associated surface-exposed proteins. When cell lysates were subjected to $^{125}I$ labelling followed by SDS-PAGE, many more protein bands could be evaluated after autoradiography. Moreover, the SDS-PAGE profile of the radiolabelled proteins of the membrane was clearly different from that of whole-cell lysates.

**Immunofluorescence assay.** Formaldehyde-fixed *M. pneumoniae* cells grown attached to chamber slides as well as live bacteria showed positive immunofluorescence reactions with anti-FP 130 K-1 antiserum or anti-FP 130 K-5 antiserum as shown in Fig. 6A to C. This result provides direct evidence for the association of the two proteins with the cell membrane with epitopes exposed on the bacterial surface which are detected by the anti-FP antisera.

**Immunoadsorption test.** To assess the surface exposure of the ORF6 gene product, antisera directed against the 40-kDa (anti-FP 130 K-1) and the 90-kDa (anti-FP 130 K-5) proteins were adsorbed to whole *Mycoplasma* cells. The antibodies eluted from the bacteria showed positive reactivity with both proteins in Western blot analysis (Fig. 7). On the basis of this result, we conclude that the antibodies raised against the two FP 130 K-1 and 130 K-5 detect surface-exposed epitopes of the 40- and the 90-kDa proteins, respectively.

## DISCUSSION

The first and essential step during infection with a surface parasite is the attachment of the pathogen to the host cell. In order to understand the pathomechanisms of bacteria, we have to learn more about the proteins which are directly or indirectly involved in adherence to mammalian cells. The fact that ORF6 belongs to the same operon as the *M. pneumoniae* attachment protein gene P1 suggests a functional interaction of the P1 protein with the ORF6 gene product, a hypothesis which needs to be proven by further characterization of the two proteins. On the basis of the DNA-derived amino acid sequence of ORF6, Inamine et al. (16) proposed a membrane protein of 130 kDa with a signal sequence of 25 amino acids and a hydrophobic membrane anchor sequence between amino acid positions 1102 and 1218. However, Sperker et al. (29) identified as gene product of the ORF6 two proteins with molecular masses of 40 and 90 kDa by the use of anti-FP antisera. Since we were not able to detect a protein of 130 kDa by using methods such as Western blot analysis and immunoprecipitation, a putative precursor protein must have a very short half-life before it might be posttranslationally modified. On the other hand, the gene product might be cotranslationally cleaved and thus a precursor protein will never be found. Pulse-chase experiments which might contribute to the identification of a precursor are hampered by the complex growth requirements of *M. pneumoniae*, making efficient radiolabelling during short pulses with amino acids difficult. To elucidate the putative cleavage site of the ORF6 gene product, we performed N-terminal amino acid sequencing of the immunoaffinity-purified 90 kDa protein. We identified up to 11 amino-terminal amino acids of the 90-kDa protein which could be directly related to the known DNA sequence ORF6. Cleavage occurs before amino acid position 455 of the DNA-deduced amino acid sequence. We cannot rule out the possibility that the 90- and the 40-kDa proteins are the products of more than one cleavage event as long as the C terminus of the 40-kDa protein is not determined, but we have convincing evidence that the 40-kDa protein represents the N-terminal part of the gene product of ORF6.

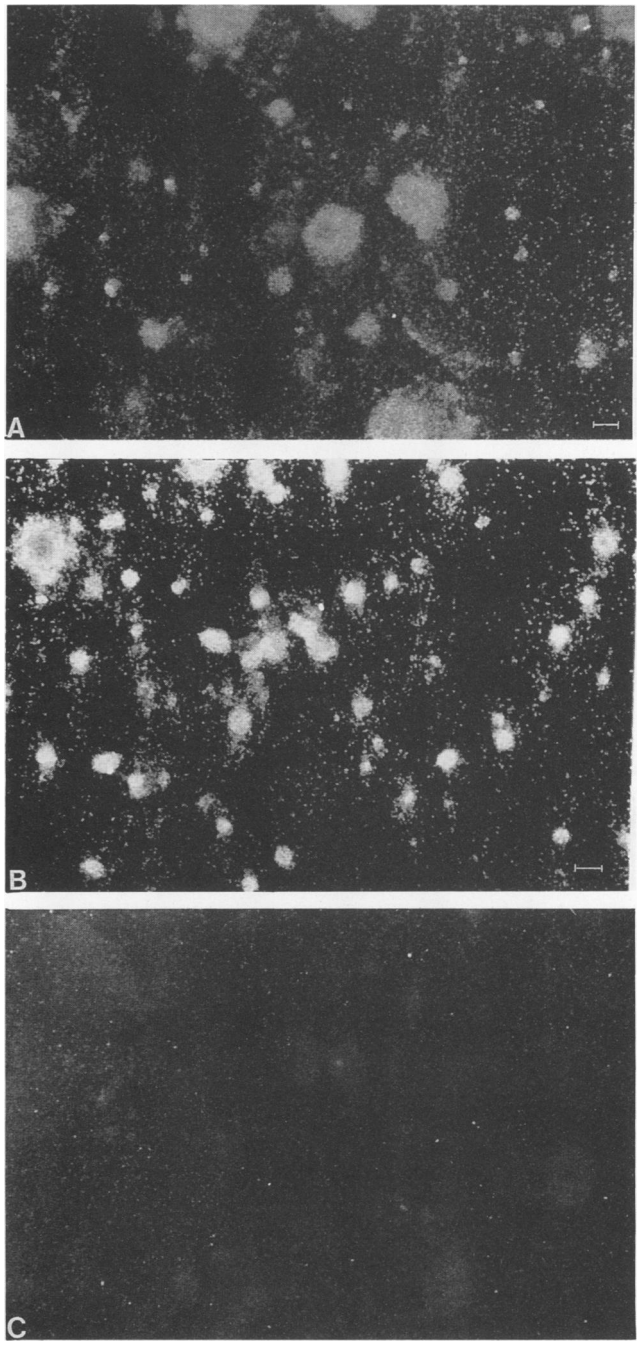


FIG. 6. Immunofluorescence of formaldehyde-fixed *M. pneumoniae* probed with anti-FP 130 K-1 (A) or 130 K-5 (B) antiserum. (C) Control with preimmune serum. Bars = μm.

We generated gene fusions of several selected DNA regions of ORF6 (nucleotide numbers of the P1 operon, 6111 to 6610 [130 K-1], 6450 to 6910, and 6450 to 7481) and expressed FP in *E. coli* M15 (UGA suppressor strain) (24a). Each antiserum raised against these FP detected exclusively

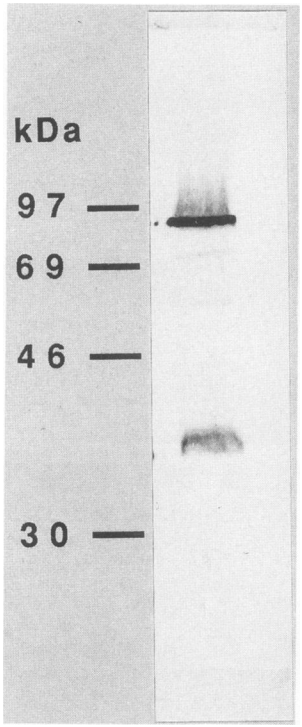


FIG. 7. Western blot analysis with *M. pneumoniae* lysates and antibodies against the 40- and 90-kDa proteins eluted from intact cells after immunoadsorption of anti-FP 130 K-1 and anti-FP 130 K-5 antisera to whole bacteria.

the 40-kDa protein, with the exception of 30% of the mouse antisera which were directed against the FP 130 K-1 and recognized not only the 40-kDa protein but also a protein ladder between 115 and 145 kDa. Since the spontaneous mutant *M. pneumoniae* M129-B176 (ATCC 29343), which was derived from the wild type and which was isolated after several in vitro passages from *M. pneumoniae* M129-B18 (ATCC 29342), lacks the 40- and 90-kDa proteins (29) but still expresses the protein ladder, we assume that the ladder proteins are encoded by genes other than ORF6. The fact that the spontaneous mutant B176 lacks both the 40- and the 90-kDa proteins suggests strongly that both proteins are encoded by one gene because it is rather unlikely that two independent mutation events on different genes would result in this wild-type-derived mutant.

In our efforts to localize the 40- and the 90-kDa proteins in *M. pneumoniae*, we performed a series of different experiments which are generally accepted to elucidate membrane association and surface exposure of bacterial proteins. We first showed that both proteins are minor proteins of the insoluble fraction of mechanically disrupted organisms. Both proteins solubilized in Triton X-100 (data not shown), which is a feature of membrane proteins. Surface proteolysis of *Mycoplasma* cells revealed that both proteins possess surface-exposed regions which carry antigenic determinants detected by the anti-FP antisera. Surface [$^{125}$I]iodide labelling of intact bacteria followed by immunoprecipitation confirmed the surface exposure of the 40- and the 90-kDa proteins. Direct evidence of the membrane association combined with surface exposure of both proteins was achieved by positive immunofluorescence reactions with formaldehyde-fixed and living bacteria by using anti-FP antisera. With immunoadsorption experiments, we were able to confirm that the antisera anti-FP 130 K-1 and anti-FP 130 K-5 recognize surface-exposed epitopes of the 40- and 90-kDa proteins, respectively. On the basis of all four experiments, the results of which were consistent, we suggest that both proteins are membrane proteins with surface-exposed regions. In addition, data from the literature support the findings.

For instance, Vu et al. (33) reported that convalescent-phase sera from humans show constant immunoreactivity with five *M. pneumoniae* proteins with molecular masses of 170, 130, 90, 45, and 35 kDa. Sperker et al. (29) confirmed the identity of the 90- and 45-kDa proteins with the ORF6 gene product. These results support our findings that both proteins exhibit necessary features of surface proteins. Partly surface-exposed membrane proteins are promising candidates for proteins involved in attachment because of their potentiality to interact with host cells. Attachment inhibition studies with anti-FP antisera which are reactive with surface-exposed epitopes of the 40- and the 90-kDa proteins might contribute to the evaluation of the function of both proteins in cytadherence. On the other hand, it is very well feasible that the ORF6 gene product plays just an accessory role during the attachment process by controlling the position of the P1 protein, which might be necessary for effective interaction with the host cell, as shown for HMW1-HMW4 by Baseman et al. and Krause et al. (3, 19). Hu et al. (14) and Sperker et al. (29) observed that the avirulent protein P1-positive *M. pneumoniae* M129-derived mutant B176 (ATCC 29343) does not express the 90- or 40-kDa protein, otherwise showing the same protein pattern in SDS. Barile et al. (2) and Izumikawa et al. (17) reported that mutant B176 fails to colonize the lungs of hamsters and shows reduced adherence to tissue culture cells, which is consistent with our results (data not shown). On the basis of these findings, we suggest a correlation between the presence of the two proteins and the proper adherence of *M. pneumoniae* to host cells. In this context, the observations of Yayoshi et al. (35, 36) are very important. They reported the protective effects of *M. pneumoniae* live vaccine on experimentally infected mice. Mutants missing an 85-kDa protein which we suggest to be identical with the 90-kDa ORF6 gene product did not exhibit features of a protective live vaccine. Thus, we are convinced that the ORF6 represents the gene for two important proteins which may play an essential role not only in adhesion to host cells but also during the immune response.

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## 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:** 2443–2449.
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. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**:251–276.
6. **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.
7. **Eckerskorn, C., W. Mewes, H. Goretzki, and F. Lottspeich.** 1988. A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. **176**:509–519.
8. **Engleberg, N. C., E. Pearlman, and B. I. Eisenstein.** 1984. *Legionella pneumophila* surface antigens cloned and expressed in *Escherichia coli* are translocated to the host cell surface and interact with specific anti-*Legionella* antibodies. J. Bacteriol. **160**:199–203.
9. **Feldner, J., U. Göbel, and W. Bredt.** 1982. *Mycoplasma pneumoniae* adhesin localized to tip structure by monoclonal antibody. Nature (London) **298**:765–767.
10. **Goding, J. W.** 1976. Conjugation of antibodies with fluorochromes. Modifications to standard methods. J. Immunol. Methods **13**:215–226.
11. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1): 285–303.
12. **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.
13. **Hu, P.-C., A. M. Collier, and J. Baseman.** 1977. Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. J. Exp. Med. **145**:1328–1343.
14. **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.
15. **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.
16. **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.
17. **Izumikawa, K., D. K. F. Chandler, and M. Barile.** 1986. *Mycoplasma pneumoniae* attachment to glutaraldehyde-treated human WiDr cell cultures (42284). Proc. Soc. Exp. Biol. Med. **181**:507–511.
18. **Krause, D. C., and J. Baseman.** 1982. *Mycoplasma pneumoniae* proteins that selectively bind to host cells. Infect. Immun. **37**:382–386.
19. **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.
20. **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.
21. **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. Beppu (ed.), Proceedings of IVth International Symposium on Genetics of Industrial Microorganisms. Kodansha Ltd., Tokyo.
22. **Küpper, H., W. Keller, C. Kurz, S. Forss, H. Schaller, R. Franze, K. Strohmeier, G. Marquardt, V. G. Zaslavsky, 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.
23. **Laemmli, U. K.** 1970. Cleave of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Marchalonis, J. J., R. E. Cone, and V. Santer.** 1971. Enzymic iodination. A probe for accessible surface proteins of normal and neoplastic lymphocytes. Biochem. J. **124**:921–927.
24a. **Proft, T., and R. Herrmann.** Unpublished data.
25. **Razin, S.** 1981. *Mycoplasmas*: the smallest pathogenic procaryotes. Isr. J. Med. Sci. **17**:510–515.
26. **Schmitt, S., G. Layh, and T. M. Buchanan.** 1987. Purification of protein IA from Neisseria gonorrhoeae using monoclonal antibody affinity chromatography. Microb. Pathog. **3**:221–225.
27. **Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves.** 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. J. Biol. Chem. **257**:10766–10769.
28. **Sorensen, K., and U. Brodbeck.** 1986. Assessment of coating efficiency in ELISA plates by direct protein determination. J. Immunol. Methods **95**:291–293.
29. **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.
30. **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.
31. **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.
32. **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.
33. **Vu, A. C., H. M. Foy, F. D. Cartwright, and G. E. Kenny.** 1987. The principal protein antigens of isolates of *Mycoplasma pneumoniae* as measured by levels of immunoglobulin G in human serum are stable in strains collected over a 10-year period. Infect. Immun. **55**:1830–1836.
34. **Wenzel, R., and R. Herrmann.** 1988. Physical mapping of the *Mycoplasma pneumoniae* genome. Nucleic Acids Res. **16**:23–36.
35. **Yayoshi, M., U. Gakuin, and E. Hayatou.** 1989. Protective effects of *Mycoplasma pneumoniae* live vaccine or its hyperimmune serum on the experimental infection in mice. J. Jpn. Assoc. Infect. Dis. **63**:684–691.
36. **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.