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Surface Proteins of *Mycoplasma hyopneumoniae* Identified from an *Escherichia coli* Expression Plasmid Library

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A genomic library of *Mycoplasma hyopneumoniae* was constructed by cloning random DNA fragments approximately 300 base pairs long in a fusion expression plasmid, pEx29, containing the N terminus of the phage MS2 polymerase under the control of the PL promoter of phage lambda. Clones that produced fusion proteins carrying surface-specific antigenic determinants were identified by using antiserum raised in a pig by intranasal inoculation of viable mycoplasmas. Rabbit antisera produced against gel-purified fusion proteins synthesized in *Escherichia coli* were analyzed by Western blotting to identify antigenically related mycoplasma components. Distinct mycoplasma proteins termed P90, P68, P50, P30, and P26 were identified. Evidence for the surface location of P90, P68, and P50 was provided by their sensitivity to trypsin and their comigration with lactoperoxidase-catalyzed iodinated proteins of intact mycoplasmas. Immune electron microscopy, performed with antiserum against the hybrid MS2-mycoplasma protein produced in *E. coli* and corresponding to P90, also showed that its antigenic determinant is associated with the mycoplasma surface.

Mycoplasma hyopneumoniae is the causative agent of enzootic pneumonia in pigs, which is the most important swine disease in the world (21, 26, 28). It is a chronic, nonfatal disease affecting pigs of all ages. Infected pigs show only mild symptoms of coughs and fever; however, the economic impact is significant because of reduced feed efficiency and reduced weight gain, the consequence of which is the marketing of undersized pigs (17). Until today efforts to control the disease by vaccination (7, 8, 16) or establishing specific-pathogen-free herds (28, 30) have not been successful.

The physical association of mycoplasmas with the host cell surface is the basis for the development and persistence of disease (29). Therefore, the identification and characterization of bacterial surface constituents mediating attachment to the host cell, and in particular those that elicit neutralizing antibodies during the course of an infection, will not only contribute towards a better understanding of the pathogenesis of the disease but could also lead to the development of genetically engineered vaccines.

Little is known about components on the mycoplasma surface contributing to host cell interactions. In *Mycoplasma pneumoniae*, protein P1 has been assigned a major role in the attachment of the parasite to target cells (4, 12). A protein antigen (P120) of *Mycoplasma hyorhinis* was recently reported to be expressed at the surface of the organism during the colonization of host cells (31). The existence of several strain-specific surface antigens of *Mycoplasma hominis* (11), *Mycoplasma arginini* (1), and *Mycoplasma pulmonis* (22) has also been reported. Recently a proteinaceous membrane fraction of *M. hyopneumoniae* was found to be capable of inducing cytopathic damage on porcine lung fibroblasts (6). The involvement of cytopathic factors in the pathogenesis of the organism, however, has not yet been determined.

As a first step to gain more insight into the molecular interactions between *M. hyopneumoniae* and its host, we attempted to characterize surface proteins of this microorganism that may be involved in the process. In this paper we describe the identification of several immunogenic surface proteins of *M. hyopneumoniae* by recombinant DNA technology.

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MATERIALS AND METHODS

Growth of *M. hyopneumoniae*. *M. hyopneumoniae* ATCC 27719 was obtained from the American Type Culture Collection. The organisms were grown in Friis medium (5) at 37°C. Culture stocks stored at −70°C were used to inoculate the medium at a 1:10 dilution. Mycoplasmas were harvested at mid-log phase (2×10^7 to 5×10^7 cells per ml), as evidenced by a slight orange-yellow color shift of the medium. The culture was centrifuged at 12,500 × *g* for 15 min at 4°C, washed twice in phosphate-buffered saline (PBS; 150 mM NaCl–10 mM sodium phosphate buffer, pH 7.4), and suspended in PBS in 1/100 the original volume.

DNA isolation. Genomic DNA from *M. hyopneumoniae* was isolated as described by Blin and Stafford (2). Plasmid pEx29 (E. Beck, unpublished data), a derivative of the expression vector pPLc24 (23), was isolated by an alkaline lysis procedure (18).

Preparation of antisera. A 10-week-old pig, free from mycoplasma infection, was intranasally inoculated with freshly grown *M. hyopneumoniae* culture (2×10^7 cells for each immunization) at 2- to 4-week intervals for a period of 3 months. Sera were collected 1 week before each inoculation; antiserum taken after the sixth inoculation (antiserum 19) was used for the immunological screening. A second pig, also 10 weeks of age, was injected once intramuscularly with 2×10^7 organisms emulsified in Freund complete adjuvant. Antiserum taken 3 weeks thereafter was used (antiserum 20).

Antisera against MS2-mycoplasma fusion proteins were produced in rabbits. A total of 200 to 500 μg of gel-purified protein was used for each animal, spread over three subcutaneous injections at 2- to 3-week intervals. After 3 months antiserum was taken and used for Western blot analyses.

Cloning of mycoplasma DNA fragments. A 20-μg sample of mycoplasma DNA was digested with 2 ng of DNase I (Boehringer, Mannheim, Federal Republic of Germany) in 200 μl of buffer containing 33 mM Tris (pH 7.6)–10 mM

$MnCl_2$–1 mM β-mercaptoethanol for 5 min at room temperature (25), giving fragments approximately 100 to 1,000 base pairs long. The ends of the fragments were filled with *Escherichia coli* polymerase I, and ^{32}P-labeled *Eco*RI linkers were added. Mycoplasma DNA was cleaved with *Eco*RI and ligated into the expression vector pEx29.

E. coli GC1 (20), a kind gift of T. F. Meyer, was grown to a density of 4×10^7 to 7×10^7/ml at 28°C and prepared for transformation by the procedure of Hanahan (10). The strain is transformed with a plasmid carrying a kanamycin resistance marker and a temperature-sensitive λ *c*I repressor gene (p*c*I857; 24), which regulates the activity of the PL promoter on pEx29. It is repressed at 28°C, whereas induction is achieved by incubating the culture at 42°C. Translation initiation is provided by the ribosome binding site and the coding sequence for some 100 N-terminal amino acids of the MS2 polymerase. Foreign DNA is inserted into the *Eco*RI site, and statistically one in six constructions will be in the correct orientation and reading frame and thus generate a fusion protein with the MS2 polymerase.

Immunoscreening of the genomic library. Approximately 3×10^5 transformants, grown at 28°C on 20 agar plates (25 by 25 cm) to colonies 1 mm in diameter, were transferred to nitrocellulose filters. Immediately after transfer, the filters were placed onto prewarmed agar plates and incubated at 42°C for 2 h. The colonies were lysed by a 15-min exposure to chloroform vapor. The filters were then air dried for 30 min, soaked for 2 h in PBS containing 2% bovine serum albumin, and incubated overnight in pig antiserum 19 (diluted 1:500 in PBS–1% bovine serum albumin–0.01% Nonidet P-40). The antiserum had been previously absorbed with an *E. coli* extract to reduce background staining of the colonies. The filters were washed in PBS containing 0.05% Nonidet P-40, incubated for 4 h with ^{125}I-labeled protein A, and rinsed extensively with PBS–0.05% Nonidet P-40. Clones showing positive signals on autoradiograms were picked in duplicate and rescreened by the same procedure.

Expression of fusion proteins in *E. coli*. Clones were grown at 28°C in LB medium containing 50 μg of ampicillin per ml and 25 μg of kanamycin per ml to a density of 2×10^9 cells per ml. The expression of fusion proteins was induced by incubating the culture (diluted 1:5 in prewarmed medium) at 42°C for 2 h under vigorous aeration (14). Bacteria samples (0.5 ml, corresponding to 2×10^8 cells) were pelleted, suspended in an equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris, pH 6.8, 10% β-mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue), boiled for 5 min, and analyzed on 12.5% SDS-polyacrylamide gels (15).

Purification of fusion proteins. Fusion proteins were prepared by the method of Küpper et al. (14). A 20-ml overnight culture grown at 28°C was diluted in 180 ml of medium and incubated at 42°C for 2 h with vigorous shaking. The cells were pelleted and washed in 30 ml of 50 mM Tris (pH 8)–100 mM NaCl and suspended in 1.6 ml of 10% sucrose–50 mM Tris (pH 8). After the addition of 0.4 ml of lysozyme (5 mg/ml) and 0.4 ml of 0.5 M EDTA, the cells were incubated at 37°C for 30 min; thereafter, 4 ml of Triton lytic mix (0.1% Triton X-100, 50 mM Tris, pH 8, and 62.5 mM EDTA) was added, and the mixture was kept for 15 min on ice and then further incubated for 30 min at 37°C. The cells were then sonicated (four bursts of 15 s) and centrifuged for 30 min at $20,000 \times g$. The pellet was first suspended in 5 ml of 1 M urea and extracted for 30 min at 37°C, then centrifuged and further extracted with 5 ml of 7 M urea (30 min, 37°C). The fusion protein solubilized in the 7 M urea supernatant was further purified by running a 12.5% preparative SDS-polyacrylamide gel. The fusion protein band was visualized by staining for 10 min with Coomassie blue (0.06% Coomassie brilliant blue, 50% methanol, and 10% acetic acid) and excised. The gel was crushed, and the protein was eluted overnight at 42°C with PBS containing 0.1% SDS. Polyacrylamide was separated by centrifugation, and the supernatant containing the fusion protein was concentrated to 1 mg/ml. The average yield from a 20-ml overnight culture was between 0.2 and 1 mg.

SDS-PAGE and Western blotting. *E. coli* extracts or mycoplasma lysates were subjected to polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (15). The Western blot procedure was carried out according to Towbin et al. (27). Proteins separated on the gels were transferred electrophoretically to nitrocellulose paper (overnight at 4°C, at 60 V or 0.3 A, in 192 mM glycine, 25 mM Tris, pH 8.3, and 20% methanol), using a Transblot apparatus (Bio-Rad), and identified with antiserum and radioiodinated protein A as described above.

Trypsin treatment of intact mycoplasmas. A 20-ml sample of a fresh mycoplasma culture was centrifuged, washed once with PBS, and suspended in 2 ml of PBS. A 1-ml portion of this suspension was incubated with 50 μg of trypsin. To the remaining 1 ml, which acted as control, no trypsin was added. After a 10-min incubation at room temperature, the cells in both tubes were centrifuged and washed once with PBS, and the pellets were suspended in sample buffer and loaded onto polyacrylamide gels. The separated proteins were either stained with Coomassie blue or transferred to nitrocellulose paper for Western blotting.

Labeling of mycoplasma proteins. Lactoperoxidase-catalyzed iodination of *M. hyopneumoniae* surface proteins was performed by the procedure of Marchalonis et al. (19). A 20-ml mid-log-phase culture was washed twice in PBS and suspended in 0.6 ml of PBS. A 0.3-ml portion of the suspension was pretreated by sonication (five bursts of 15 s) and served as a control. The labeling of whole cells and sonicated cells was carried out by the addition of the reagents (dissolved in PBS) in amounts and sequence as follows: 5 μl of KI (5×10^{-5} M; Merck), 10 μl of lactoperoxidase (0.2 mg/ml; Sigma), 50 μCi of carrier-free ^{125}I (New England Nuclear Corp.), and 3 μl of H_2O_2 (9×10^{-4} M). After 5 min, another 3 μl of H_2O_2 was added, and the reaction was continued for 5 min more. Sample buffer was added to the radioiodinated samples and processed for gel electrophoresis.

Labeling of total mycoplasma proteins was performed with 20-ml cultures grown in Friis medium in the presence of 250 μCi of [^{35}S]methionine (Amersham). Cells were harvested at mid-log phase and subjected to PAGE and autoradiography as described above.

Immune electron microscopy. Frozen thin-sectioning and antibody labeling were carried out by the method of Griffiths et al. (9). A mycoplasma cell pellet from a 10-ml culture was fixed in 4% formaldehyde, infused with 2.3 M sucrose, and frozen in liquid nitrogen. Sectioning was carried out with glass knives. Thin sections were thawed, transferred to Formvar/carbon-coated, 100-mesh copper grids, floated on 10-μl immunoglobulin G fractions (diluted 1:10 in 1% fetal calf serum in PBS), and incubated at room temperature for 30 min. The grids were rinsed five times with PBS for a total of 30 min, then transferred to a 5-μl drop of gold-protein A solution (1 mg/ml, a kind gift of G. Griffiths), diluted 1:30 in PBS–1% fetal calf serum. The reaction was allowed to take place for 20 min at room temperature. The grids were further

rinsed five times for 30 min in PBS and finally four times for 5 min with distilled water. The grids were then stained with 2% uranyl acetate and embedded in a 1.5% methylcellulose solution. Sections were visualized in an electron microscope.

RESULTS

Characterization of antimycoplasma antisera raised in pigs. Antisera against mycoplasma proteins were raised in pigs by two different routes: by the inhalation of intact mycoplasmas (antiserum 19) and by intramuscular injection of intact mycoplasmas suspended in Freund complete adjuvant (antiserum 20). By Western blot analyses of total mycoplasma proteins, both antisera recognized a similar set of approximately 11 protein antigens (Fig. 1, lanes 2 and 4). To ascertain that the proteins recognized by the antisera were specifically located on the surface, the Western blot was compared to an autoradiograph of ^{125}I-labeled proteins of intact mycoplasmas iodinated by the lactoperoxidase method (lane 5). Many of the proteins that reacted with both antisera in the Western blot comigrated with those that were iodinated in the intact bacteria. Evidence that iodination was being confined to cell surface proteins was provided by comparing the iodination of intact mycoplasmas with that of a sonicated cell extract (lanes 5 and 6, respectively). In contrast to the results with intact cells, where only a discrete number of proteins were labeled (approximately 10), the iodination of the extract resulted in the labeling of many more proteins. Furthermore, its profile is similar not only to an extract of in vivo [^{35}S]methionine-labeled cells (lane 7) but also to total mycoplasma proteins stained with Coomassie blue (lane 8). Thus we conclude that the antisera were mainly directed against cell surface components.

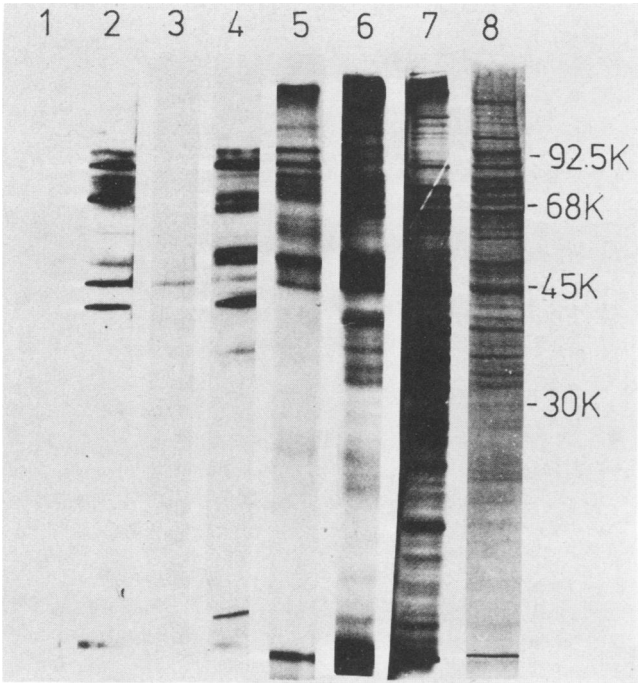

FIG. 1. Specificity of the pig antisera 19 and 20. Mycoplasma proteins were separated by PAGE, transferred to nitrocellulose paper, and treated with the following sera: antiserum 19 (lane 2), antiserum 20 (lane 4), preimmune sera to antisera 19 and 20, respectively (lanes 1 and 3). These blots were compared with autoradiographs of protein gels of intact mycoplasmas labeled with ^{125}I (lane 5), sonicated mycoplasma extract labeled in vitro with ^{125}I (lane 6), or total mycoplasma proteins labeled in vivo with [^{35}S]methionine (lane 7). Total mycoplasma proteins stained with Coomassie blue are shown in lane 8. The molecular weights (10^3) are indicated on the right using molecular weight standards (Sigma).

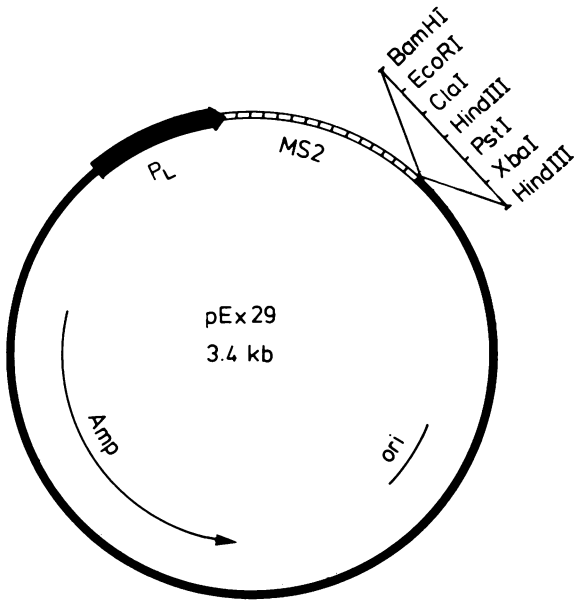

FIG. 2. Expression vector pEx29 used for constructing the genomic library. This is a derivative of the expression vector pPLc24. The original *Eco*RI site between the λ PL promoter and the MS2 region (encoding 100 N-terminus amino acids) was deleted, and a polylinker was introduced into the *Bam*HI and *Hin*dIII sites, as indicated. The origin of replication (ori) and the region coding for β-lactamase (Amp) are indicated.

Construction of a genomic library. To screen for individual surface antigens with the specific pig antiserum, mycoplasma genes had to be expressed in *E. coli*. Initial attempts to detect autonomous expression of mycoplasma genes from large DNA fragments (5 to 10 kb), cloned either in plasmid pBR322 or in a phage λ vector, failed. This led us to clone the genome into the plasmid pEx29, which expresses foreign DNA sequences fused to a gene that is highly expressed in *E. coli*. It makes use of the PL promoter of phage λ to provide efficient transcription of the foreign DNA inserted (Fig. 2).

We aimed at cloning short random fragments of mycoplasma DNA coding for one or more epitopes of a surface antigen. Thus mycoplasma DNA was treated with DNase I to provide fragments averaging 300 base pairs long, rather than being cleaved with restriction enzymes. Synthetic *Eco*RI linkers were added, and the fragments were inserted into the vector. A 1-pmol amount of fragment (calculated from the radioactivity of added linkers) ligated to 1 pmol of vector resulted in approximately 5×10^4 transformants; ca. 75% of the clones carried inserts.

Immunoscreening of the genomic library. A total of 3×10^5 colonies of *E. coli* GC1 cells containing MS2-mycoplasma expression plasmids were screened immunologically with antiserum 19 for the expression of mycoplasma surface antigens (for details see Materials and Methods). Approximately 40 clones gave positive signals of different intensities. Ten were finally selected on the basis that they expressed MS2-mycoplasma fusion proteins in quantities

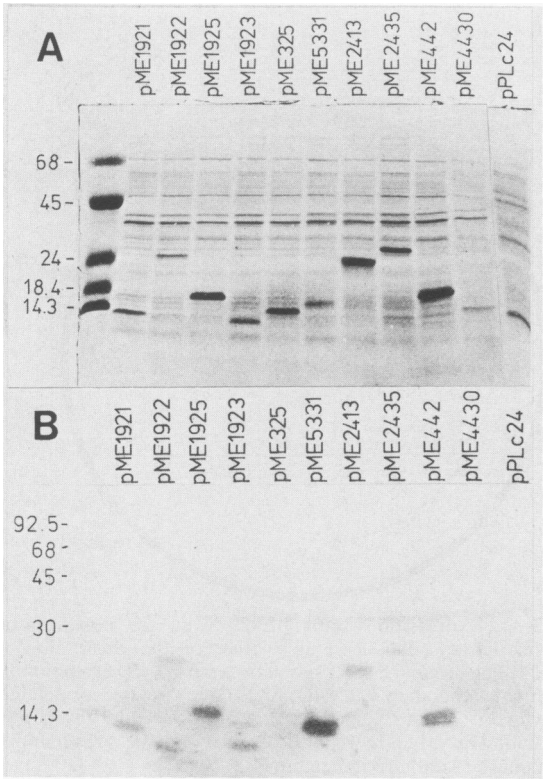

FIG. 3. Specificity of the fusion proteins expressed in *E. coli*. (A) Total cell extracts of the 10 clones selected from the immunoscreening of the library were analyzed by PAGE and stained with Coomassie blue. pPLc24, the vector from which pEx29 is derived and which expresses the same 11K protein as pEx29, was included in the outermost lane of the same gel as a control. A further clone run in the series, which did not synthesize a stable fusion protein upon induction, was omitted from the gel. (B) A second sample of the cell extracts of the 10 positive clones and pPLc24 were run in a second gel. The proteins were transferred to nitrocellulose filters and incubated with antiserum 19. Bound antibody was detected with ^{125}I-labeled protein A and autoradiographed. Fusion proteins were seen to react with the antiserum to varying intensities, independent of the quantities of fusion proteins synthesized. ^{14}C-labeled molecular weight standards (Amersham) are shown on the left.

which made up 10 to 20% of the total proteins synthesized in *E. coli*. Figure 3A shows the Coomassie blue-stained gel of extracts of the induced *E. coli* clones with their corresponding fusion protein bands. The molecular weights of the fusion proteins ranged from 11,000 (11K) to 28K. The fusion proteins of two clones, pME1921 and pME1923, were also 11K, like that of the MS2 polymerase. The DNA inserts of the two clones, each of which was ca. 150 bp in length, were then sequenced and found to contain stop codons after only nine and six amino acids, respectively. This explains why no obvious size increase was observed for the fusion proteins produced.

In a Western blot of the induced *E. coli* clones, the fusion proteins reacted with antiserum 19 to various extents (Fig. 3B). The strengths of the signals did not always correlate to the amounts of proteins synthesized. Antiserum 19 did not recognize the 11K protein of pPLc24 in the Western blot. The antiserum, however, appeared to react with a protein migrating around 30K in all clones, including pPLc24, throughout the Western blot. The reaction is therefore regarded as nonspecific. The rest of the clones provided fusions that either were synthesized only in small amounts (<10%) or were unstable. This would pose problems in the preparation of fusion proteins as well as that of antisera in rabbits; these clones thus have not been further characterized.

Identification of specific mycoplasma proteins using anti-fusion protein antisera raised in rabbits. *E. coli* extracts were enriched for the fusion proteins as described in detail above. The proteins were separated by PAGE and eluted from the polyacrylamide. These proteins were then tested for homogeneity by PAGE before being used for the immunization of rabbits. The titers of the various antisera obtained were between 1:10^3 and 1:10^4 in an enzyme-linked immunosorbent assay. The antisera were tested by Western blot analysis for the presence of antibodies against specific mycoplasma proteins. Figure 4 shows the positive antisera: anti-1922 antiserum reacted primarily with a protein designated P90 according to its molecular weight of 90K; anti-2413 picked up essentially two proteins, P68 and P26 (molecular weights 68K and 26K, respectively), while anti-442 recognized P50 (molecular weight 50K). Anti-1921 recognized a protein with a molecular weight of 30K, termed P30. Anti-1925 also reacted with a protein of the same molecular weight (not shown). All of these proteins comigrated with the mycoplasma bands stained strongly by antiserum 19, indicated by the arrows in the figure, except for P26, which correlated only with a minor band (see below). Preimmune

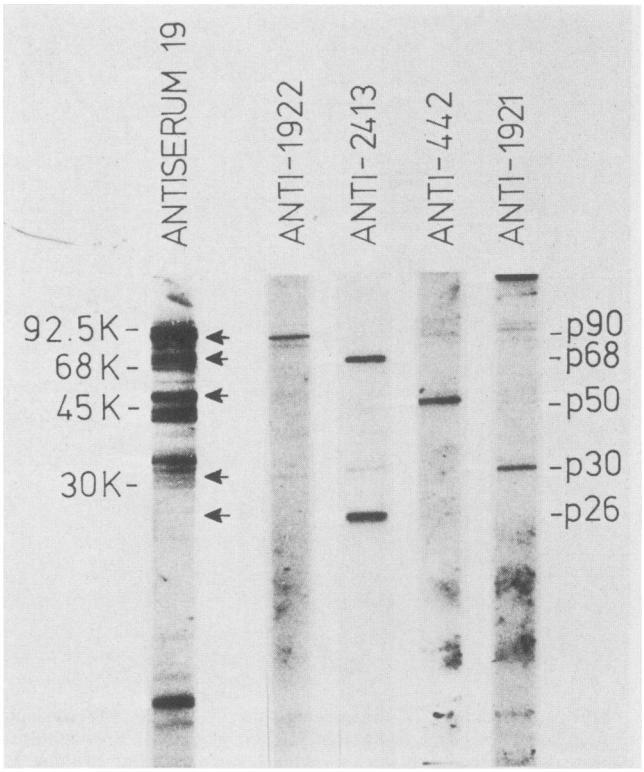

FIG. 4. Western blots of *M. hyopneumoniae* proteins using specific antisera. Nitrocellulose strips prepared as described for Fig. 1 were treated with various antisera. The mycoplasma profile stained by antiserum 19 is used as a reference. Mycoplasma proteins recognized by the anti-fusion protein antisera are indicated by the arrows, and their approximate molecular weights, shown on the right, are based on comparison with molecular weight standards (left).

sera from rabbits immunized with the various fusion proteins did not stain any mycoplasma proteins (not shown). These results demonstrate that mycoplasma antigens generated from fusion proteins in *E. coli* induce specific antibodies capable of reacting with discrete *M. hyopneumoniae* components.

Surface location of the identified proteins revealed by trypsin digestion. We attempted to correlate the mycoplasma surface proteins recognized by the anti-fusion protein antisera with those that were sensitive for a trypsin digestion. By treating intact mycoplasmas with trypsin we observed that several high-molecular-weight proteins were selectively degraded to lower-molecular-weight ones (see Coomassie blue-stained gel of Fig. 5). These proteins, being accessible to trypsin, are presumably localized on the surface, exposing one or more trypsin-sensitive sites to the outside. Subjecting such extracts of trypsin-treated intact mycoplasmas to Western blot analyses with anti-1922, anti-2413, and anti-442, we observed that the determinants associated with P90, P68, and P50 had been eliminated by trypsin (Fig. 5). These proteins also comigrated with proteins which can be iodinated, indicating clearly that they are exposed on the surface. The other two proteins, P30, recognized by anti-1925 (Fig. 5) and anti-1921 (not shown), and P26, originating from anti-2413, did not appear to be accessible or sensitive to the protease.

Immune electron microscopy. More direct evidence for the surface location of the cloned antigens was provided by in situ labeling of mycoplasmas with specific antibodies. Frozen-thawed thin sections of formaldehyde-fixed mycoplasmas were attached to the grids before incubation with antibody and protein A-colloidal gold complex. The electron micrographs in Fig. 6 demonstrate the binding of gold particles (i.e., of antiserum 19) mainly to the membrane of the mycoplasmas (Fig. 6A). Binding of the antiserum against the fusion protein corresponding to P90, anti-1922, was also observed (Fig. 6B). Antisera against other fusion proteins have not yet been tested. Antiserum 20 provided membrane labeling similar to that of antiserum 19 (not shown). The absence of gold was observed for both preimmune sera (shown only for antiserum 19 in Fig. 6C).

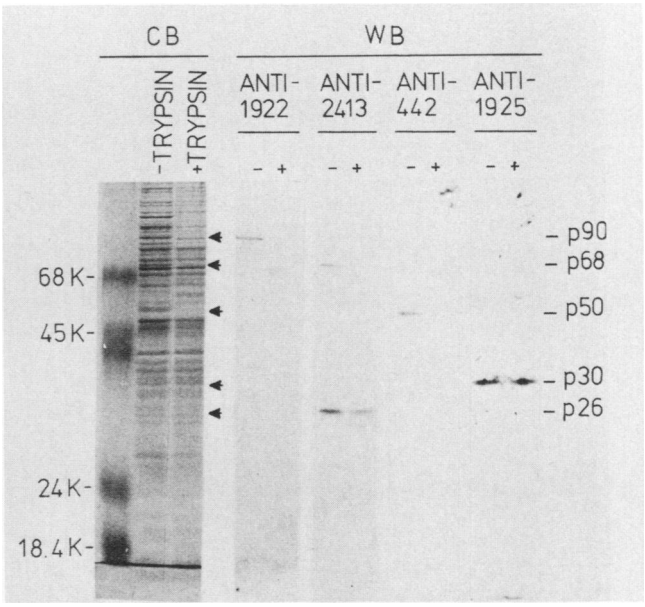

FIG. 5. Trypsin sensitivity of the cloned surface antigens. Intact mycoplasma cells (corresponding to 1 ml of culture) were incubated in the absence or presence of trypsin (50 μg/ml), and the proteins were thereafter separated by PAGE and either stained with Coomassie blue (CB) or transferred to nitrocellulose filters for Western blot analyses (WB). The blots were incubated with anti-fusion protein antisera, as indicated; the arrows show the positions of the proteins that were recognized by the antisera.

DISCUSSION

M. hyopneumoniae surface protein antigens have been identified in this study. The specificity of the antiserum used to select clones encoding antigenic determinants was based on the following lines of evidence. Antibodies were produced in a pig infected by inhalation of viable mycoplasmas. (The immune response stimulated by exposure to intact cells is assumed to be similar to a respiratory tract infection, so that antibodies elicited are believed to recognize mainly surface-exposed antigens.) The proteins recognized by this antiserum in a Western blot could also be correlated to those that were iodinated in intact cells. Immune electron microscopy using pig antiserum and gold particles coupled to protein A demonstrated that the antibodies bind predominantly to the outside of the mycoplasma membrane.

This latter approach, which provides the most direct evidence for the surface location of the proteins, was also used for anti-1922 antiserum directed against the fusion product corresponding to P90. The exposure of P90 and two other proteins, P68 and P50, to the surface was also confirmed because of their susceptibility to trypsin. Whereas the surface location of these three proteins cannot be doubted, that of P30 and P26 is less obvious. First of all, both proteins are not digested by trypsin. Their insensitivity to trypsin is not necessarily an argument against their surface exposure, however, since it is conceivable that some surface proteins incorporated into the membrane have no sites available for trypsin digestion. Second, P30 does not comigrate with an iodinated surface protein. Even though lactoperoxidase-catalyzed iodination is used as a criterion for surface location of a protein, it cannot be ruled out that P30 may represent a minor surface component or one too poor in tyrosine residues to be labeled well. P26 comigrates with an iodinated protein. It also correlates with one of the surface proteins recognized only very weakly by antiserum 19 in the Western blot. On the other hand, the signal picked up by the antiserum against the fusion protein in the Western blot is strong. This could be explained by the possibility that the epitope expressed on the surface of the mycoplasma may be a weaker immunogen in a pig than when it is injected into a rabbit as a fusion protein of *E. coli*.

P26 is also one of the two proteins recognized by the antifusion protein 2413 antiserum, the other being P68. These could be two unrelated proteins sharing a common antigenic determinant. Alternatively, P68 could be a precursor of P26, or the latter a breakdown product of the former. The second possibility is supported by Southern blot analysis, in which only one genomic fragment hybridizes to the clone (data not shown). Peptide mapping may also be performed to characterize their relationship.

In a Western blot analysis, both pME1921 and pME1925 recognize a 30K protein. Southern blotting has shown that the two clones belong to different restriction fragments. It was established by analysis of the nucleotide sequences that the cloned fragments share no sequence homology at the DNA or predicted protein levels (data not shown). Thus the two clones may correspond either to different regions of the same gene or to two different genes coding for proteins of the same molecular weight.

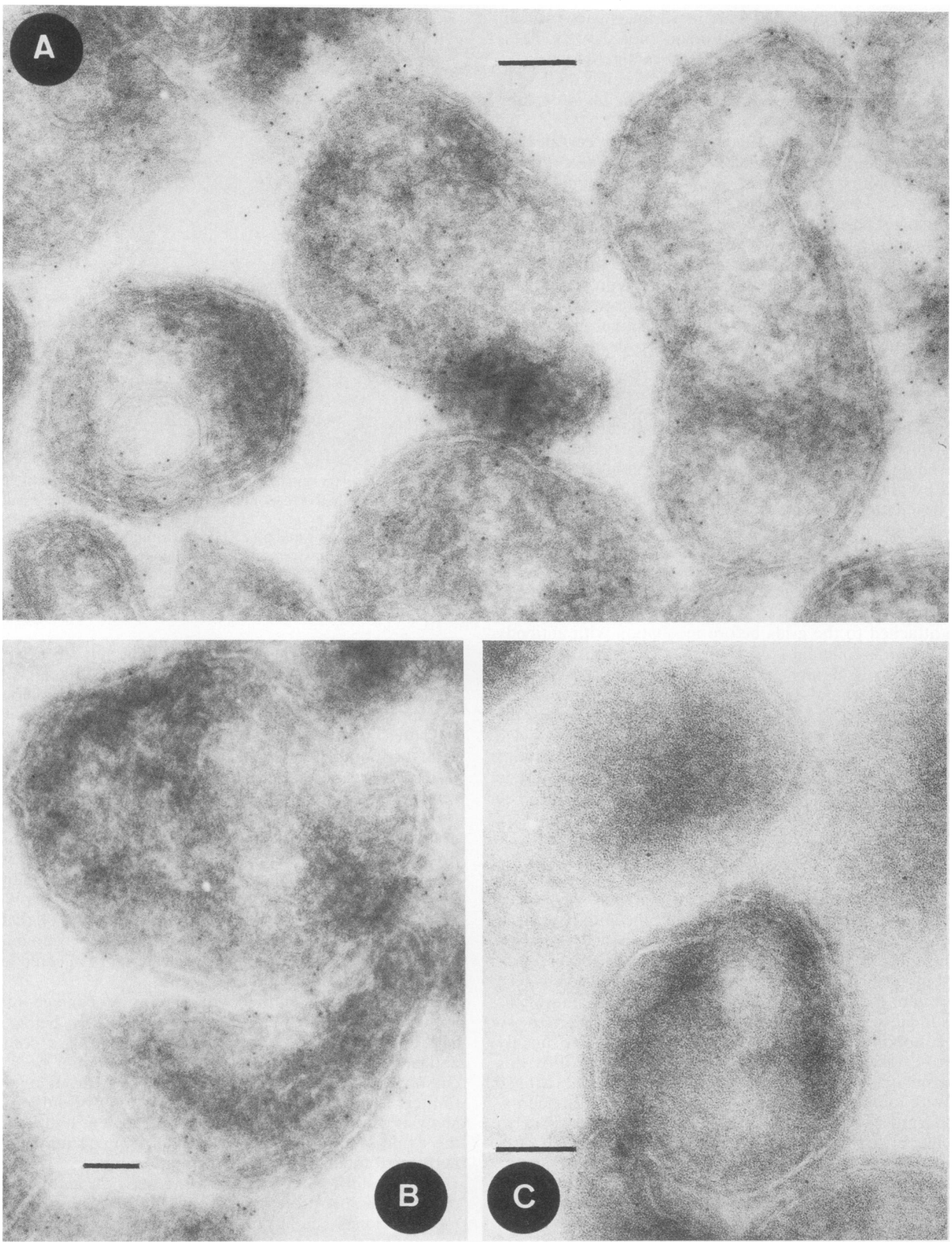

FIG. 6. Localization of antigenic structures on the surface of mycoplasmas. (A) Electron micrograph of a frozen thin section of *M. hyopneumoniae* incubated with antiserum 19 and gold-labeled protein A. Note the attachment of gold particles almost exclusively to the surface of the organism in the presence of surface-specific antibodies, antiserum 19. (B) Note the presence of gold particles on the surface (albeit to a lesser extent) with an antibody raised specifically against the fusion protein 1922. (C) No labeling is observed when mycoplasmas are incubated with pig preimmune serum. Bars, 0.1 μm.

The surface proteins as characterized in this study have not all been cloned. This could be for reasons other than the poor immunogenicity of certain proteins or the insufficient screening of the genomic library. It is very likely that the remaining 30 clones identified in the colony blotting may carry information for some additional surface antigens. The characterization of these clones has been hampered by the fact that they have not reacted positively with the antiserum in the Western blot. The reason for this finding could be due either to the instability of the fusion proteins or to their being in the native state in the colony blotting but denatured in the Western blot procedure.

The recombinant plasmids coding for surface-specific antigens can now be used as probes to localize their genes on the chromosome. Considering the lack of genetic markers in mycoplasmas, the identification of genes encoding strain-specific proteins will aid in the construction of a genetic map of *M. hyopneumoniae*.

The identification of immunogenic surface proteins is a first step towards the understanding of cell-to-cell interactions. To elucidate the functions of the mycoplasma proteins identified in this work, antibodies against the complete gene products or parts of them can be produced and used in adherence studies, as already done for *M. pneumoniae* (3), or in studies associated with virulence (13). Finally, should one or more proteins prove to be surface antigens responsible for protective immunity, they could then serve as a basis for the development of synthetic vaccines.

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