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Two-Dimensional Gel Electrophoretic Comparison of Proteins from Virulent and Avirulent Strains of *Mycoplasma pneumoniae*

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The protein composition of the virulent M129 strain of *Mycoplasma pneumoniae* was compared to that of its homologous avirulent strain by the use of standard one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Forty-nine individual *M. pneumoniae* cell proteins were resolved by this method, and the virulent strain was shown to possess a single high-molecular-weight protein not present in avirulent cells. Variability in the resolution of this particular protein in one-dimensional gels prompted the application of two-dimensional gel electrophoresis to the analysis of *M. pneumoniae* cell proteins. The sequential use of isoelectric focusing in the first dimension and sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the second dimension permitted the resolution of at least 142 individual *M. pneumoniae* cell proteins. Application of nonequilibrium pH gradient electrophoresis in the first dimension achieved the resolution of at least 20 additional basic proteins. Three proteins which are synthesized only by cells of the virulent strain, and not by the homologous avirulent strain, were identified by these two-dimensional gel electrophoresis techniques.

Mycoplasma pneumoniae, a pathogen of the human respiratory tract, has been shown to parasitize and produce cell injury in respiratory epithelium (3, 7, 8). Using tracheal organ culture as a model system, it has already been established that the capacity of virulent *M. pneumoniae* to cause damage to respiratory epithelium is dependent upon the fulfillment of at least two criteria. The first critical step in the experimental pathogenesis of *M. pneumoniae* infection is the attachment of this organism by its specialized organelle-like tip structure to sialidase-sensitive receptor sites on the surface of respiratory epithelial cells (4, 15). Once *M. pneumoniae* has attached to the host cell, only viable metabolically active organisms demonstrate pathological potential, evidenced first by gross alterations in macromolecular synthesis, followed by ciliostasis and more severe cytopathology (7, 8). Relatively little is known about the attachment mechanism employed by *M. pneumoniae*, or about those factors which mediate the disruptive effects on both macromolecular synthetic processes and cellular integrity of *M. pneumoniae*-infected respiratory epithelium. Previous data from this laboratory have implicated at least one membrane-bound proteinaceous structure on the surface of *M. pneumoniae* in the attachment of this parasite to tracheal ring epithelium (9).

An avirulent strain of *M. pneumoniae* M129 has been obtained by co-workers (10), employing serial broth passage of the virulent organism. This avirulent strain does not cause histological pneumonia in hamsters (10), and will not attach to or cause cytopathology in tracheal ring epithelial cells in vitro (7). In an attempt to further confirm the identity of the trypsin-sensitive proteinaceous attachment factor(s) on the surface of *M. pneumoniae*, we employed one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to compare the proteins of the virulent, attaching strain to those of the avirulent, nonattaching strain. Gel electrophoretic comparisons of the protein composition of various mycoplasma strains (16, 17), and of strains of *M. pneumoniae* in particular (6, 10), have been performed previously, utilizing the standard technique of one-dimensional gel electrophoresis. We have determined that this type of protein analysis system is not always capable of discerning slight qualitative differences between mycoplasma cell protein samples which might be critical in establishing the role of particular protein species in virulence. We therefore utilized the exquisitely sensitive technique of two-dimensional polyacrylamide gel

electrophoresis (12) for the identification of protein similarities and differences between virulent and avirulent strains of *M. pneumoniae*.

MATERIALS AND METHODS

Organisms. The *M. pneumoniae* strain (M129) used in this study was originally isolated from a patient with mycoplasma pneumonia (10). This organism retained virulence during early broth passages as determined by its ability to produce histological pneumonia in hamsters after intranasal inoculation (10). The 10th broth passage of this organism (M129-B10) was designated as the virulent strain for this study. Subsequent to the 169th broth passage, this strain became avirulent, based on its inability to cause histological pneumonia in hamsters (10). The 181st broth passage level (M129-B181) was designated as the avirulent organism for this study.

Culture medium and growth conditions. Cultures of virulent and avirulent *M. pneumoniae* were grown at 37°C in 32-oz. (ca. 0.95-liter) glass prescription bottles containing 70 ml of Hayflick medium (10). Cultures of both mycoplasma strains were allowed to grow for 48 to 96 h, until the phenol red indicator in the growth medium became orange-yellow in color.

Cell harvesting procedure. The glass-adherent cells of the virulent strain were scraped into the spent culture medium by means of a rubber policeman to harvest these cells in a manner identical to that employed for the avirulent strain, which does not attach to glass, but grows in suspension in the culture medium. The suspending medium was then spun at 15,000 × *g* for 20 min at 4°C. The resultant cell pellet was resuspended in 10 ml of cold phosphate-buffered saline (PBS) (pH 7.2), and spun at 12,000 × *g* for 10 min at 4°C. The mycoplasma cells were then washed twice more with PBS by this procedure.

Trypsin treatment. The final washed pellet obtained from a single culture bottle was suspended in 10 ml of cold PBS in a 30-ml glass test tube. Trypsin (Sigma, Type XI, diphenylcarbamylchloride-treated, 7,500 α-*N*-benzoyl-L-arginine ethyl ester U/mg) was then added to a final concentration of 25 μg/ml. Cells were incubated in the presence of trypsin at 37°C for 12 min. Trypsinization was terminated by the addition of 20 ml of PBS containing 20 kallikrein inhibitor units (19) of the protease inhibitor Aprotinin (Sigma Chemical Co., St. Louis, Mo.) per ml, and the tube was plunged into ice water. The cells were then spun at 12,000 × *g* for 20 min at 4°C, and washed once more with 10 ml of the cold Aprotinin solution. The cells were then washed once with cold PBS, and the final pellet was prepared for cylindrical SDS-PAGE by previously published methods (9).

SDS-PAGE. Discontinuous SDS-PAGE of mycoplasma cell proteins was performed in cylindrical tubes as described previously (9).

Preparation of cell samples for two-dimensional gel electrophoresis. The disruption of *M. pneumoniae* cells and the solubilization of membrane proteins were accomplished by a modification of the procedure of Ames and Nikaido (1). The final washed cell pellet from a single culture bottle was uniformly suspended in 190 μl of 0.05 M tris(hydroxymethyl) aminomethane-hydrochloride buffer (pH 6.8) and 15 μl of 2-mercaptoethanol in a test tube (12 by 75 mm). A 53-μl volume of SDS (10%, wt/vol) and 15 μl of 2-mercaptoethanol were then added to the cell suspension. The contents of the tube were mixed well and allowed to stand at room temperature for 15 min. The tube was then heated at 100°C for 4 min and cooled to room temperature, and 95 μl of the resultant preparation was added to 55 mg of urea in a new tube. After dissolution of the urea at 37°C, 100 μl of the resultant preparation was added to 205 μl of lysis buffer (1). A 50- to 60-μl volume of this solution, representing approximately 75 μg of protein, was used for two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. Isoelectric focusing (IEF) of proteins in cylindrical gels was accomplished by the method of O'Farrell (12), using ampholines with the pH range 3.5 to 10. A total of 7,700 V-h was employed for IEF. Nonequilibrium pH gradient electrophoresis (NEPHGE) was performed by the procedure of O'Farrell et al. (13), using pH 3.5 to 10 ampholines and an electrophoresis period of 2,000 V-h. Both IEF and NEPHGE gels were equilibrated for 30 min in SDS sample buffer (12). SDS slab gel electrophoresis was performed by the method of Ames and Nikaido (1), using a 10% polyacrylamide separating gel (0.75 mm thick) with a 2-cm-high, 4% polyacrylamide stacking gel. Protein staining with Coomassie blue was accomplished by the method of O'Farrell (12). Chemicals and reagents for two-dimensional gel electrophoresis were obtained from the same sources used by O'Farrell (12). Molecular weight standards for both one- and two-dimensional electrophoresis were obtained from Bio-Rad Laboratories, Rockville Centre, N. Y. For two-dimensional electrophoresis, the molecular weight standards were run only in the second-dimension SDS slab gel by the method of Ames and Nikaido (1).

Iodination of mycoplasma cells. Intact cells of the virulent strain were labeled with ^{125}I by the lactoperoxidase method as described previously (9), with the exception that unlabeled carrier iodine was added to a final concentration of 1 mM. Carrier free ^{125}I (~17 Ci/mg) was obtained from New England Nuclear, Boston, Mass. Proteins from the iodinated cells were then prepared and separated on IEF and NEPHGE two-dimensional gels by the standard methods described above. Autoradiography was performed as described previously (9).

RESULTS

One-dimensional gel electrophoresis of *M. pneumoniae* proteins. Preliminary experiments employing one-dimensional SDS-PAGE showed that this gel electrophoretic system was capable of resolving 49 individual *M. pneumoniae* protein bands. These studies established that the protein profiles of the virulent M129-B10 strain and the avirulent M129-B181 strain were almost identical (Fig. 1). In addition, the avirulent strain was shown to possess a protein with a relative abundance and molecular weight essentially identical to that of the protein des-

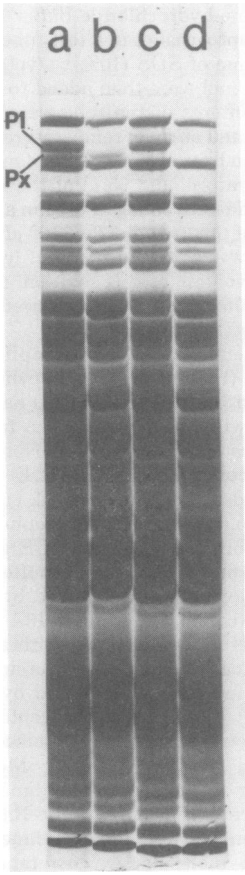

FIG. 1. *SDS-PAGE of proteins from virulent and avirulent strains of M. pneumoniae with and without exposure to trypsin. Cells were incubated in the presence or absence of trypsin as described in the text. (a) virulent cells minus trypsin; (b) virulent cells plus trypsin; (c) avirulent cells minus trypsin; (d) avirulent cells plus trypsin. The proteins designated as P1 and P_x are indicated by the appropriate arrows.*

ignated as the trypsin-sensitive attachment factor P1 in a previous study (9). The P1-like protein of the avirulent strain was also shown to be readily removed from cells of this strain by trypsin (Fig. 1).

This study also revealed that the virulent strain possessed a single high-molecular-weight protein not present in the avirulent strain. This protein (P_x) banded just beneath P1 in 7.5% polyacrylamide gels, and was relatively insensitive to trypsin cleavage under the conditions employed in this experiment (Fig. 1). Resolution of P_x as distinct from P1 was not always reproducible, however, as P_x did not appear as a separate entity in approximately half of the non-trypsin-treated samples of virulent strain cell proteins analyzed by means of one-dimensional gel electrophoresis. In contrast, the P_x protein was never seen in any of the numerous avirulent strain gels prepared during this study.

Two-dimensional gel electrophoresis of *M. pneumoniae* proteins. The difficulty encountered in the reproducible resolution of P_x indicated that a gel electrophoretic technique with increased resolving power was required for the reliable identification of protein differences between virulent and avirulent mycoplasma strains. In view of limited data concerning virulence factors possessed by *M. pneumoniae*, the elucidation of protein species differences between homologous virulent and avirulent strains might provide a potential basis for the identification of proteinaceous virulence factors. A combination of isoelectric focusing and molecular weight-based protein separation methods, as embodied in the O'Farrell two-dimensional gel electrophoresis technique (12), was selected for use.

The lack of a peptidoglycan-containing cell wall structure facilitated analysis of *M. pneumoniae* total cell protein, as it was determined that heating a concentrated suspension of mycoplasma cells at 100°C for 4 min in the presence of 2% (wt/vol) SDS and 10% (vol/vol) 2-mercaptoethanol resulted in essentially complete lysis of these membrane-bound procaryotes, with the concomitant solubilization of membrane-bound proteins (1). Two-dimensional IEF-SDS gel analysis of proteins from cells of the virulent strain prepared in this manner revealed that this technique could resolve up to 142 individual mycoplasma cell proteins (Fig. 2a), using Coomassie blue staining for protein detection. The tremendous resolving power of this two-dimensional gel system is immediately obvious, as only 49 mycoplasma protein bands could be detected on standard one-dimensional SDS-PAGE gels (Fig. 1). In accordance with standard convention (12), the IEF-SDS gel appears with the basic end on the left, the acidic end on the right, and with proteins of decreasing molecular weight running from top to bottom. The standard IEF-SDS gel employed here generates an effective pH gradient from about pH 4.0 on the acid end to about pH 8.0 on the basic end.

The use of NEPHGE-SDS gels was required to effectively resolve those proteins with very basic isoelectric points. The increased resolution of proteins possessing relatively basic isoelectric points from the virulent strain in NEPHGE-SDS gels is evidenced by the fact that essentially all of the protein spots to the left of, and including, the protein spot labeled C (Fig. 3a) appear as blurred streaks on the extreme left (basic) end of the IEF-SDS gel (Fig. 2a). The use of NEPHGE-SDS gels permitted the resolution of

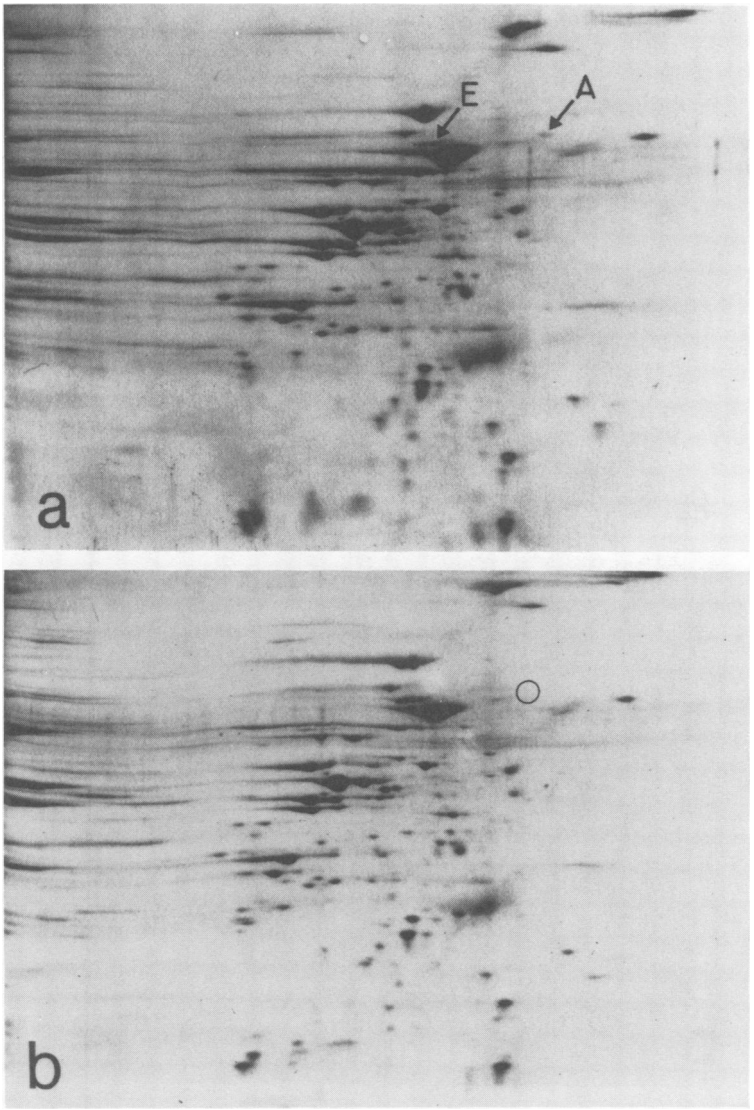

FIG. 2. *IEF-SDS electrophoretic analysis of proteins from virulent and avirulent strains of M. pneumoniae. (a) Virulent M. pneumoniae. The protein spot A possessed by the virulent strain and lacking in the avirulent strain is indicated by an arrow. The protein spot E, which represents a culture medium protein, is indicated by the appropriate arrow. (b) Avirulent M. pneumoniae. The absence of protein spot A is indicated by the circle.*

161 individual *M. pneumoniae* cell proteins, as detected by Coomassie blue staining.

Identification of protein differences between virulent and avirulent strains. Analysis of photographic enlargements of IEF-SDS and NEPHGE-SDS gels of cell proteins from the virulent M129-B10 strain and the avirulent M129-B181 strain revealed a number of qualitative protein differences between these two strains. Protein spot A (molecular weight 72,000) appears on the IEF-SDS gel of the virulent strain (Fig. 2a) and is not present on the IEF-SDS gel of the avirulent strain (Fig. 2b). Other differences in the protein composition of the virulent and avirulent strains were identified by the resolution of proteins via the use of NEPHGE-SDS gels. The virulent strain possessed protein spot B (molecular weight 85,000) (Fig. 3a), which is not present in the avirulent strain (Fig. 3b). It is of interest to note that this particular protein spot has no corresponding spot in the same approximate pH range in the IEF-SDS gel (Fig. 2a). This anomaly probably reflects the fact that, as opposed to IEF-SDS

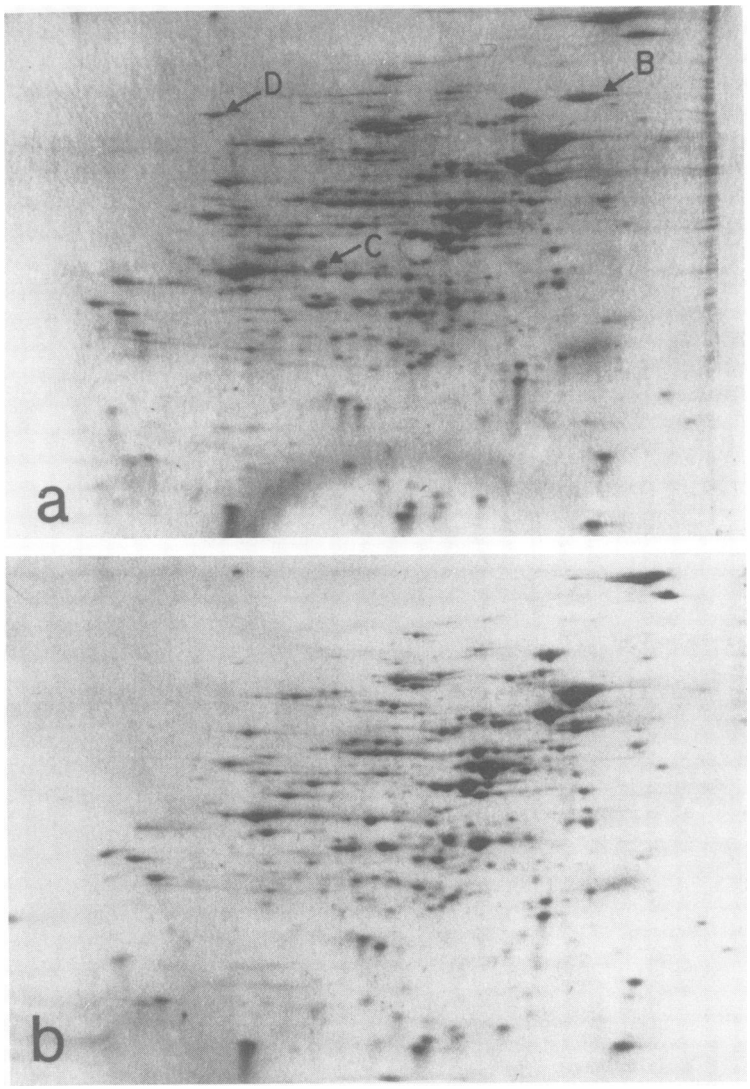

FIG. 3. *NEPHGE-SDS electrophoretic analysis of proteins from virulent and avirulent strains of M. pneumoniae. (a) Virulent M. pneumoniae. The protein spots B and C possessed by the virulent strain and lacking in the avirulent strain are indicated by the appropriate arrows. Protein spot D is also indicated by an arrow in this figure. (b) Avirulent M. pneumoniae.*

gels, the parameters of protein separation in the first dimension of the NEPHGE-SDS system involve more than just isoelectric points alone (13). In addition, the protein spot C (molecular weight 37,00) (Fig. 3a) is associated with virulent *M. pneumoniae*, but not with the avirulent strain (Fig. 3b). Protein spot D (molecular weight 82,000), which is present in the virulent strain (Fig. 3a) and apparently lacking or much decreased in relative abundance in the avirulent strain (Fig. 3b), represents the single protein whose appearance on NEPHGE-SDS gels is quite variable between experiments. Therefore, it has not been conclusively determined whether this protein represents a qualitative or quantitative protein difference between these virulent and avirulent strains of *M. pneumoniae*.

Detection of a culture medium protein. Two-dimensional gel electrophoresis of various fractions of the mycoplasma culture medium has established that protein spot E (molecular weight 68,000) (Fig. 2a and b) is a protein contaminant of these *M. pneumoniae* cells, derived from the agamma horse serum component of

Hayflick medium (10). The presence of serum proteins in preparations of mycoplasma cells, noted in previous studies (5, 21), may be due to either co-precipitation of this serum protein with mycoplasma cells during the harvesting procedure (21), or may be caused by specific adherence of this serum protein to cells of *M. pneumoniae* (18). Co-electrophoresis of samples of Hayflick medium and purified horse serum albumin has tentatively identified this protein contaminant of the *M. pneumoniae* cells as horse serum albumin (data not shown).

Identification of membrane surface proteins. Intact cells of the virulent strain were labeled with radioactive iodine (^{125}I) by the lactoperoxidase method, and proteins from the labeled cells were analyzed on IEF-SDS and NEPHGE-SDS gels. Many fewer proteins were revealed by autoradiographic procedures (Fig. 4), relative to Coomassie blue-stained preparations (Fig. 2 and 3), and these iodinated proteins are presumed to be exposed on the external membrane surface of *M. pneumoniae* (14). The virulent strain-specific protein B was identified

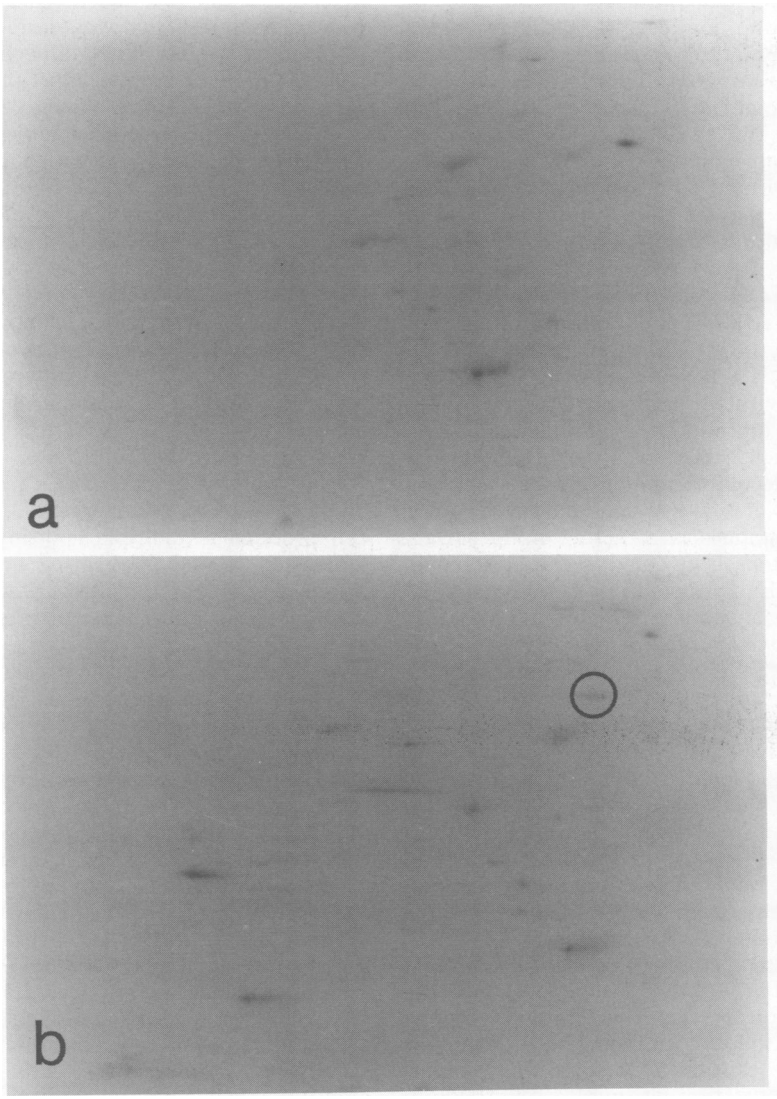

FIG. 4. *Two-dimensional gel autoradiograms of proteins from intact cells of the virulent strain of M. pneumoniae labeled with ^{125}I. (a) IEF-SDS gel; (b) NEPHGE-SDS gel. The virulent strain-specific protein B is circled in (b).*

as a membrane-surface protein by this technique (Fig. 4). In addition, the virulent strain-specific protein B has been shown to be removed or altered by trypsin treatment of intact mycoplasma cells (data not shown).

DISCUSSION

The relative paucity of information concerning virulence determinants of *M. pneumoniae* has hindered the development of an effective vaccine to protect against this respiratory tract pathogen. The existence of a proteinaceous attachment factor (P1) on the membrane surface of virulent *M. pneumoniae* (9) prompted examination of the homologous avirulent strain, which does not attach to respiratory epithelium (3, 15), to determine if this trypsin-sensitive attachment factor was lacking, or in some way modified, in the avirulent strain. One-dimensional gel electrophoretic comparison of proteins from these virulent and avirulent strains of *M. pneumoniae* showed that the avirulent strain did possess a protein apparently identical to P1 in both molecular weight and trypsin sensitivity (Fig. 1). The existence of this P1-like protein in the avirulent strain does not rule out a functional role for P1 in the attachment of *M. pneumoniae* to host cells. It is possible that the P1-like protein of the avirulent strain has been altered by spontaneous mutation to a nonfunctional state, an event which would not necessarily alter this protein's molecular weight or trypsin sensitivity. Alternatively, it is conceivable that there is more than one proteinaceous structure on the surface of *M. pneumoniae* involved in the attachment mechanism. Equally plausible is the possibility that a previously undetected protein on the surface of *M. pneumoniae* is responsible for the trypsin-sensitive attachment mechanism. In the latter two instances, the relatively low resolving power of the one-dimensional gel electrophoretic system employed in the previous study (9) might have prevented identification of this protein(s).

The use of the recently developed technique of two-dimensional gel electrophoresis has firmly established that the virulent M129-B10 strain of *M. pneumoniae* differs from its homologous avirulent strain by a minimum of three different proteins (A, B, and C in Fig. 2 and 3). In addition, the membrane surface location and trypsin sensitivity of the virulent strain-specific protein B indicate that this protein could be involved in the trypsin-sensitive attachment mechanism(s) utilized by virulent *M. pneumoniae* in its colonization of respiratory epithelium. That these differences between the virulent and avirulent strains were not detected in an earlier study (10) can be attributed to the use of the inherently less sensitive one-dimensional gel electrophoresis technique. A different study which found a qualitative protein difference between virulent and avirulent strains of *M. pneumoniae* employed an avirulent strain which was not derived from the virulent strain used for comparative purposes (6). The significance of this particular finding is difficult to interpret, since it has already been shown that different strains of another *Mycoplasma* species, *M. gallisepticum*, differ from one another in qualitative protein content (17).

Using Coomassie blue staining as the protein detection technique, at least 160 proteins from preparations of *M. pneumoniae* cells could be resolved by the use of two-dimensional gel electrophoresis. This number most probably represents a lower limit of resolvable *M. pneumoniae* cell proteins. Studies with soluble proteins from *Escherichia coli* showed that about 400 proteins can be detected on an IEF-SDS gel with Coomassie blue staining. The use of radioactively labeled amino acids and autoradiographic procedures increased the number of detectable proteins to 1,100 (12). We are currently developing methodology for the efficient intrinsic radioisotope labeling of *M. pneumoniae* proteins, and it is possible that additional differences between these virulent and avirulent strains of *M. pneumoniae* may be detected by the use of radiolabeled protein preparations.

One potential artifact which may be present on these two-dimensional gels of *M. pneumoniae* cell proteins is multiple spotting in the first dimension caused by heating proteins in the presence of SDS (20). This artifact, as possibly represented by the cluster of proteins beneath and to the left of protein spot C in Fig. 3a, is unavoidable due to the requirement for heating of the *M. pneumoniae* cells in the presence of SDS to obtain complete cell lysis and solubilization of membrane-bound proteins. Nonetheless, the protein spot patterns obtained using the standard cell lysis technique are completely reproducible and, thus, these few possible artifactual spots do not in any way interfere with the detection of specific protein differences between the virulent and avirulent strains.

Homologous virulent and avirulent strains of microorganisms may differ from one another in a number of parameters, including their qualitative and quantitative protein, carbohydrate, and lipid compositions. The use of two-dimensional electrophoresis has greatly advanced our ability to resolve minute qualitative protein differences between virulent and avirulent strains of *M. pneumoniae*. With proper methodology, this technique can also be used to detect quan-

titative protein differences between strains (2, 11). When coupled with the use of appropriate model systems, such as tracheal organ culture (3, 7) for the study of microbial virulence, the use of two-dimensional gel electrophoresis should significantly enhance our ability to identify, interpret, and understand proteinaceous microbial virulence mechanisms.

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