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Protein Variability among Strains of *Mycoplasma pulmonis*

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The proteins of *Mycoplasma pulmonis* were examined by two-dimensional polyacrylamide gel electrophoresis and immunoblotting. Comparison of profiles from silver-stained two-dimensional polyacrylamide gel electrophoresis of 18 strains of *M. pulmonis* allowed identification of five proteins that were strain variable. These variable proteins were not dependent on the medium used to grow the organisms and were shown by reaction with serum samples from naturally infected rats and mice to be produced in vivo and not to be medium components. Identification of strain-variable proteins may lead to an explanation of the differences in properties found among *M. pulmonis* strains.

Murine respiratory mycoplasmosis (MRM) caused by *Mycoplasma pulmonis* is one of the most common and important naturally occurring diseases of laboratory mice and rats (2). *M. pulmonis* is also the etiologic agent of a naturally occurring genital disease of rodents (1). MRM is easily reproducible; experimental *M. pulmonis* infections are used as models of other mycoplasmal diseases of humans and animals and for the study of the pathogenesis of chronic infectious respiratory disease (1). MRM has been well characterized morphologically in both rats and mice, and the cellular nature of the disease is partially understood (2). However, the molecular mechanisms of MRM and other mycoplasmal diseases have not been well elucidated.

Nichols and Kenny have previously shown that at least one *M. pulmonis* antigen (or antigen complex) varies among the four strains studied (13). *M. pulmonis* strains can also vary in several properties, including virulence, mitogenicity, resistance to phagocytosis, and hemadsorption (8, 12, 16). Identification of proteins that vary among strains may lead to identification of proteins involved in these processes. The purpose of our study was to identify the proteins of a large number of *M. pulmonis* strains by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and to determine, with this technique, which proteins vary among strains.

The 18 strains of *M. pulmonis* used in this study were from various tissues of rats and mice and from diverse geographic locations. Strains UAB 5782C, UAB 6510, UAB 8145D, UAB T, and UAB 3794 were obtained from G. H. Cassell and M. K. Davidson, Department of Microbiology, University of Alabama at Birmingham, Birmingham; strain PG34(ASH) is ATCC 19612; strain OGATA T was obtained from G. Ogata, Department of Veterinary Public Health, Azabu University, Fuchinobe Sagamihara, Kanagawa 229, Japan; strains 66 and PETER C were obtained from G. Taylor, Agricultural Research Council, Institute for Research on Animal Diseases, Compton Newbury, Berks RG16 0NN, England; and strains M1, WRAIR, NELSON C, 7MC-A-1, GINSBURG, NEGRONI, JB, KON, and PG-22(M50) were obtained from J. G. Tully, Mycoplasma Section, Laboratory for Molecular Biology, Frederick Cancer Research Facility, Frederick, Md. Four media were used for growth of *M. pulmonis*. Broth A was a slight modification of one recommended for the growth of murine mycoplasmas (3). The medium was buffered with 13.0 g of *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.3; Research Organics, Cleveland, Ohio) per liter to reduce acid precipitation of medium components. Broth B was identical to broth A except that 20% fetal calf serum was used in lieu of horse serum. Broth C was a dialyzed medium using Frey mycoplasma broth base and was prepared as described elsewhere (11). Broth D was identical to broth A except that a different base was used (*Mycoplasma* broth base without crystal violet; GIBCO Laboratories, Grand Island, N.Y.) and no HEPES was added. The pH was adjusted to 7.8.

Cultures of each strain were harvested and washed three times in phosphate-buffered saline by centrifugation at 12,000 × *g* for 10 min at 4°C. The protein content of each culture was estimated by the method of Bradford (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard. Samples were prepared and 2D PAGE was done by the method of O'Farrell (14). Twenty micrograms of protein was loaded per gel and focused for 16 h at 300 V and for 1 h at 800 V. Second dimensions were obtained in 10% resolving and 4% stacking gels. Protein profiles were visualized by silver staining (10).

M. pulmonis UAB 5782C, a low-passage strain isolated from naturally occurring MRM and grown in media A, B, C, and D, was analyzed by 2D PAGE and showed essentially identical results regardless of the medium used. Occasional quantitative differences were seen, but there were no obvious qualitative differences. In addition, UAB 5782C harvested at various phases of the growth cycle showed no qualitative differences. Because of overall higher protein yields obtained from cultures in late logarithmic phase (between 10 and 14 mg/liter with no consistent variation among strains), all organisms for subsequent experiments were harvested in late logarithmic phase in broth A.

2D PAGE profiles from 18 strains of *M. pulmonis* showed that the majority of proteins (over 250) resolved between pHs 4.5 and 7.0 with molecular masses of 10 to 150 kilodaltons and were common to all 18 strains, suggesting limited protein heterogeneity. Variable proteins were found in two areas, one acidic and one basic (Fig. 1). Three of these variable proteins (proteins 2, 3, and 5) correspond to proteins which were previously shown to be surface exposed (7). A composite of the variations seen in the basic area is shown in Fig. 2. These proteins are in an area of approxi-

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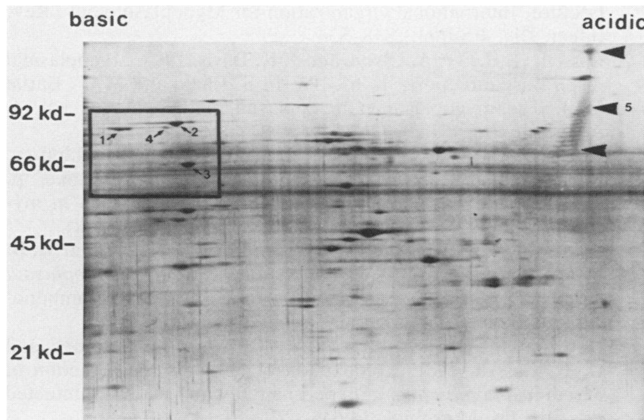

FIG. 1. Proteins of *M. pulmonis* UAB 5782C were separated by 2D PAGE and silver stained. Orientation of the isoelectric-focusing dimension is indicated (basic and acidic). Molecular weights are given in kilodaltons (kd). The box indicates the area of basic protein variation (spots 1 to 4), and arrows show the variable acidic protein complex (arrow 5).

mately 60 to 90 kilodaltons with isoelectric points of 6.0 to 6.5. The variation at the acidic end of the gel consisted of an unusual protein complex with proteins which varied symmetrically by molecular weight and pI. This complex did not appear to be a protein degradation product, as the presence of protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin A, and aprotinin) during sample preparation had no effect on the pattern. Among the 18 strains, some showed no indication that this complex was present, while others had one or two such complexes.

2D PAGE profiles of UAB 5782C were immunoblotted with enzyme-linked-immunosorbent-assay-positive serum samples from four rats and four mice naturally infected with *M. pulmonis* (confirmed by cultural isolation). These samples were chosen from breeder colonies, each with no known relationship to the other breeder colonies, that were separated geographically (Alabama, Maryland, New York, Oklahoma, and California). Proteins were transferred to nitrocellulose (Bio-Rad) by the method of Towbin et al. (17). Nonspecific binding sites were blocked by incubation in phosphate-buffered saline containing 10% fetal calf serum and 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co., St. Louis, Mo.). Mouse and rat serum samples were diluted 1:200 in broth A and reacted with nitrocellulose blots. Specifically bound antibody was identified by using biotinylated goat anti-mouse immunoglobulin G (Sigma) or anti-rat immunoglobulin G (Sigma) followed by avidin-peroxidase. Peroxidase activity was visualized with 4-chloro-1-naphthol (HRP color development reagent; Bio-Rad). Figure 3 is representative of all serum samples tested; many of the common and all of the variable proteins (1 to 5) were recognized, indicating that these were produced in vivo in both rats and mice and were not medium components.

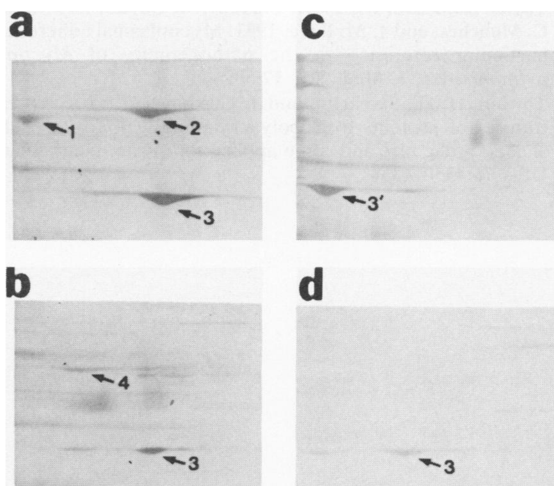

FIG. 2. Enlargement of the basic variable proteins (box in Fig. 1) found among strains of *M. pulmonis*. Proteins were separated by 2D PAGE and silver stained. (a) UAB 5782C; (b) PG34(ASH); (c) 66; (d) JB.

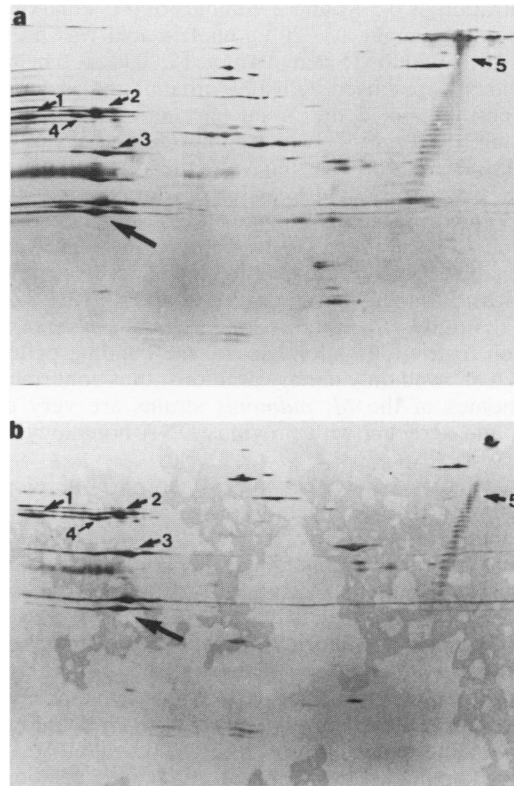

FIG. 3. Immunoblots of *M. pulmonis* UAB 5782C reacted with serum samples from naturally infected animals. Proteins were separated by 2D PAGE, transferred to nitrocellulose, and reacted with sera from a naturally infected rat (a) or mouse (b). Small numbered arrows (1 to 5) indicate reactions with strain-variable proteins. The large arrow indicates reaction with a major group of strain-constant proteins.

DNAs from 14 of the *M. pulmonis* strains were examined by using restriction endonucleases and separation by electrophoresis. Washed *M. pulmonis* cells in phosphate-buffered saline were lysed by the addition of sodium dodecyl sulfate (Bio-Rad) to a final concentration of 1.0%, and DNA was isolated by multiple extractions with a phenol-chloroform mixture to yield a clean interphase. The phenol-chloroform mixture was prepared by mixing equal volumes of phenol saturated with 10 mM Tris hydrochloride, 1 mM EDTA (Sigma), and 100 mM NaCl buffer (pH 8.0). The final aqueous phase was extracted with ether, and nucleic acid was precipitated by the addition of sodium acetate to a

concentration of 0.3 M and 2 volumes of 0°C ethanol. After incubation overnight at −20°C, nucleic acid was harvested by centrifugation for 15 min at 4°C at 15,000 × *g*. The nucleic acid pellets were rinsed with 70% ethanol and suspended at a concentration of 2 mg of nucleic acid per ml in buffer consisting of 10 mM Tris hydrochloride and 1 mM EDTA (pH 8.0). DNA was digested with the restriction endonucleases *Pst*I, *Eco*RI, *Bgl*II, and *Mbo*I (containing 20 μg of RNase A per ml) as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). DNA digests were analyzed in 0.8% agarose gels. The electrophoresis buffer was 89 mM Tris-borate–2 mM EDTA (pH 8.0) containing 0.5 μg of ethidium bromide (Sigma) per ml of buffer. For each restriction endonuclease, the banding patterns of the DNA showed only minor variations, thus confirming that the genomes of the *M. pulmonis* strains are very closely related and agreeing with previous DNA homology studies (15).

Contradictory evidence exists concerning the degree of heterogeneity to be expected among *M. pulmonis* strains. Functional serologic tests, particularly metabolic or growth inhibition assays, suggest that there is a large degree of heterogeneity among strains (6, 9). In contrast, the enzyme-linked immunosorbent assay, which primarily recognizes protein and does not depend on antibody function, suggests that limited heterogeneity is present (4, 5, 11). Our results also suggest limited protein heterogeneity among strains. The few variable proteins thus far identified could actually be homologous proteins (as suggested by Fig. 3) that have undergone amino acid substitutions or genetic deletions that cause different pIs (e.g., proteins 3 and 3′ in Fig. 2) or changes in molecular weight which may or may not alter function. Therefore, further examination of these variable proteins is warranted. Immunological identity can be analyzed by using monospecific, polyclonal antisera or monoclonal antibodies to these specific antigens. Use of specific immunological probes for in vitro and in vivo functional assays will determine if these variable proteins are responsible for any of the strain differences mentioned earlier. These studies will provide the experimental tools necessary to begin to understand the molecular mechanisms of *M. pulmonis* disease pathogenesis.

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