

Protein and Antigen Heterogeneity among Strains of *Mycoplasma fermentans*

C. T. K.-H. STÄDTLANDER,* C. ZUHUA, H. L. WATSON, AND G. H. CASSELL
Department of Microbiology, Schools of Medicine and Dentistry, University of Alabama
at Birmingham, Birmingham, Alabama 35294

Received 7 March 1991/Accepted 18 June 1991

The proteins and antigens of three strains of *Mycoplasma fermentans* were compared with those of a mycoplasma, designated "*Mycoplasma incognitus*," recently identified in tissues of AIDS patients. Previous studies have shown that "*M. incognitus*" is most likely not a new species but rather a strain of *M. fermentans*. In the present study, one- and two-dimensional electrophoretic analysis demonstrated the expected similarity between these mycoplasmas, but it also demonstrated several distinct protein differences. Nine proteins were identified as strain variable by two-dimensional gel electrophoresis. Also, immunoblot analysis using rabbit antiserum against the type strain of *M. fermentans* (strain PG 18) documented the occurrence of size heterogeneity in at least one and possibly two other antigens.

Mycoplasma infections in human adults are generally noninvasive and nonfatal (2). An organism initially identified as "*Mycoplasma incognitus*" (9, 12) has recently been linked with renal and central nervous system complications in patients with AIDS (1, 13), as well as with an acute fatal respiratory disease in otherwise healthy adults (10). Further serologic and genomic analyses indicate that this organism is not a new species of the genus *Mycoplasma* but rather a strain of *Mycoplasma fermentans* (12, 22).

Since its original isolation from the human urogenital tract in 1950 (21), *M. fermentans* has been isolated infrequently from humans. Early serologic studies, however, suggest that antibody to this organism is common in adolescents and young adults (19, 23). Attempts to recover *M. fermentans* (incognitus strain) from clinical material indicate that it may be difficult to cultivate on artificial media (12). Furthermore, electron microscopic data suggest, albeit do not alone prove, that in contrast to other species of mycoplasmas, *M. fermentans* (incognitus strain) can be found intracellularly (1, 9, 10, 11, 14). These findings could in part explain the apparent discrepancy regarding the prevalence of *M. fermentans* as determined by serologic and cultural data. The present investigation was undertaken to identify the proteins of *M. fermentans* (incognitus strain) by two-dimensional gel electrophoresis using isoelectric focusing (IEF-2D-PAGE) and two-dimensional nonequilibrium-pH-gradient electrophoresis (NEPHGE-2D-PAGE) and to compare them with the proteins and antigens of three reference strains of *M. fermentans*. Information obtained could be useful for the development of better serologic methods and perhaps for a better understanding of the organism's pathogenic potential.

The mycoplasmas used in this investigation were obtained from the following sources: *M. fermentans* (incognitus strain) was provided by S.-C. Lo (American Registry of Pathology, Armed Forces Institute of Pathology, Washington, D.C.), and *M. fermentans* PG 18, K 7, and MT-2 were obtained from J. G. Tully (National Institute of Allergy and Infectious Diseases). Strains PG 18, K 7, and MT-2 were grown in modified Friis medium (5) (supplemented with

porcine serum) basically as described elsewhere (7), except that Cefobid (cefoperazone sodium; Roerig Pharmaceuticals) was used as the antibiotic (250 µg/liter). This medium was buffered at pH 7.4 with 13 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per ml. Cultures of *M. fermentans* incognitus were grown in SP-4 medium (25) supplemented with fetal bovine serum. In some experiments, all strains were grown in both media to determine whether different protein or antigen expression may result from the differences in growth media. Mycoplasmas were harvested and washed in Dulbecco's phosphate-buffered saline (3), and protein concentrations were estimated by the method of Lowry et al. (15).

IEF-2D-PAGE or NEPHGE-2D-PAGE was performed basically by the method of O'Farrell et al. (17, 18) and as described previously (27, 29). In brief, 20 µg of protein was loaded per first-dimension gel and run for 24 h at 400 V, followed by 1 h at 1,000 V (IEF-2D-PAGE) or 3 h at 500 V (NEPHGE-2D-PAGE). For the separation of proteins in the second dimension, tube gels were grafted horizontally onto the top of a slab gel (10% resolving gel, 4% stacking gel, 0.75 mm thick) and protein profiles were visualized by silver staining (16) with the GELCODE Color Silver Stain Kit (Pierce).

IEF-2D-PAGE permitted the resolution of at least 140 protein spots, and about 90 proteins were identified by NEPHGE-2D-PAGE. The majority of the protein spots were common to all three reference strains of *M. fermentans* and also to the incognitus strain. By overlay comparison of individual gels, it was found that some proteins were absent from one or more strains (nine numbered spots in Fig. 1). The number of proteins that varied among the reference strains of *M. fermentans* was not higher than the number that varied between the incognitus strain and individual reference strains of *M. fermentans*. Slight differences in the concentrations of individual proteins (not indicated in figures) were also observed. This protein analysis is in agreement with previous studies on the genetic relatedness of the incognitus strain to *M. fermentans* (12, 22) which concluded that "*M. incognitus*" is a strain of *M. fermentans*.

To analyze the antigenic relationships between the *M. fermentans* reference strains and the incognitus strain, one-

* Corresponding author.

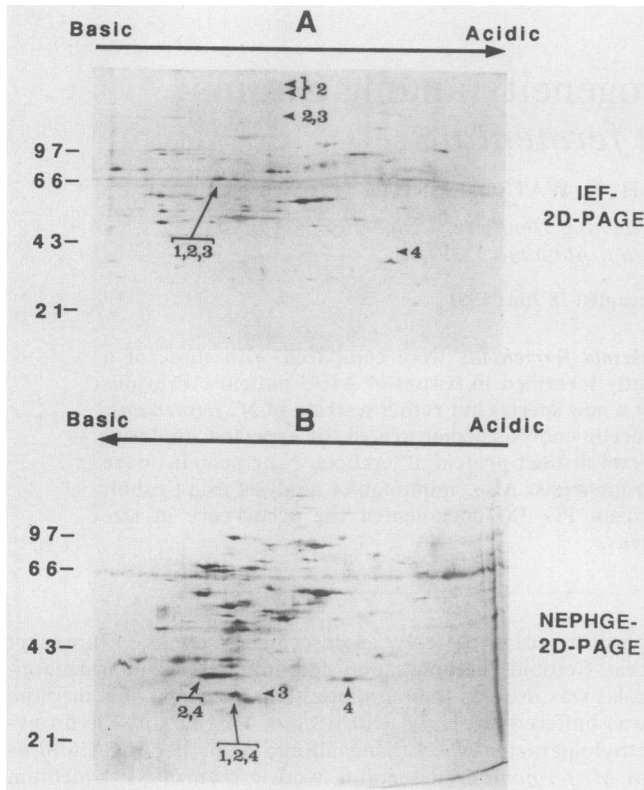


FIG. 1. Two-dimensional electrophoretic analysis of proteins from *M. fermentans* reference strains and *M. fermentans* incognitus as visualized by silver stain. Twenty micrograms of protein was loaded per gel. Representative protein patterns are shown in panels A and B. (A) An IEF-2D-PAGE gel of *M. fermentans* (incognitus strain). About 140 individual proteins were identified. (B) A NEPHGE-2D-PAGE gel of *M. fermentans* PG 18. About 90 individual proteins were identified. The arrows indicate the positions of proteins that vary among *M. fermentans* reference strains and *M. fermentans* incognitus (a total of nine for both panels). The number(s) associated with each arrow indicates which strain had proteins in the corresponding position, as follows. 1, *M. fermentans* incognitus; 2, *M. fermentans* K 7; 3, *M. fermentans* MT-2; 4, *M. fermentans* PG 18. Molecular masses (in kilodaltons) are shown to the left.

dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8) and immunoblotting were utilized as previously described (28). Separated proteins were either visualized with Coomassie blue stain (17) or transferred to nitrocellulose (24) for immunoblotting. Immunological reactions were performed with a rabbit anti-*M. fermentans* PG 18 antiserum (1:500 dilution; obtained from J. G. Tully) that was prepared in Hayflick's medium (6) containing horse serum. Positive reactions were visualized with peroxidase-labeled conjugates (Sigma) as described previously in detail (29). Controls for the immunoblot reactions included reaction of the anti-PG 18 rabbit antiserum with immunoblots of the media in which the organisms were grown and reaction of a nonimmune rabbit serum with immunoblots of the different *M. fermentans* strains. No reactions were detected for these controls.

The SDS-PAGE banding patterns of the three reference strains of *M. fermentans* and of the incognitus strain visualized by Coomassie blue are shown in Fig. 2A and reveal essentially identical banding profiles. However, with careful

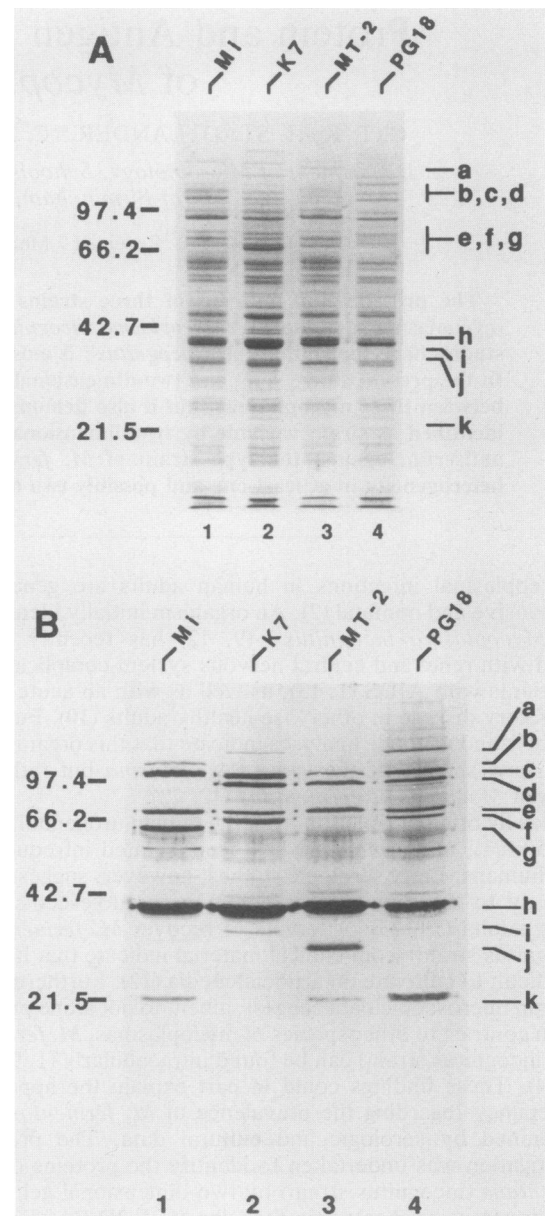


FIG. 2. SDS-PAGE and immunoblot analysis of *M. fermentans* incognitus and three reference strains of *M. fermentans*. Proteins in panel A (20 μ g per well) were separated by SDS-PAGE (4% stacking gel, 10% resolving gel) and visualized by Coomassie blue stain. Proteins in panel B (10 μ g per well) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with rabbit anti-*M. fermentans* PG 18 antiserum. The antibody-reactive bands in panel B were visualized with peroxidase-labeled conjugates. Lanes (both panels): 1, *M. fermentans* incognitus (Mi); 2, *M. fermentans* K 7; 3, *M. fermentans* MT-2; 4, *M. fermentans* PG 18. Molecular masses are shown to the left (in kilodaltons). Letters to the right of panel A indicate areas containing subtle differences between strains, slight differences in the intensities of certain bands, or slight shifts in molecular masses. These differences are more easily detectable in panel B; letters in this panel correspond to the same areas indicated in panel A. The immunoblot patterns were unchanged whether organisms were grown in Friis or SP4 medium.

TABLE 1. Immunoblot reactions of *M. fermentans* strains with rabbit antiserum to strain PG 18

Antigen ^a	Reaction with the following strain ^b :			
	M.i. ^c	K 7	MT-2	PG 18
a	—	+	—	+
b	+	—	—	—
c	+	+	+	+
d	—	+	+	+
e	+	+	+	+
f	—	+	±	+
g	+	—	—	—
h	+	+	+	+
i	—	+	—	±
j	—	—	+	—
k	+	—	+	+

^a Letters indicate antigen bands shown in Fig. 2.

^b +, presence of the band; —, absence of the band; ±, weak reaction with the band.

^c *M. fermentans incognitus*.

examination, slight differences in the concentrations of single bands as well as possible molecular mass differences could be detected.

Although there have been other studies showing serologic relatedness of the incognitus strain with *M. fermentans* strains (12, 22), none of these have used techniques which would allow identification of specific antigens. In the present study, immunoblot analysis, using polyclonal antiserum against the *M. fermentans* type strain PG 18, revealed distinct differences among the strains (Fig. 2B). These differences in molecular mass are indicated by the letters to the right of the panel, and it can be easily seen that no two strains are identical. Table 1 summarizes these findings. It should be emphasized that all reactive bands in all strains were identified with antiserum to the type strain PG 18, suggesting the size heterogeneity of the observed antigens. For example, MT-2 has a band (j) which is not detectable in the incognitus strain or the other two *M. fermentans* reference strains. However, this band must contain epitopes identical to epitopes of at least one antigen of strain PG 18. Additional differences include two bands (b and g) found only in the incognitus strain, whereas two other bands (d and f) were absent only from this strain. With the exception of MT-2, which contained a single unique band (j), all strains contained bands which were either present or absent in two or more strains.

To more specifically address the antigen size heterogeneity observed in Fig. 2B, monospecific antibodies against bands j and h of strain MT-2 (Fig. 2B, lane 3) were obtained and reacted with the other strains. For this purpose, 500 µg of protein of a fresh culture of MT-2 was loaded on a slab gel (0.75 mm thick, without using a comb), and the separated MT-2 proteins were then transferred to nitrocellulose. After the blocking of nonspecific binding sites (29), the blot was reacted with the hyperimmune serum (1:500 dilution) against strain PG 18 for 3 h at 37°C and washed. Vertical strips were cut from the right and left sides of the blot and were reacted with a 1:1,000 dilution of biotinylated goat anti-rabbit immunoglobulin G (Sigma) for 1 h. The strips were washed, reacted with avidin-peroxidase conjugate, and developed as described elsewhere (29). These two developed strips were used to identify the positions of bands j and h of MT-2 on the remaining undeveloped nitrocellulose. Two horizontal strips, one containing band j and one containing band h,

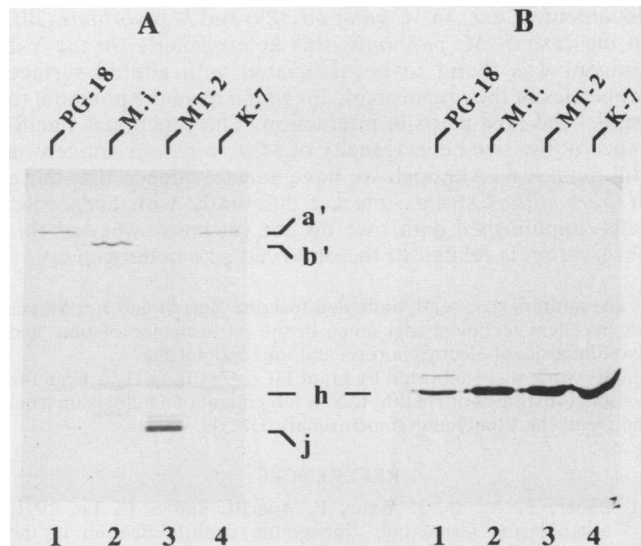


FIG. 3. SDS-PAGE immunoblots of *M. fermentans incognitus* and three reference strains of *M. fermentans* reacted with a monospecific antibody against band j (panel A) and band h (panel B) of *M. fermentans* MT-2. Reactive bands in both panels were visualized with peroxidase-labeled conjugates, as described in the text. Lanes (both panels): 1, *M. fermentans* PG 18; 2, *M. fermentans incognitus* (M.i.); 3, *M. fermentans* MT-2; 4, *M. fermentans* K 7. Letters indicate areas of reactive bands comparable to those described in the legend to Fig. 2.

were cut from the undeveloped nitrocellulose and placed for 30 min in a tube containing a low-pH buffer (50 mM glycine HCl-0.15 M NaCl, pH 2.34) for eluting the monospecific antibody against bands j and h from the nitrocellulose. The solution was immediately neutralized by adding a high-pH buffer (containing 50 mM Tris HCl-0.5 M NaCl, pH 9.0). The antibody solution diluted in blocking buffer was then used for reaction with SDS-PAGE-separated proteins of all four *M. fermentans* strains.

As shown in Fig. 3A, the monospecific antibody to band j reacted with band j of MT-2 and with higher-molecular-mass bands (97 to 100 kDa) of the other strains. There were also some faint reactions at approximately 40 kDa. The reaction of the monospecific antibodies to band h, which appeared to be common to all strains (Fig. 2B), is shown in Fig. 3B. As expected, the h band reacted in all strains, but there was an additional band with a slightly higher molecular mass which reacted in strains PG 18 and MT-2 (Fig. 3B, lanes 1 and 3). These results (particularly in Fig. 3A) document an epitopic relationship between different-sized antigens of the different strains.

The results of the present study establish further evidence that "*M. incognitus*" is a strain of *M. fermentans*. More importantly, although all *M. fermentans* strains (including the incognitus strain) revealed obvious similarities in their protein and antigen profiles, there was size heterogeneity among their antigens. However, the protein and antigenic patterns of *M. fermentans incognitus* were not more unique than those of the *M. fermentans* reference strains. Therefore, if *M. fermentans incognitus* is a novel pathogen as compared with other *M. fermentans* strains, the properties responsible are not obviously detected with the techniques used in the current study.

Antigen size heterogeneity among mycoplasmas has been

documented, e.g., in *M. pulmonis* (29) and *M. hyorhinis* (20). In the case of *M. pulmonis*, this heterogeneity (in the V-1 antigen) was found to be associated with altered surface properties of the organism (4, 26) which have the potential to impact the host-parasite interaction. The functional significance of the size heterogeneity of *M. fermentans* antigens is still unknown. Although we have some evidence that these *M. fermentans* strains interact differently with eucaryotic cells (unpublished data), we do not yet know whether this observation is related to the observed size heterogeneity.

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