Antigenic Distinctiveness of Mobiluncus curtisi and Mobiluncus mulieris

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A total of 26 Mobiluncus strains (17 M. curtisi and 9 M. mulieris strains) were compared serologically by double immunodiffusion and immunoblotting against antisera prepared against representative isolates of each species. All strains from the same species were strongly reactive with homologous antiserum but generally weakly reactive with antiserum to the heterologous Mobiluncus spp. The antisera did not react with strains of the unrelated genera Campylobacter, Succinivibrio, Wolinella, Actinomyces, Anaerobiospirillum, and Anaerovibrio.

Motile curved anaerobic bacteria isolated from the vaginas of women with bacterial vaginosis have recently been classified as belonging to the genus Mobiluncus (7). Their fermentation products include succinic, acetic, and lactic acids, and they have an atypical, multilayered, gram-positive cell wall structure (7). These organisms are thought to be associated with bacterial vaginosis, although their role in the disease process, if any, remains to be elucidated (1, 4, 6-8). Two species have been proposed: M. curtisi and M. mulieris. Previous work in a number of laboratories has shown that the two species can be distinguished on the basis of morphological and biochemical differences and are not highly related to any previously described genus (1, 4, 6-8). We have shown by DNA homology studies that members of the same species share >75% of their DNA sequences in common, whereas DNA relatedness between the two species ranges from 9 to 25% (7). More recently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein patterns of whole-cell extracts were shown to be species specific (1). These data indicate a high degree of relatedness between strains within a species. Strains within a species normally share most antigenic determinants, whereas cross-reactions between species in the same genus varies with the organisms being studied.

In this study, we examined the antigenic relationships of a selected group of well-characterized strains of the genus Mobiluncus by using two common serological techniques. We also examined the antigenic relationship between Mobiluncus and other genera that share some morphological or metabolic characteristics in common but share no detectable DNA sequences with Mobiluncus (6, 7).

MATERIALS AND METHODS

Bacterial strains. Twenty-one of the Mobiluncus strains, including the type strain M. curtisi ATCC 35241 and M. mulieris ATCC 35243 strains of the other genera used in this study, have been previously described in detail (1). Two Mobiluncus strains isolated by Sweden by P.-A. Mardh and three strains isolated in the United States by M. Ohm-Smith were also included in the study (7).

Preparation of cell extracts. Organisms were grown in 300 ml of Columbia broth (BBL Microbiology Systems, Cockeysville, Md.) with 2% defibrinated rabbit serum (GIBCO Laboratories, Grand Island, N.Y.) for 7 to 10 days at 37°C. Cells were harvested by centrifugation at 4°C, washed twice in 0.05 M phosphate-buffered saline (pH 7.4), and resuspended in 0.062 M Tris buffer (pH 6.8) (0.5 to 1.0 ml, depending on the size of the cell pellet), and glass beads (diameter, 0.1 mm) were added in a ratio of 3:4 (beads by weight)/Tris buffer [by volume]). The cell pellets were vortexed for 4 min. Cell debris, unbroken cells, and glass beads were pelleted by centrifugation at 10,000 x g for 20 min. The supernatant fluids were removed and stored at −20 or −70°C (1).

Preparation of antisera. Antisera were prepared against two strains of each of the two species of Mobiluncus: M. curtisi BV-13-6 and ATCC 35242 and M. mulieris ATCC 35243 and BV-64-5. Each strain was grown in 3.5% brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 2% rabbit serum for 7 to 10 days at 37°C. Cells were harvested by centrifugation at 4°C and washed twice in 0.05 M phosphate-buffered saline. The organisms were adjusted to 2 x 10^7 CFU/ml and mixed with 3% Formalin. The cells were incubated at room temperature overnight and washed with phosphate-buffered saline. The mixture was diluted 1:1 with complete Freund adjuvant and used to inoculate the rabbits. A 1-ml portion, divided among 10 sites, was injected into each rabbit subcutaneously. Three subcutaneous boosters (10^6 CFU) in phosphate-buffered saline without adjuvant were given at 2-week intervals. Blood samples were collected before the first injection and 2 weeks after the last injection. Sera were removed and stored at −20°C. The crude immunoglobulin G fraction of the hyperimmune serum was purified by using ammonium sulfate precipitation as described by Harboe and Ingild (3). The final volume was adjusted to the same volume as the original serum.

SDS-PAGE. SDS-PAGE was performed in 10% acrylamide with a 5% stacking gel in a dual vertical slab gel modified to run four gels at once (Bio-Rad Laboratories, Richmond, Calif.) (2). Cell extract samples were adjusted to contain between 6 and 15 μg of protein per lane. The gels were stained in 25% isopropyl alcohol–10% acetic acid–0.05% Coomassie blue R250 (Bio-Rad Laboratories) overnight. The

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gels were then destained in 25% isopropyl alcohol-10% acetic acid (2).

**Double immunodiffusion.** Double immunodiffusion was performed on glass slides with 0.5% agarose in 0.025 M Chex-Bicine buffer (pH 8.6) as previously described (5). Samples (10 μl) of the IgG fraction were tested against cell extracts containing a total of 30 to 80 μg of protein. In some experiments, the antigen samples were boiled for 1 h before being tested.

**Immunoblotting.** Replicate SDS-PAGE gels were immunoblotted overnight with the Bio-Rad Transblot Cell by the method of Towbin (9) as modified by Erwin and Kenny (2). The nitrocellulose was blocked by incubation for 1 h at 37°C in NET (0.15 M NaCl, 5.5 mM tetrasodium EDTA, 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 0.025% gelatin, 0.1% Triton X-100 (pH 7.3)) and stored in the same solution at 4°C until used. The blots were stained with one of the four rabbit antisera at 1:320 dilution, washed, and incubated in horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories, Westchester, Pa.) (1:500 in NET-1% Triton). The color was developed in 0.005 mM TES-0.15 M NaCl (pH 7.3) containing 0.015% 4-chloro-1-naphthol (0.3%, wt/vol, in methanol) and 0.6% hydrogen peroxide as previously described (2, 9).

**RESULTS**

**Double immunodiffusion.** At least six lines were observed when homologous antisera and cell extracts from the same species were used in double immunodiffusion (Fig. 1, panels A and D). Undiluted antisera were used; however, the antisera could be diluted 1:64 and still show some precipitin lines against homologous cell extracts. The strongest precipitin bands occurred when undiluted antisera were used with 3 to 8 μg of protein from cell extracts. One or two precipitin lines developed when the antisera against *M. curtisi* were tested against extracts from *M. mulieris* or when *M. mulieris* antisera were tested against extracts of *M. curtisi* (Fig. 1, panels B and C). No lines were seen when antisera was tested against cell extracts of organisms with morphological or biochemical similarities to *Mobiluncus* spp. (1, 6, 7). No lines of precipitation were seen when antisera were tested against *Actinomyces bovis* ATCC 13683, *Wolinella* sp. strain Tanner 286, *Anaerobiospirillum succiniciproducens* ATCC 29305 (Fig. 1, panel A and D), or *Anaerovibrio lipolytica* ATCC 33276, *Campylobacter fetus* subsp. *veneraealis* ATCC 19438, *Campylobacter concisus* FDC 484, and *Succinivibrio dextrinosolvens* Bryant 24 (7). The use of boiled antigen reduced the number of precipitin lines to one which may be the common cross-reactive antigen seen when the antisera and extracts were of the opposite species.

**Immunoblots.** We have previously shown that members of the same *Mobiluncus* sp. have very similar protein profiles on SDS-PAGE gels (1). Replicate immunoblots were stained with the four antisera; 15 to 18 bands were stained in all the homologous strains (Fig. 2). However, the number of bands stained was strain dependent and varied slightly with the antiserum used (Fig. 2). The overall pattern was the same between different strains within a species. The cross-reactions between the two species were variable among the different strains. In general, only a few bands (2 to 5) were weakly stained when reactions between the two species
were compared. A few strains showed strong cross-reactions; however, the total number of reactive bands was fewer than when homologous antisera were used on the same cell extract (Fig. 2, lane C). The staining patterns were reproducible among different gels and could be easily used to identify unknown cell extracts to the species level. Very faint, nonspecific, or no staining was seen with cell extracts from the other genera.

**DISCUSSION**

The genus *Mobiluncus* has only recently been studied in detail. On the basis of the genetic and biochemical data, we predicted that members of the same species would share a large number of cross-reactive antigens, whereas cross-reactions between species would be rather limited.

In this study, we examined the antigenic relationships between *M. curtisi* and *M. mulieris* strains by using two analytical serological techniques. We found that antiserum raised against a single strain of *M. curtisi* or *M. mulieris* reacted strongly with all strains of that species, but the cross-reaction with heterologous strains was generally limited to a few antigenic determinants, whereas almost no reactions were observed with genetically unrelated species. The use of boiled antigen in double immunodiffusion tests reduced the number of precipitin lines to one which may be the common cross-reactive antigen seen when the antisera and extracts were of the opposite species. This suggests that most of the antigenic determinants detected were proteins or contained protein moieties, whereas the heat-stable antigen could be a cell wall component.

*M. curtisi* has tentatively been divided into two subspecies on the basis of nitrate reduction and migration through soft agar (7). The two *M. curtisi* strains used to prepare antisera represented one from each subgroup. All the *M. curtisi* strains were identified to the subspecies level; however, we found no difference in the number of precipitin lines produced or the number of bands stained by immunoblots when the same or opposite subspecies antisera was used against the same cell extract. These results indicate that the two analytical serological techniques could not distinguish between the proposed subspecies of *M. curtisi* (7).

Either of the two methods described can be used to distinguish between homologous and heterologous *Mobiluncus* strains and would also differentiate *Mobiluncus* from other genera. The work presented here confirms the antigenic relationship predicted by the DNA homology studies. Work is currently in progress to further characterize these reactive antigens in the hope of better understanding this group of bacteria.

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**LITERATURE CITED**