Antigenic Distinctiveness of *Mobiluncus curtisii* and *Mobiluncus mulieris*

MARILYN C. ROBERTS,^{1*} ELLEN J. BARON,^{2†} SYDNEY M. FINEGOLD,² AND GEORGE E. KENNY¹

Department of Pathobiology, University of Washington, Seattle, Washington 98195¹; Medical Service, Veterans Administration Wadsworth Medical Center, Los Angeles, California 90073²; and University of California Los Angeles School of Medicine, Los Angeles, California 90024³

Received 14 January 1985/Accepted 25 February 1985

A total of 26 *Mobiluncus* strains (17 *M. curtisii* 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 antisera but generally weakly reactive with antisera 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 vaginæ 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. curtisii* 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 ranged 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. curtisii* 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 × 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. curtisii* 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 × 10⁹ 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 (10⁹ 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

* Corresponding author.

† Present address: North Shore University Hospital, 300 Community Drive, Manhasset, NY 11030.

FIG. 1. Double immunodiffusion using (c) *M. curtisii* ATCC 35242 antiserum (A and B) and (m) *M. mulieris* antiserum (C and D). Cell extracts: 1, *Anaerobiospirillum succiniciproducens* ATCC 29305; 2, *Actinomyces bovis* ATCC 13683; 3, 4, and 9, *M. curtisii* ATCC 35241, 383B, and 6114, respectively; 5 through 8, *M. mulieris* 364K, SV52T, SV58S, and SV45ES respectively; 10, *Wolinella* sp. Tanner 286 (1, 7).

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 Ches-Bicine buffer (pH 8.6) as previously described (5). Samples (10 μ l) of the IgG fraction were tested against cell extracts containing a total of 30 to 80 μ g 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 antiserum 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 μ g of protein from cell extracts. One or two precipitin lines developed when the antisera against *M. curtisii* were tested against extracts from *M. mulieris* or when *M. mulieris* antisera were tested against extracts of *M. curtisii* (Fig. 1, panels B and C). No lines were seen when antiserum 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 *Anaerobivrio lipolytica* ATCC 33276, *Campylobacter fetus* subsp. *venerealis* 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

FIG. 2. Immunoblotting with *M. curtisii* ATCC 35242 antiserum. Lanes A through C, *M. mulieris* 364K, 2473, and 2473, respectively; and lanes D through J, *M. curtisii* 6114, 9252, 311-9, BV416-10, BV105-4, BV424-9, and BV306-7, respectively. The numbers indicate the locations of the molecular weight markers in relation to the stained bands.

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. curtisii* and *M. mulieris* strains by using two analytical serological techniques. We found that antiserum raised against a single strain of *M. curtisii* 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. curtisii has tentatively been divided into two subspecies on the basis of nitrate reduction and migration through soft agar (7). The two *M. curtisii* strains used to prepare antisera represented one from each subgroup. All the *M. curtisii* 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. curtisii* (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.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI12192 from the National Institutes for Health.

We thank A. L. Erwin for technical advice.

LITERATURE CITED

1. Baron, E. J., H. M. Wexler, and S. M. Finegold. 1984. Biochemical and polyacrylamide gel electrophoretic analyses of vaginosis-associated anaerobic curved rods, p. 65-69. In P. Mardh and D. Taylor-Robinson (ed.), *Bacterial vaginosis*. Almqvist and Wiksell International, Stockholm.
2. Erwin, A. L., and G. E. Kenny. 1984. *Haemophilus influenzae* type b isolates show antigenic variation in a major outer membrane protein. *Infect. Immun.* 46:570-577.
3. Harboe, N., and A. Ingild. 1973. Immunization, isolation of immunoglobulins, and estimation of antibody titer, p. 161-164. In N. H. Axelsen, J. Kroll, and B. Weeks (ed.), *A manual of quantitative immunoelectrophoresis*. Universitetsforlaget, Oslo.
4. Holst, E., A. Skarin, and P.-A. Mardh. 1982. Characteristics of anaerobic comma-shaped bacteria recovered from the female genital tract. *Eur. J. Clin. Microbiol.* 1:310-316.
5. Kenny, G. E. 1983. Agar precipitin and immunoelectrophoretic methods for detection of mycoplasmic antigen, p. 441-456. In S. Razin and J. G. Tully (ed.), *Methods in mycoplasmaology*, vol. 1. Academic Press, Inc., New York.
6. Roberts, M. C., S. L. Hillier, F. D. Schoenknecht, and K. K. Holmes. 1984. Nitrocellulose filter blots for species identification of *Mobiluncus curtisii* and *Mobiluncus mulieris*. *J. Clin. Microbiol.* 20:826-827.
7. Spiegel, C. A., and M. Roberts. 1984. *Mobiluncus* gen. nov., *Mobiluncus curtisii* subspecies *curtisii* sp. nov., *Mobiluncus curtisii* subsp. *holmesii* subsp. nov., and *Mobiluncus mulieris* sp. nov., curved rods from the human vagina. *Int. J. Systematic Bacteriol.* 34:177-184.
8. Sprott, M. S., H. R. Ingham, R. S. Pattman, R. L. Eisenstadt, G. R. Short, H. K. Naranj, P. R. Sisson, and J. B. Selkan. 1983. Characteristics of motile curved rods in vaginal secretions. *J. Med. Microbiol.* 16:175-182.
9. Towbin, H., F. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76:4350-4354.