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Evidence that Mycoplasmas, Gram-Negative Bacteria, and Certain Gram-Positive Bacteria Share a Similar Protein Antigen

TSUGUO SASAKI

Department of General Biologics Control, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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It was demonstrated that mycoplasmas, gram-negative bacteria, and certain gram-positive bacteria share a similar protein antigen with a molecular weight ranging from 42,000 to 48,000. Western blotting (immunoblotting) with an antibody specific to a 43-kDa membrane protein of *Mycoplasma fermentans* showed the existence of this protein antigen in all *Mycoplasma* spp. tested (14 species), *Acholeplasma laidlawii* (1 strain), and gram-negative bacteria (8 species) but only in *Staphylococcus aureus* of four gram-positive species tested. Neither *Ureaplasma urealyticum* nor mammalian cell cultures showed any cross-reactions with this antibody. These proteins were found in both cytoplasmic and membrane fractions of mycoplasma cells but were not exposed on the surface of mycoplasmal or bacterial cells.

In the course of studies on the characterization of membrane proteins of *Mycoplasma hominis* (8), a specific antibody directed against a 45-kDa membrane protein, which is one of the major membrane-associated proteins of *M. hominis*, showed strong cross-reactions with membrane-associated proteins of about 42 to 48 kDa of some *Mycoplasma* species. The cross-reactivity of a specific antibody against a 43-kDa membrane protein of *M. fermentans* with some mycoplasmal and bacterial strains was investigated. The cross-reactive antigen (referred to as the 45,000-molecular-weight [45K] protein), with an apparent molecular weight of 42,000 to 48,000, depending on the species, was found in all *Mycoplasma* spp. and gram-negative bacteria tested and in *Staphylococcus aureus*, which is a gram-positive bacterium. Although the function and localization of the 45K protein in these microorganisms have not been established, the results of Western blots (immunoblots) of mycoplasmas, bacteria, and mammalian cell cultures with the antibody specific to the 45K protein of *M. fermentans* are reported here.

The mycoplasmal and bacterial strains used are listed in Table 1. *Mycoplasma* spp. and *Acholesplasma laidlawii* were grown in a glucose or arginine broth medium consisting of 2.1% (wt/vol) PPLO broth base (Difco), 10% (vol/vol) horse serum, 0.002% (wt/vol) phenol red, and either 0.25% (wt/vol) glucose (glucose broth) or 0.25% (wt/vol) arginine monohydrochloride (arginine broth). The final pHs of the glucose and arginine broths were adjusted to 7.7 and 7.1, respectively. *Ureaplasma urealyticum* was cultured in Shepard fluid medium U-9 modified to contain only 5% (vol/vol) horse serum (10).

Bacterial strains were cultured in brain heart infusion broth (Difco) at 31°C for 10 h. Mycoplasmas grown in 1 liter of each growth medium at 37°C were centrifuged (16,000 × *g* for 40 min) when their pHs began to change. The cell pellets were suspended in 200 ml of phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 16,000 × *g* for 40 min. The pellets were washed twice by centrifugation, resuspended in a minimal amount of PBS, sonicated at 20 kHz for 3 min, and stored frozen at −30°C until used. Frozen mycoplasma stocks were thawed and centrifuged at 100,000 × *g* for 60 min, the pellets (membrane fraction) (6) were suspended to 3.0 mg of protein per ml in PBS, and an equal volume of sampling buffer (2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris [pH 6.8]) was added. The supernatant was concentrated by ultrafiltration with a Centrimex tube (KB D-31; Sanko Junyaku Co., Tokyo, Japan) and used as the cytoplasmic fraction. Samples were then heated at 100°C for 5 min. Samples of bacterial strains were prepared by centriguation at 3,000 × *g* for 10 min, washed with PBS, irradiated with a UV lamp for 120 min, and sonicated at 20 kHz for 3 min. Bacterial samples were then suspended in the sampling buffer and heated at 100°C for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) was then carried out by the method of Laemmli (5), with 10 and 5% acrylamide in the separating and stacking gels, respectively. Electrophoretic transfer of the separated components to nitrocellulose sheets (HAWP 324FO; Millipore) and subsequent immunological staining were performed by modifications of the method of Towbin et al. (11), as previously described (7).

The 45K proteins of *M. hominis* and *M. fermentans* were purified by SDS-PAGE. After the 45-kDa protein band was stained with Coomassie blue, it was cut out of the gels, homogenized in sampling buffer, and subjected again to SDS-PAGE. These procedures were repeated twice more. The 45-kDa protein band cut out of the gels was homogenized in PBS and injected intraperitoneally into BALB/c female mice (5 weeks old). The mice were given booster injections twice intraperitoneally with the same protein antigen at 2-week intervals. Two weeks after the third injection, the mice were bled, and the serum was used as monospecific antibodies (MsAb) against the 45K proteins of *M. hominis* (MsAb-H) and *M. fermentans* (MsAb-F).

Figure 1 shows immunoblots of 14 *Mycoplasma* spp., *A. laidlawii*, and 3 serovars of *U. urealyticum* with MsAb-H and MsAb-F. MsAb-H showed cross-reactions with 13 mycoplasmas but not with *M. mycoides* subsp. *mycoides* or *M. pneumoniae*. Although MsAb-H showed cross-reactions with two protein bands of *M. arthritidis* and *M. buccale*, MsAb-F showed cross-reactions with only a single protein band of all *Mycoplasma* spp. tested. Neither MsAb-H nor MsAb-F showed any cross-reaction with *U. urealyticum*. The molecular weights of the cross-reactive antigen of seven *Mycoplasma* spp. and *A. laidlawii* were about 42,000 to 48,000, depending on the *Mycoplasma* sp. (Fig. 2). MsAb-F also reacted with all gram-negative bacteria tested and *S.*

TABLE 1. Mycoplasmal and bacterial strains used

Organism
Mycoplasmas
M. arginini G230
M. arthritidis PG6
M. buccale CH20247
M. fermentans PG18
M. hominis PG21
M. lipophilum MaBy
M. orale CH10299
M. primatum HRC292
M. salivarium PG20
M. hyorhinis PG29
M. mycoides subsp. *mycoides* PG1
M. neurolyticum type A
M. pneumoniae Mac
M. pulmonis G34
A. laidlawii PG8
U. urealyticum serotype I
U. urealyticum serotype IV
U. urealyticum serotype VIII
Gram-positive bacteria
B. subtilis ATCC 6633
S. aureus ATCC 25923
S. epidermidis ATCC 12228
S. faecalis CG110
Gram-negative bacteria
A. sobria 620-90
E. coli ATCC 25922
P. vulgaris ATCC 13315
P. aeruginosa ATCC 27853
S. typhimurium ATCC 14028
S. sonnei HW383
V. cholerae 0116
Y. pseudotuberculosis 4b

aureus, which is a gram-positive bacterium (Fig. 3). Cultured cells such as Vero (African green monkey kidney), 3T6-Swiss albino (mouse embryo), WI-38 (human lung), and SP2/0 (mouse myeloma) and growth medium for mycoplasmas did not show any cross-reactions with MsAb-F (data not shown). Figure 4 shows the immunoblot of cytoplasmic and membrane fractions from *M. pneumoniae*, *A. laidlawii*, *M. fermentans*, and *M. hominis* with MsAb-F. The 45K protein was found in both cytoplasmic and membrane fractions of mycoplasma cells and seemed to be a major component of mycoplasmas, judging from Coomassie blue staining. Although immunofluorescence and immunoperoxidase staining procedures were performed on the colonies of several mycoplasma strains, *Escherichia coli*, and *S. aureus* with MsAb-F, the staining results were negative (data not shown). These results suggest that the 45K protein is not exposed on the surface of these microorganisms.

There have been some reports of monoclonal antibodies (MAbs) which might recognize the epitopes of the 45K protein of mycoplasmas (1–3). Their blotting patterns for mycoplasmas also differed from species to species, and the relative positions of the 45K protein of each species on the immunoblot were consistent with the blotting results shown in Fig. 1 and 2. Cimolai et al. (2) reported a MAb (OC2F5) to a 43-kDa protein of *M. pneumoniae*, which cross-reacted with an antigen with a similar molecular mass from both *M.*

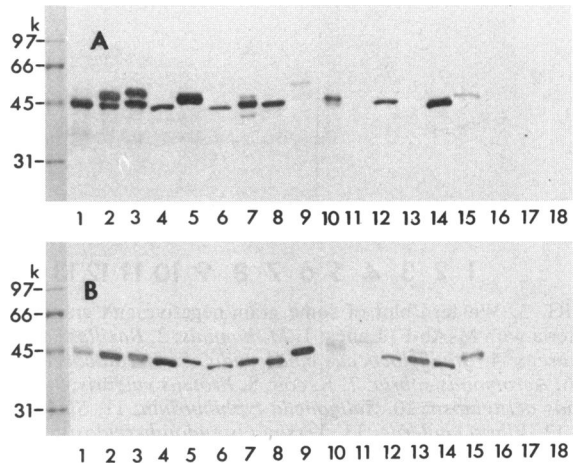

FIG. 1. Western blot of *Mycoplasma* spp., *A. laidlawii*, and *U. urealyticum* with MsAbs against the 45K protein of *M. hominis* (MsAb-H) (A) and *M. fermentans* (MsAb-F) (B). Lanes: 1, *M. arginini*; 2, *M. arthritidis*; 3, *M. buccale*; 4, *M. fermentans*; 5, *M. hominis*; 6, *M. lipophilum*; 7, *M. orale*; 8, *M. primatum*; 9, *M. salivarium*; 10, *M. hyorhinis*; 11, *M. mycoides* subsp. *mycoides*; 12, *M. neurolyticum*; 13, *M. pneumoniae*; 14, *M. pulmonis*; 15, *A. laidlawii*; 16, *U. urealyticum* serotype I; 17, *U. urealyticum* serotype IV; 18, *U. urealyticum* serotype VIII. Molecular weight markers are phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and bovine carbonic anhydrase (31,000) (89-0499; Bio-Rad Laboratories).

genitalium and *A. laidlawii*. Dudler et al. (3) reported a MAb (DD9) reacting with the 43.5-kDa protein of *M. hyorhinis* but not with that of *M. arginini* or *M. orale*. However, they sequenced the gene (p37 gene) for the 43.5-kDa protein of *M. hyorhinis* and showed that rabbit antiserum directed against a synthetic N-terminal peptide of the 43.5-kDa protein of *M. hyorhinis* recognized proteins from *M. arginini* and *M. orale* as well as from *M. hyorhinis*. Furthermore, Dudler et al. and Gilson et al. suggested that the p37 gene is part of an operon encoding two additional proteins which are highly similar to components of the periplasmic binding-protein-dependent transport systems of gram-negative bacteria (3, 4). Blazek et al. (1) have recently reported a MAb (CCM-2) reacting with the 45K protein of seven standard *Mycoplasma* spp. and all mycoplasmas isolated from cell cultures. I have not yet established a MAb reacting with the 45K protein of all *Mycoplasma* spp. used in this study, but some established MAbs showed diverse cross-reactions with some species.

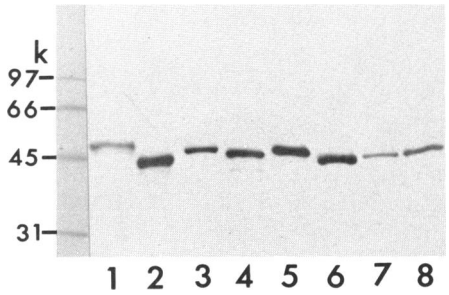

FIG. 2. Western blot of seven *Mycoplasma* spp. and *A. laidlawii* with MsAb-F. Lanes: 1, *A. laidlawii*; 2, *M. arthritidis*; 3, *M. hominis*; 4, *M. orale*; 5, *M. salivarium*; 6, *M. fermentans*; 7, *M. pulmonis*; 8, *M. pneumoniae*.

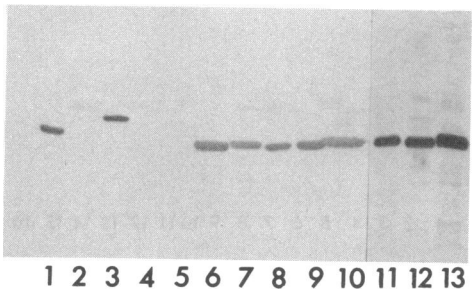

FIG. 3. Western blot of some gram-negative and gram-positive bacteria with MsAb-F. Lanes: 1, *M. hominis*; 2, *Bacillus subtilis*; 3, *S. aureus*; 4, *Staphylococcus epidermidis*; 5, *Streptococcus faecalis*; 6, *Aeromonas sobria*; 7, *E. coli*; 8, *Proteus vulgaris*; 9, *Pseudomonas aeruginosa*; 10, *Salmonella typhimurium*; 11, *Shigella sonnei*; 12, *Vibrio cholerae*; 13, *Yersinia pseudotuberculosis*.

These results suggest that the 45K protein has various antigenic epitopes.

A considerable amount of the 45K protein was detected in both cytoplasmic and membrane fractions (Fig. 4), but this protein may not be very antigenic. Cross-reactive antibodies against the 45K protein could not be observed in humans naturally infected with *M. pneumoniae* or in mice immunized with cell lysates of *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. salivarium*, or *M. orale* (data not shown).

To investigate whether the 45K protein found in mycoplasmas, gram-negative bacteria, and *S. aureus* is related to the membrane-bound complex of several permeases from gram-negative bacteria (3, 4), the N-terminal amino acid sequence of the purified 45K protein from *A. laidlawii*, *M. fermentans*, *M. hyorhinis*, *E. coli*, and *S. aureus* and the function of the 45K protein in these microorganisms are now under investigation.

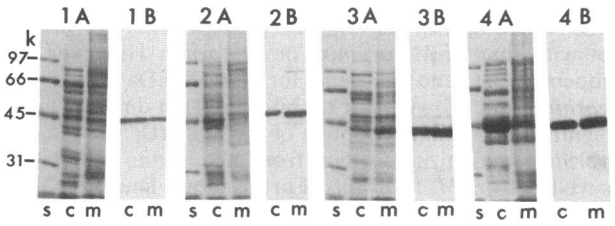

FIG. 4. SDS-PAGE and Western blot analysis of cytoplasmic (lanes c) and membrane (lanes m) fractions from *M. pneumoniae* (lanes 1), *A. laidlawii* (lanes 2), *M. fermentans* (lanes 3), and *M. hominis* (lanes 4). (A) Cytoplasmic and membrane fractions of mycoplasmas and molecular weight markers (lanes s) were separated by 10% PAGE, transferred to nitrocellulose sheets, and stained with Coomassie blue. (B) Western blot of cytoplasmic and membrane fractions of mycoplasmas with MsAb-F. Arrows indicate the protein band corresponding to the 45K protein.

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