Comparison of *Mycoplasma arthritidis* Strains by Enzyme-Linked Immunosorbent Assay, Immunoblotting, and DNA Restriction Analysis

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Received 26 January 1995/Returned for modification 21 March 1995/Accepted 31 May 1995

Twenty *Mycoplasma arthritidis* strains or isolates were compared by a combination of enzyme-linked immunosorbent assay by an antiserum adsorption technique, Western immunoblotting, and restriction analysis of chromosomal DNA. Antigenic markers that defined strains related to strains 158p10p9, PG6, and H606 were identified. In addition, restriction analysis allowed all 20 strains to be divided into six groups. Results of restriction analysis corresponded generally with antigenic similarities, although the former did not allow grouping with as fine a precision as the latter. However, intrastrain antigenic variability, which is common among many *Mycoplasma* species, including *M. arthritidis*, introduced a complicating factor into our attempts at antigenic analysis. While serologic and antigenic analyses remain useful, we recommend that they be used with caution and in combination with other techniques for identifying and characterizing new isolates and newly acquired strains. Combinations of these techniques have proven to be useful in our laboratory for quality control and for uncovering interesting relationships among strains subjected to animal passage and their less virulent antecedents and among strains originally classified as the same but obtained from different sources and maintained, sometimes for decades, in different laboratories.

Mycoplasma arthritidis causes an acute, self-limiting, systemic infection in rats, with migratory polyarthritis as a primary manifestation. This disease rarely occurs under natural conditions but is readily induced by the intravenous or intraperitoneal injection of *M. arthritidis* (6). Examination of reports from different laboratories that used different *M. arthritidis* strains indicates no dramatic differences in the nature of the diseases induced by the known virulent strains (4, 8, 16, 17, 19, 24, 43, 47), although differences in the relative degree of arthritogenicity are prominent (16, 17, 19, 22, 53). In recent years several research groups, including our own, have begun to look more closely at those *M. arthritidis* cell components actually involved in host-parasite interaction. These studies have uncovered cross-reactivities between *M. arthritidis* and *Mycoplasma pulmonis* (31) and between *M. arthritidis* and various rat tissues (41, 45). In addition, antigens that serve as targets for metabolism-inhibiting antibodies (50) and two surface proteins involved in adherence to host tissues (52) were identified. Dreesse et al. (10–12) showed that several *M. arthritidis* surface proteins undergo rapid spontaneous size and phase variations both in vivo and in vitro, which could possibly complicate immune recognition. However, much of this work was done with single strains of *M. arthritidis*, and most studies with multiple strains were not specifically designed for comparative purposes. Therefore, little is known about the relationships

among strains isolated from different sources and maintained in different laboratories and, consequently, about the relevance of data derived from single strains to the species as a whole.

Previously, we reported (51) some differences in antigenic specificity among four strains, while Stadländer and Watson (44) showed protein and antigenic variabilities among a set of six strains. We have now expanded our earlier observations to include 20 *M. arthritidis* strains, which we examined for differences in their antigenic profiles by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting and for differences in the restriction endonuclease cleavage patterns of chromosomal DNA.

Our goal was to identify convenient markers that would allow us to define relationships among isolates and to more easily keep track of the individual strains used in our laboratory for purposes of quality control. In addition, it was important to determine the actual stability of any such markers in individual members of a strain, and hence the reliability of any identification or quality control scheme based on these properties. This final point illustrates a very real concern, considering that variation in surface proteins seems to be a common property of several *Mycoplasma* species (37, 38, 40), including *M. arthritidis* (10–12).

(Portions of this work were presented at the 94th General Meeting of the American Society for Microbiology [57] and at the 9th and 10th International Congresses of the International Organization for Mycoplasmaology [55, 56].)

MATERIALS AND METHODS

***M. arthritidis*.** The strains of *M. arthritidis* used in the present study and their sources are summarized in Table 1; the term “strain” will be used throughout this report for the sake of convenience, but many of these probably more properly fit the designation “isolate.” In cases in which the same strains were obtained from different sources or were maintained in different laboratories, they were given individual designations and were treated separately. Stock cultures of each strain were prepared by growing mycoplasmas in modified Edward medium (48) sup-

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TABLE 1. *M. arthritis* strains used in the study and their origins

Strain	Origin (reference)
158 ^a	Cloned from PG27 (Campo), human isolate (4, 13)
158p10 ^a	158 passaged 10 times through rats (16)
158p10p9 ^a	158p10 passaged 9 times through mice (5)
ATCC 14152 ^b	ATCC number for Campo (PG27), human isolate (14, 33)
14152 ^a	Obtained by Cole et al. (4) from ATCC ca. 1967
23192 ^b	ATCC number for Campo (PG27), human isolate (14, 33)
14152p13 ^a	14152 passaged 13 times through rats (16)
ATCC 14124 ^b	Jasmin, rat lymphosarcoma isolate (22)
14124 ^a	Obtained by Cole et al. (4) from ATCC ca. 1967
Jasmin ^c	Rat lymphosarcoma isolate (22)
14124p10 ^a	14124 passaged 10 times through rats (16)
PG6 ^a	Rat joint isolate (16, 34)
19611 ^b	ATCC number for PG6 (34)
H606 ^a	Mouse brain passaged (16, 28)
13988 ^a	ATCC number for H606
H39 ^c	Strain 39, human prostate isolate (14, 27)
Tru ^c	Rat Hardarian gland isolate (7)
JR3 ^c	Rat joint isolate (46)
ISR1 ^c	Rat labyrinthitis isolate (26)
O7 ^d	Human cervical isolate (27, 28, 42)

^a Obtained from Barry C. Cole, Division of Rheumatology, University of Utah, in 1985.

^b Purchased from ATCC in 1991.

^c Obtained from Gail H. Cassell, Department of Microbiology, University of Alabama, in 1991.

^d Obtained from Paul F. Smith, Department of Microbiology, University of South Dakota, in 1991.

plemented with 7.5% (vol/vol) horse serum; they were stored frozen in 1-ml aliquots at -70°C. To avoid cross-contamination, stocks were prepared one at a time. Each culture was reidentified as *M. arthritis* by epi-immunofluorescence (9) with fluorescein isothiocyanate-labeled rabbit antiserum prepared against strain 158p10p9 (48).

Filter-cloned lineages of strains 158p10p9, PG6, and H606 were prepared by filtering 48-h broth cultures through 0.45-μm-pore-size filters, plating 200 μl of filtrates diluted 1:1,000 onto modified Edward agar plates (identical to modified Edward broth except for the addition of 1.4% [wt/vol] Bacto Agar [Difco Laboratories, Detroit, Mich.]), and expanding individual colonies in 5 ml of broth medium. After 24 to 48 h of incubation, the 5-ml cultures were frozen in 1-ml aliquots at -70°C for use as stock cultures. For PG6 and H606, the filtration and cloning processes were performed once, while for 158p10p9 they were repeated a total of three times for each lineage (52).

Antisera. Rabbit antisera were prepared against each strain as described previously (51-54); the resulting antibody responses were directed largely against surface antigens. Monoclonal antibodies (MAbs) G9a and 7a (isotype immunoglobulin G2A [IgG2A]), directed against putative adhesins MAA1 and MAA2 from strain 158p10p9, respectively, were also described previously (52). A third MAbs (isotype IgG1), directed against an antigen that migrates at 74 kDa in strain H606 but at a slightly higher molecular mass range in the other strains tested, was prepared similarly from mice immunized with strain 158p10p9.

Serologic comparison of *M. arthritis* strains. Samples of 250 μl of each rabbit antiserum were adsorbed with 4 mg (protein) of intact cells of strains 158p10p9 and PG6 as described previously (51). Unadsorbed antisera and samples similarly adsorbed with equal concentrations of the homologous strains served as controls. Adsorbed and unadsorbed rabbit antisera were then tested by ELISA for their activities against their homologous strains (49). This method was used in an earlier study to determine relationships among strains 158p10p9, 14124p10, PG6, and H606 (51).

In a separate experiment, the abilities of intact cells from strains 158, 158p10, and 158p10p9 to adsorb antibody activity from each of the three corresponding antisera were also tested. The relationships among these three strains were assessed by determining by ELISA the antibody titers of adsorbed and unadsorbed antisera against their homologous antigens. The relationships between 14124 and 14124p10 and between 14152 and 14152p13 were determined similarly.

Western and colony immunoblotting. Twenty micrograms of total protein from each strain was electrophoresed on 7.5% (wt/vol) T (acrylamide) and 2.7% (wt/vol) C (*N,N'*-methylene-bisacrylamide) sodium dodecyl sulfate (SDS)-polyacrylamide gels in a model SE Hoefer slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's instructions. Separated proteins were transferred to nitrocellulose membranes in

a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.) as described by the manufacturer. Membranes were blocked for 1 h with phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (PBST) and were exposed to the appropriate rabbit antiserum diluted 1:40 or MAb diluted 1:1,600 (for MAbs G9a and 7a) or 1:100 (for anti-p74) in PBST and then to peroxidase-conjugated anti-rabbit or anti-mouse IgG (heavy and light chain specific; Organon Teknica, Cappel Division, Durham, N.C.) diluted 1:1,600 in PBST and finally to a substrate consisting of 4-chloro-1-naphthol and H₂O₂. Blots were rinsed six times with PBST between each step.

Colony blotting was performed as described by Kotani and McGarrity (25) and Rosengarten and Wise (39). Briefly, autoclaved nitrocellulose membranes were placed onto agar surfaces containing *M. arthritis* colonies and were left in place for 30 min. The blots were then blocked with PBST and were stained as described above for Western immunoblots.

Restriction endonuclease digestion of mycoplasmal DNA. DNA was extracted from each strain by pelleting 40- to 42-h 100-ml cultures, resuspending the cell pellet in 1.0 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TE buffer), and lysing the cells with 1% (vol/vol; final concentration) SDS. RNase A (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μg/ml, and the sample was then incubated for 30 min at 37°C and extracted twice with Tris-buffered phenol (pH 8.0), once with phenol-chloroform (1:1), and once with chloroform. After ethanol precipitation, the DNA was resuspended in 50 to 100 μl of TE buffer and was stored at -20°C until use. DNA samples were digested with restriction endonucleases *Bam*HI, *Pst*I, *Sal*I, *Kpn*I, and *Spe*I (New England Biolabs, Beverly, Mass.; Stratagene Cloning Systems, La Jolla, Calif.; United States Biochemical, Cleveland, Ohio) according to the manufacturers' instructions and were electrophoresed on 0.55% (wt/vol; for *Spe*I digests) or 0.40% (for all other digests) agarose gels. Electrophoretically separated DNA fragments were visualized under UV light after staining with ethidium bromide by standard procedures (2).

RESULTS

Serologic relationships of *M. arthritis* strains to 158p10p9 and PG6. Rabbit antisera against each of the 20 strains were adsorbed with intact cells of strain 158p10p9 as described in Materials and Methods and were tested by ELISA against their homologous strains. These results were compared with those for unadsorbed antisera and antisera similarly adsorbed with the homologous antigens. Each adsorption experiment was performed at least twice for confirmation. Adsorption with the homologous strains consistently reduced their titers at least 64-fold. On the basis of their relationships to 158p10p9, the 20 strains could tentatively be divided into three groups consisting of (i) strains capable of adsorbing only a small amount (2- to 8-fold reduction in titer) of antibody from anti-158p10p9 (158, Tru, PG6, 19611, ATCC 14124, ISR1), (ii) strains showing an intermediate relationship (16-fold reduction in titer) to 158p10p9 (O7, 23192, H39, 14152, ATCC 14152, Jasmin, JR3, 13988), and (iii) strains capable of adsorbing nearly as much antibody (32- to 128-fold) from anti-158p10p9 as the homologous strain (158p10, 158p10p9, 14152p13, 14124, 14124p10, H606) (Table 2). In a similar experiment, each strain was compared with the *M. arthritis* type strain, strain PG6. Four of the six strains most distantly related to 158p10p9 (Tru, PG6, 19611, and ATCC 14124) were closely related to PG6 (256- to 1,024-fold reduction in anti-PG6 titer). Otherwise, these groupings correlated poorly with those defined by relatedness to 158p10p9 (Table 2).

Assignment of strains to different groups by this method could not always be done with great precision, because single-dilution differences are not particularly meaningful. However, our primary goal here was to confirm that *M. arthritis* strains differed antigenically and to estimate approximate degrees of relatedness. This technique proved useful in that regard and was used several times throughout the study as an initial step to determine whether differences existed among strains or sub-strains prior to more detailed analysis of the antigens involved (see below).

Relationships among *M. arthritis* strains assessed by Western immunoblotting; demonstration of phase variability of one surface marker by colony blotting. Figure 1 shows elec-

TABLE 2. Antigenic and DNA analysis of 20 strains

Strain	Restriction group ^a	Relatedness to ^b :		158p10p9 markers ^c	PG6 markers ^d	H606 markers ^e	MAA1
		158p10p9	PG6				
158 ^f	I	2	16	—	—	—	—
PG6	I	4	1,024	—	+	—	+
19611 ^g	I	4	512	—	+	—	+
Tru	I	4	256	—	+	—	+
23192 ^h	I	16	128	—	—	—	—
14152	I	16	16	—	—	—	—
ATCC 14152 ^h	I	16	32	—	—	—	—
H39	I	16	16	—	—	—	—
O7	I	16	64	—	—	—	—
14152p13	II	32	32	+	—	—	+
14124	II	32	32	+	—	—	+
14124p10	II	32	16	+	—	—	+
158p10p9	II	64	2	+	—	—	+
158p10	II	128	16	+	—	—	+
ATCC 14124 ⁱ	III	8	256	—	—	—	+
Jasmin	III	16	32	—	—	—	+
JR3	IV	16	32	—	—	—	+
ISR1	V	8	2	—	—	—	+
13988 ^j	VI	16	2	±	—	+	+
H606	VI	32	2	+	—	+	+

^a Results of DNA restriction analysis.^b Fold decrease in antibody titer against the homologous strain after adsorption with strain 158p10p9.^c Possession of antigens p51 and MAA2.^d Possession of antigens p120-140 and p42.^e Possession of antigens p74 and lower-molecular-mass version of MAA2.^f Derived from PG27 (Campo).^g ATCC designation for PG6.^h ATCC designations for Campo.ⁱ ATCC designation for Jasmin.^j ATCC designation for H606.

trophoretically separated whole-cell lysates from all 20 strains exposed to rabbit anti-158p10p9 (Fig. 1A) and to rabbit anti-PG6 (Fig. 1B) antisera. A second set of blots, prepared from different stock cultures of the same 20 strains, gave similar results. Potential markers were identified for a 158p10p9-like group of seven strains (158p10p9, 158p10, 14152p13, 14124, 14124p10, H606, and 13988), a PG6-like group of three strains (PG6; ATCC 23192, the American Type Culture Collection [ATCC] designation for strain PG6; and Tru), and an H606 group of two strains (H606 and ATCC 13988, the ATCC designation for H606).

(i) **The 158p10p9-like group.** A band in the 51-kDa range (p51, see arrow in Fig. 1A) appeared in six of these strains, 158p10, 158p10p9, 14152p13, 14124p10, ATCC 14124, and H606 (Fig. 1A; the band in the 158p10p9 lane is partially obscured on this blot but can be seen more clearly on the 158p10p9 lanes in Fig. 2A [lanes 3 to 14] and 2C [lane 2]). This antigen was also recognized by anti-H606 (Fig. 2C) but not by anti-PG6 (Fig. 1B and 2B), indicating that it was at least partly strain specific. Another marker for this set of strains was a prominent antigen, previously identified as a putative adhesin and designated MAA2 (52), which migrated just below the 66-kDa marker in strains H606 and 13988 and just at 66 kDa in the other five strains (Fig. 1A; see arrows). Its distribution among the 20 strains is better shown on the immunoblot in Fig. 3, on which mycoplasmal proteins were exposed to MAA2-

specific MAb 7a (Fig. 3B). Six of the seven MAA2-positive strains in Fig. 3B were shown by adsorption assay to be closely related to 158p10p9 (32- to 128-fold reduction in titer), suggesting that MAA2 may be an immunodominant antigen for this group. H606 was included among these six strains; the seventh was strain 13988, which is also classified as strain H606 (Table 1). Adsorption with 158p10p9 reduced the titer for anti-13988 16-fold, whereas it reduced the titer for anti-H606 32-fold, which arbitrarily placed 13988 in the "intermediate" serologic group; however, its possession of MAA2 suggests a closer relationship.

Distribution of a second putative adhesin, MAA1, is also shown on Fig. 3A, on which mycoplasmal antigens were immunoblotted against MAb G9a. Although all seven 158p10p9-like strains also possessed MAA1, it was not confined to this group, and its presence did not correlate with any of the strain-specific markers identified in the present study or with serologic relatedness to either 158p10p9 or PG6 (Table 2).

To confirm surface expression of the immunodominant marker MAA2, the seven 158p10p9-like strains were subjected to colony blotting with MAb 7a. Results with strain 158p10p9 are shown in Fig. 4; similar results were obtained with the other "positive" strains. Although the majority of colony replicas were positive, each positive replica showed the emergence of negative sectors and vice versa. This indicated that MAA2 was phase variable, undergoing rapid on-off switching within progeny derived from single cells. Strains failing to react with MAb 7a by Western immunoblotting also showed uniformly negative colony blots with no sectoring, with the exception of PG6, which showed a very small percentage of weakly positive colonies (data not shown). This could be due to a cross-reacting antigen or the expression of only a very small amount of MAA2 by PG6. Whatever the reason, this observation may explain why antiserum directed against PG6 recognized MAA2 on Western immunoblots of heterologous strains (Fig. 1B; see arrows).

(ii) **The PG6-like group.** The PG6-like group showed a unique banding pattern in the 120- to 140-kDa range, designated p120-140; this set of antigens was recognized by anti-PG6 (Fig. 1B; see arrows) but not by anti-158p10p9 (Fig. 1A) or anti-H606 (Fig. 2C), indicating that these may be strain specific. A second prominent marker for the PG6-like group was a band designated p42 (Fig. 1A and B; see arrows), which was not seen in the other 17 strains. This marker was useful because of its size, although it was not a unique antigen, because it was also recognized by antisera against 158p10p9 and H606 (Fig. 1A, and 2C, respectively; see arrows).

(iii) **The H606 group.** A size-variable antigen migrating in the 74-kDa range (p74) in the two H606 strains (H606 and 13988) was identified as a marker for this group (Fig. 1 and 2; see arrows). This protein appeared in a slightly larger form in the other 18 strains; this was confirmed by immunoblotting with the anti-p74 MAb (data not shown). A second marker for the H606 group was their possession of a lower-molecular-mass version of putative adhesin MAA2 than that seen in other MAA2-positive strains (Fig. 1, 2, and 3B).

(iv) **JR3.** Strain JR3 showed an unusual high-molecular-mass smear containing antigens that cross-reacted with 158p10p9 (Fig. 1A) but not with PG6 (Fig. 1B). This antigen was not subjected to further examination in the present study.

Relationships among *M. arthritis* strains assessed by DNA restriction analysis. DNA from each of the 20 strains was digested with *Bam*HI, *Sal*I, *Pst*II, and *Kpn*I (data not shown). On the basis of the similarities in cleavage patterns with all four enzymes, the strains could be divided into six groups. In Table 2, restriction groupings are compared with serologic

FIG. 1. Western immunoblot analysis of 20 *M. arthritidis* strains. Whole-cell lysates were loaded onto a 7.5% (wt/vol) T (acrylamide) SDS-polyacrylamide gel at a protein concentration of 20 μ g per lane. After electrophoresis, proteins were transferred to a nitrocellulose membrane and were exposed to hyperimmune rabbit antiserum against *M. arthritidis* 158p10p9 (A) or PG6 (B); this was followed by exposure to peroxidase-conjugated anti-rabbit IgG and a 4-chloro-1-naphthol- H_2O_2 substrate. Molecular mass markers and the positions of potential strain markers are indicated on the right.

relatedness to 158p10p9 and PG6 and possession of the antigenic markers described above. Strains found by antigenic and serologic analyses to be similar also shared similar restriction patterns. All members of restriction groups II and VI possessed the 158p10p9-like markers MAA2 and p51. The H606

strains formed a subset of the larger 158p10p9-like group, differing from the others by restriction pattern and the possession of p74 and a lower-molecular-mass version of MAA2. Restriction group I was the largest group and contained strains that were only distantly to moderately related to 158p10p9 by

FIG. 2. Western immunoblotting analysis of clonal lineages derived from three *M. arthritidis* strains (arrows across the top indicate clone derivations). Whole-cell lysates were electrophoresed at 20 μ g (total protein) per lane on 7.5% (wt/vol) T (acrylamide) SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and exposed to the appropriate rabbit antiserum, peroxidase-conjugated anti-rabbit IgG, and a 4-chloro-1-naphthol- H_2O_2 substrate. (A) Eleven clones (lanes 3 to 13) derived from strain 158p10p9, the parent strain (lane 14), and strains H606 (lane 1) and PG6 (lane 2) were exposed to hyperimmune rabbit antiserum against strain 158p10p9. The clone in lane 4 was subcloned from that in lane 3, and the one in lane 6 was subcloned from that in lane 5. Clones designated LC1, SCR, and LCR are in lanes 3, 4, and 6, respectively. Molecular mass markers are on the left, and potential strain markers are on the right. (B) Nine clones (lanes 3 to 11) derived from strain PG6, the parent strain (lane 12), and strains H606 (lane 1) and 158p10p9 (lane 2) were exposed to hyperimmune rabbit antiserum against strain PG6. Molecular mass markers are on the left, and potential strain markers are on the right. (C) Ten clones (lanes 3 to 12) derived from strain H606, the parent strain (lane 13), and strains PG6 (lane 1) and 158p10p9 (lane 2) were exposed to hyperimmune rabbit antiserum against strain H606. Molecular mass markers are on the right, and potential strain markers are on the left.

adsorption assay. All three of the PG6-like strains (markers p120-140 and p42) were in this group. Groups III, IV, and V contained only one to two members each and were also serologically and antigenically distinct from 158p10p9.

Stability of strain-specific markers. Because some of the surface antigens of *M. arthritidis* have been shown in the present study (Fig. 4) and by others (10, 12) to undergo both phase and size variations, filter-cloned isogenic progeny of strains 158p10p9, PG6, and H606 were examined for the stability of expression of the antigens identified above as potential strain markers. To begin with, the 158p10p9-derived clones were tested for differences in their abilities to adsorb antibody from anti-158p10p9 antiserum. Most of the clones adsorbed antibody at least as well as the parent strain (128- to 512-fold reduction in titer), but three, designated LC1, SCR, and LCR,

were less effective. In repeated experiments, LC1 and SCR consistently reduced the titer only 32- to 64-fold, and LCR consistently reduced the titer 4- to 8-fold.

Eleven 158p10p9 clones were immunoblotted against anti-158p10p9 antiserum (Fig. 2A). The 158p10p9-like markers MAA2 and p51 were present in all 11 clones; the former was confirmed by immunoblotting with MAb 7a (data not shown). However, the three clones showing reduced abilities to adsorb antibody from anti-158p10p9 were missing some antigens present in the other clones. LC1 (Fig. 2A, lane 3) was described previously as a low-adherence variant missing putative adhesin MAA1 (Fig. 2A; see arrow); SCR (Fig. 2A, lane 4) was subcloned from it (52). In addition, LCR (Fig. 2A, lane 6) was missing an antigen migrating just below the 42-kDa marker. This antigen may correspond to one of several phase-variable antigens in the 37- to 43-kDa range recently described by Droege et al. (10) in the related strain, strain 158p10. In any case, loss of these antigens may explain the loss of adsorption ability.

Nine PG6 clones and 10 H606 clones were also examined by immunoblotting against their homologous antisera (Fig. 2B and C, respectively). The p42 and p120-140 markers were present in all nine PG6 clones (Fig. 2B; see arrows), and all 10

FIG. 3. Western immunoblot analysis of 20 *M. arthritis* strains for possession of putative adhesins MAA1 and MAA2. Whole-cell lysates were loaded onto a 7.5% (wt/vol) T (acrylamide) SDS-polyacrylamide gel at a protein concentration of 20 μ g per lane. After electrophoresis, proteins were transferred to a nitrocellulose membrane and exposed to MAb G9a (A), specific for protein MAA1, or MAb 7a (B), specific for protein MAA2; this was followed by exposure to peroxidase-conjugated anti-mouse IgG and a 4-chloro-1-naphthol- H_2O_2 substrate.

H606 clones showed the p51, p74, and MAA2 markers (Fig. 2C); the last two markers were confirmed by immunoblotting with MAbs 7a and anti-p74 (data not shown). However, the clone in lane 8 of Fig. 2C expressed only a small amount of MAA2. We confirmed by colony blotting that this clone was derived from a progeny cell in which expression of MAA2 was switched "off" (data not shown). Only about one-fourth of the colonies derived from this lineage expressed the positive phenotype. The "disappearance" of MAA2 from this particular clone was accompanied by the disappearance of a protein designated p46 (Fig. 2C; see arrow) and the appearance of a heavy band below the 42.7-kDa marker (unmarked in Fig. 2C), although it is not possible to determine from these experiments whether this was coincidental.

To determine whether antigenic variability among filter-

cloned lineages of 158p10p9, PG6, and H606 was reflected in changes in DNA restriction patterns, DNA from all clones plus the parent strains was digested with *Spe*I. Restriction patterns within each strain were identical (data not shown). For confirmation, 158p10p9 clones were also digested with *Bam*HI, *Sal*I, *Pst*I, and *Kpn*I, with similar results.

Analysis of strains subjected to serial passage in animals. Among the 20 strains used in the study were three groups consisting of a parent strain and one or two animal-passaged derivatives (Table 1). These were designated the 158 strains, consisting of strain 158 (parent), strain 158p10 (passaged 10 times through rats), and strain 158p10p9 (passaged an additional nine times through mice) (5, 16); the 14124 strains, consisting of strain 14124 (parent) and strain 14124p10 (passaged 10 times through rats) (16); and the 14152 strains, consisting of strain 14152 (parent) and strain 14152p13 (passaged 13 times through rats) (16).

Results of the experiments described above and summarized in Table 2 indicated that strain 158 was among those most distantly related to 158p10p9, even though the latter was originally derived from the former by serial passage through rats (16) and mice (5). This prompted us to look more closely at these strains for changes that may have resulted from this procedure. Rabbit antisera against strains in each group were adsorbed with intact cells from the other strains within the same group. Each experiment was performed at least twice, with similar results. Strains 158p10 and 158p10p9 both adsorbed similar amounts of antibody from anti-158p10 and anti-158p10p9, reducing their titers 64- to 128-fold, but these strains reduced the titer of anti-158 by only 4- and 2-fold, respectively, and adsorption with strain 158 reduced the titers of anti-158p10 and anti-158p10p9 by 16- and 4-fold, respectively. This indicated that there were differences in surface antigens between 158 and its rat- and mouse-passaged derivatives but that those derivatives were antigenically very similar to each other. There were similar differences between strain 14152 and 14152p13 (adsorption with heterologous strains reduced the

FIG. 4. Colony blot of *M. arthritis* 158p10p9 exposed to MAb 7a, specific for putative adhesin MAA2. (A) Colonies derived from variants in which expression of MAA2 was switched off, showing emergence of progeny expressing the on phenotype. (B) Colony derived from a variant in which expression of MAA2 was switched on, showing the emergence of progeny expressing the off phenotype.

titers of anti-14152 and anti-14152p13 16- and 32-fold, respectively, compared with reduced titers of 256- and 128-fold for homologous strains, respectively) but not between strains 14124 and 14124p10.

Western immunoblotting showed that serologic differences were accompanied by differences in antigenic profiles. Strain 158 was missing both the p51 (Fig. 1A) and MAA2 (Fig. 1A and 3B) markers that distinguish the 158p10p9-like group, as well as MAA1, which was expressed by both of its animal-passaged derivatives (Fig. 1A and 3A). MAA1, MAA2, and p51 were also missing from 14152 but were present in 14152p13 (Fig. 1A and 3). However, Western immunoblots of serologically identical strains 14124 and 14124p10 were also identical, as were 158p10 and 158p10p9 (Fig. 1A).

These altered relationships were also reflected at the DNA level. Restriction patterns of antigenically similar strains 158p10 and 158p10p9 were identical to each other but were different from that of strain 158. Similar differences were seen between strains 14152 and 14152p13, while antigenically identical strains 14124 and 14124p10 were the same (Table 2).

Because in vivo passage through animals apparently resulted in alteration of surface antigens of derivatives of strains 158 and 14152 and because those alterations were also reflected in changes at the DNA level, we were interested in determining the stability of DNA restriction patterns to in vitro subculture. We digested DNA from frozen low-passage samples of strains 158, 158p10, and 158p10p9 estimated to have undergone fewer than 10 in vitro passages since their acquisition or (for the last two strains) their isolation from rats or mice, samples from more recently prepared stock cultures in current use in our laboratory, estimated to have undergone at least one to six additional passages in broth medium, and samples deliberately subjected to 53 additional broth transfers with restriction endonucleases *Bam*HI, *Pst*I, *Sal*I, and *Kpn*I. In vitro passage induced no discernible changes in restriction patterns (data not shown).

Comparison of samples of the same strains obtained from different sources or maintained in different laboratories. The 20 strains used in the present study contained four sets that were originally designated as or derived from the same strains but that had been maintained in different laboratories, sometimes for decades. Included among these were two PG6 strains (PG6 and 19611), two H606 strains (H606 and 13988), a set of four strains originally derived from Campo or PG27 (158, 23192, 14152, ATCC 14152) (4, 13, 14, 33), and a set of three strains originally derived from Jasmin (Jasmin, 14124, ATCC 14124) (4, 22). No changes in antigenic composition or restriction patterns were seen between PG6 and 19611 or between H606 and 13988 (Fig. 1; Table 2), even though the latter members of each pair were just recently obtained from ATCC, while the former members of each pair were maintained at the University of Utah and later at the University of South Dakota for more than 25 years. The four strains derived from Campo also showed essentially no differences in either antigenic makeup (Fig. 1A) or restriction patterns, nor did two Jasmin-derived strains, Jasmin and ATCC 14124, obtained from the University of Alabama and ATCC, respectively, although a third Jasmin-derived strain, strain 14124, obtained from ATCC 28 years ago, is no longer quite the same as the other two in either antigenic makeup (Fig. 1) or restriction pattern (Table 2).

DISCUSSION

Since it has been possible to identify and categorize strains and serogroups within other mycoplasmal species by means of

serologic, electrophoretic, and chromosomal analyses (1, 3, 15, 18, 20, 21, 23, 29, 30, 32, 35, 36), we were prompted to apply these techniques to *M. arthritis*. Until recently, *M. arthritis* had not been analyzed in this manner, and the applicability of techniques used for other species to this organism was by no means certain. However, work by other investigators several decades ago suggested that it should be possible. For instance, Tully (46), in 1965, noted that the rat joint isolate JR3 and the avirulent type strain PG6 were not completely cross-reactive by immunofluorescence. We have now confirmed that they are indeed antigenically and genetically different. Five years later Golightly-Rowland et al. (16), in one of the largest, most systematic examinations of *M. arthritis* to date, demonstrated dramatic differences in arthritogenicity among strains and enhancement of the arthritogenicities of low-virulence strains by serial passage through rats as well as virulence-associated differences in growth rate and susceptibility to the effects of neutralizing antibodies. That study was the source of strains 158p10, 14124p10, and 14152p13 used in the present investigation. It represented the first attempt to systematically differentiate *M. arthritis* strains and formed the basis for our present work.

In a more recent study (51), we demonstrated serologic differences among a set of four strains, 158p10p9, 14124p10, PG6, and H606, using an antiserum adsorption assay similar to the one used in the present study. We have now confirmed that this technique may be useful on a larger scale, although it is probably more reliable for determining approximate rather than precise degrees of relatedness. It is also apparent that antigenic variability within a strain can profoundly affect these results. Therefore, this technique should not be used alone for strain identification or differentiation, especially if strains are routinely cloned on acquisition, but rather, if it is used at all, it should be in combination with restriction analysis and Western immunoblotting. Its primary usefulness may be in the actual demonstration of variation of immunodominant antigens within strains.

Interstrain antigenic variability was easily demonstrated by Western immunoblotting. While this technique also dramatically confirmed considerable intrastain variability, we were able to identify several potentially useful and apparently stable strain markers, some of which may be strain-specific antigens, while others are shared antigens showing strain-specific size variation. At least two of these, p51 and p120-140, have been observed by other investigators but have not previously been confirmed as strain markers.

A broadly diffuse high-molecular-mass band in strains PG6 and Trudeau (referred to in our study as strain Tru) and named protein II by Stadtländer and Watson (44) is probably the same as our p120-140 antigen. It also corresponds to the acidic ladder antigen no. 9 described in our earlier strain differentiation study; this antigen appeared prominently on two-dimensional Western immunoblots of PG6 and did not cross-react with 158p10p9, 14124p10, or H606 (51). However, a monospecific antiserum against it prepared by Stadtländer and Watson (44) did cross-react with strains JR3 and ISR1, although not with strains H39 and Jasmin. Among a set of five *M. arthritis* strains studied by Droege et al. (10), p120-140-like antigens were seen only in PG6, while a 48- to 52-kDa three-band pattern, containing our antigen p51, was seen only in strain 158p10; the latter has now been subjected to additional antigenic and biochemical analyses. We have now shown that other strains not among those tested by Stadtländer and Watson (44) and by Droege et al. (10) also possessed these sets of antigens, that their presence correlated with antigenic relatedness to PG6 and 158p10p9 and with similarities in re-

striction patterns, and that they appeared to be stably expressed, thus confirming their usefulness as strain markers.

In addition, we have identified two other markers, MAA2, which appeared to be unique to the 158p10p9-like set, and p74, which defined an H606-like subgroup within this set. The H606 strains were further distinguished from other strains in the 158p10p9-like group by their possession of a lower-molecular-mass version of MAA2. MAA2, which may be involved in cytoadherence (52), was also seen in our previous strain comparison study (51), although we did not recognize its significance at the time. In that study it could be seen as an intensely staining, slightly basic protein on two-dimensional Western immunoblots of strains 158p10p9 and H606, while it was prominently missing from PG6 (Fig. 3U, 5U, and 4U, respectively, in the report by Washburn and Hirsch [51]). While p74 appeared to be stably expressed by cloned derivatives of H606, MAA2, although apparently an immunodominant antigen, was phase variable, and clones showing the off phenotype showed reduced reactivity with the MAA2-specific MAb 7a by Western immunoblotting. MAA2 should still be useful as a marker but should be used with caution, especially when testing cloned populations.

Restriction analysis of chromosomal DNA proved to be the most reliable form of strain differentiation, although it lacked the fine specificity of antigenic analysis. However, restriction patterns were not subject to variation among clonally derived lineages or to alteration induced by up to 53 in vitro subcultures. These results indicate that restriction analysis can be used for the initial placement of an *M. arthritis* isolate within a larger group, after which analysis by SDS-polyacrylamide gel electrophoresis and Western immunoblotting can define its relationship to other strains with greater precision. It is, in fact, important that such analyses be performed on new isolates and newly acquired strains and for routine purposes of quality control to guard against cross-contamination with or of commonly used laboratory strains.

Two examples of the utility of combined antigenic and restriction analysis of *M. arthritis* were presented in this report. The first was a systematic comparison of strains subjected to rat and mouse passage to enhance their virulence (16). In a preliminary study, serologic analysis by cross-adsorption and two-dimensional Western immunoblotting showed that strains 158p10 and 158p10p9 were no longer antigenically closely related to their parent strain, strain 158 (55). Further analysis, presented here, showed that while 158p10 and 158p10p9 were essentially identical, they were quite different from 158 in both antigenic and restriction profiles. Restriction analysis of low-, mid-, and high-passage samples of these strains showed identical results, indicating that they had not become contaminated during manipulation in our laboratory. Similar differences were seen between strains 14152 and 14152p13 but not between strains 14124 and 14124p10. Explanations for concomitant alterations at both the protein and DNA levels include (i) the possibility of a mutation leading to the development and overgrowth of a more highly virulent variant, (ii) in vivo selection from a mixed population of a more highly virulent and an antigenically or genetically different variant, or (iii) isolation of an indigenous *M. arthritis* strain rather than the low-virulence strain originally injected. The present study and recent reports from other laboratories have confirmed that individual strains of *M. arthritis* are capable of considerable antigenic variation both in vitro (12) and, more importantly, in vivo (10, 11). Therefore, in vivo selection of a favorable phenotype is a reasonable hypothesis. The change in restriction pattern is harder to explain, since we have shown that these do not appear to vary with antigenic changes in vitro, although selec-

tion of a virulent genotype from a largely avirulent mixed population remains a possibility. Considering the length of time that has passed since the original animal passages were performed, it may not be possible to determine precisely what happened to these strains. However, additional rat passage experiments with isogenic lineages could help to answer some of the remaining questions.

Combined antigenic and restriction analysis also proved to be useful in characterizing and comparing strains, collected in our laboratory, that originated from the same source but that had been maintained in separate institutions, sometimes for several decades. These analyses confirmed that strain H606 and ATCC 13988 (the ATCC number for H606) were essentially identical, in spite of more than 25 years of separate maintenance. Restriction group III strains are both classified as Jasmin, and the present study confirmed their identities as well. It also confirmed the close relationship among type strain PG6, ATCC 19611 (the ATCC number for PG6), and Tru, obtained from the University of Alabama and described as a more recent isolate from a healthy rat (7), and the relationship among the Campo-derived strains (158, 23192, 14152, ATCC 14152), which were all found in restriction group I. However, the version of ATCC 14124 obtained from ATCC more than 25 years ago (designated simply 14124) is no longer quite the same by serology or restriction analysis as two newer (to us) samples of the same strain, ATCC 14124 and Jasmin.

In conclusion, we have shown that strains of *M. arthritis* isolated from different sources differed sufficiently to allow them to be grouped according to their antigenic and chromosomal characteristics, and we have identified several markers for three sets of strains that can be used for this purpose. Others that will help to further define additional sets of strains are no doubt present; however, in identifying them, it is essential to keep in mind the propensity of this species for antigenic variation, which can complicate antigenic analysis unless great care is taken to identify stable markers. Quality control in the past has consisted of periodic reidentification of isolates and strains as *M. arthritis* by ELISA-, growth inhibition-, or immunofluorescence-based methods, but until now no markers were available to guard against cross-contamination of strains. We hope that this work will help to alleviate that deficiency. These techniques have already proven to be highly useful in our laboratory for purposes of quality control and for characterizing and uncovering interesting relationships among animal-passaged strains and strains that were obtained from different sources.

ACKNOWLEDGMENTS

We thank Richard Duman for assistance in preparation of many of the media and reagents used in the study.

This work was supported in part by grants from the University of South Dakota School of Medicine Parson's Endowment Fund, the University of South Dakota General Research Fund, Public Health Service grant RO1 37924 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, the National Science Foundation EPSCoR Program under grant OSR-9108773, and the South Dakota Future Fund.

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