A Comparative Study of Bovine and Ovine *Haemophilus somnus* Isolates

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ABSTRACT

Bacterial isolates (including 17 *Haemophilus somnus* isolates and an *H. somnus*-like isolate) from asymptomatic or diseased cattle and sheep, were evaluated for markers associated with virulence and host predilection. The isolates were separated into 6 distinct biovariants, 3 for sheep and 3 for cattle, based on reactions in a battery of 21 test media. Three bovine isolates associated with disease caused hemolysis of bovine blood. The rest of the isolates did not hemolyze either bovine or ovine erythrocytes. Protein profiles of all *H. somnus* isolates were similar with the exception of the major outer membrane proteins (MOMPs). The MOMPs of isolates associated with disease in cattle had a relative molecular weight of approximately 41 kDa compared with 33 kDa for the MOMPs of isolates from asymptomatic cattle. The MOMPs from sheep isolates were either slightly higher or lower than the 41 kDa MOMPs of bovine isolates. Major antigens detected by Western blotting were similar in all isolates except the *H. somnus*-like isolate. An immunodominant 40 kDa antigen was conserved in all *H. somnus* isolates. Antibodies to this antigen have previously been found to be protective in cattle and may also be protective for sheep. Marked differences between cattle and sheep isolates were revealed by use of restriction enzyme analysis, which separated the isolates into 12 ribotypes and 15 unique DNA profiles. Thus, cattle and sheep isolates in this collection had distinctive differences in biochemical reactions, MOMP profiles, and DNA analyses. Such differences have potential value for epidemiological studies and may also be used to evaluate host specificity of *H. somnus* isolates.

RÉSUMÉ

Une évaluation d'indicateurs de virulence ou de prédilection d'espèce-hôte a été effectuée sur 18 isolats bactériens (17 isolats d'*Haemophilus somnus* et un isolat apparenté à *H. somnus*) provenant de bovins et de moutons malades ou asymptomatiques. Les isolats ont été séparés en six variants biochimiques distincts, trois pour les bovins et trois pour les moutons, à la suite des résultats obtenus dans 21 tests biochimiques. Trois isolats bovins provenant d'animaux malades ont causé une hémolyse d'érythrocytes bovins. Les autres isolats n'hémolysaient pas les érythrocytes bovins ou ovins. Les profils protéiniques de tous les isolats d'*H. somnus* étaient similaires à l'exception des protéines majeures de la membrane externe (MOMPs). Le poids moléculaire des MOMPs d'isolats originant d'animaux malades était de 41 kDa comparativement à 33 kDa pour les isolats provenant de bovins asymptomatiques. Le poids moléculaire des MOMPs des isolats ovins était légèrement supérieur ou inférieur à celui de 41 kDa des isolats bovins. Les antigènes majeurs mis en évidence par électrophorèse et transfert sur membrane de nitrocellulose étaient similaires pour tous les isolats à l'exception de l'isolat apparenté à *H. somnus*. Un antigène immunodominant de 40 kDa était retrouvé chez tous les isolats d'*H. somnus*. Des anticorps dirigés contre cet antigène ont été démontrés antérieurement comme protecteur chez les bovins et pourraient l'être également chez les ovins. L'utilisation d'endonucléases de restriction a permis de séparer les isolats en 12 ribotypes et en 15 profils d'ADN en plus de mettre en évidence des différences marquées entre les isolats bovins et ovins. Les isolats bovins et ovins analysés dans cette étude montraient des différences dans leurs réactions biochimiques, leurs profils de MOMPs et d'ADN. Ces différences ont une certaine valeur pour des études épidémiologiques et peuvent aussi être utilisées pour évaluer la spécificité d'hôte des isolats d'*H. somnus*.

(Traduit par Docteur Serge Messier)

INTRODUCTION

Isolation of an organism identified as *Histophilus ovis* was first reported in Australia in 1956 (1). Two years later, it was reported that a similar organism, identified as *Haemophilus agni*, was isolated from sheep with septicemia in the United States (2). In 1960, an organism with similar characteristics was isolated from bovine cases of thrombotic meningoencephalitis (TME) (3). This organism was later given the name *Haemophilus somnus* (4). It was subsequently

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concluded that all 3 organisms were closely related and possibly representative of a single taxon (5).

Although these organisms have not been reported to naturally infect or cause disease in any other animal host, a variety of disease conditions have been associated with infection of cattle and sheep. In addition to TME, *H. somnus* is recognized as the cause of respiratory disease (6,7), septicemia (8,9), metritis (10), abortion (11,12), mastitis (13), arthritis (9), and myocarditis (8,14,15) in cattle. Diseases that have been associated with infection of sheep with *H. agni*, *H. ovis* or *H. somnus* include septicemia (2,16), epididymitis (17,18), abortion (19), meningitis (20), vulvitis (21), and mastitis (1). Due to the similarity of these organisms, those isolated from sheep in North America are now commonly identified as *H. somnus* (5,20, 22). Asymptomatic vaginal and preputial carrier animals are common in both cattle and sheep (19,23–26). Disease variance in the 2 animal species has been indicative of diversity among the organisms. The diversity among organisms identified as *H. somnus* includes variances in virulence (20, 27), IgG binding (28), outer membrane protein profiles (28,29), and serum susceptibility (30). In addition, 21 different biotypes, 16 ribotypes, and 33 restriction endonuclease analysis (REA) types have been identified in a group of 105 bovine isolates (31). Although variance in virulence is recognized, correlation of identifiable characteristics or factors with differences in host and tissue predilection is not well defined.

Therefore, we have evaluated organisms from a variety of ovine and bovine disease conditions as well as preputial samples from sheep and cattle in North America in an attempt to identify factors that may be associated with host predilection and virulence. The results are presented and discussed in this report.

MATERIALS AND METHODS

BACTERIAL ISOLATES

A total of 18 isolates, 17 of which were previously identified as *Haemophilus somnus* and 1 *H. somnus*-like isolate, including 6 from cattle and 12 from sheep, were evaluated in this study. Bovine isolates included 1 from each of the following: abortion (649), pneumonia (2336), myocarditis (90-1574), and meningoencephalitis (8025), and 2 from preputial samples (1P, 129PT) from asymptomatic bulls. Isolates from sheep included 3 from cases of septicemia (2032, 2041, 2184), 3 from orchitis and/or epididymitis (L1203B, L1256-4, 86-840-B), and 5 preputial samples (67P, 93P, 81P, 35P, A8). One additional isolate (1190) was from a lymph node of a sheep that died, without association of this organism with disease or death of the animal.

Table I. Biovariant differentiation of *Haemophilus somnus* isolates and an *H. somnus*-like isolate (1190)

	Biovariant numbers					
	1[a]	2	3	4	5	6
Biochemical	Test reactions[b]					
Production of:						
Ornithine decarboxylase	+	+	−	+	+	−
β-glucosidase	+	±[c]	±	+	+	−
α-fucosidase	−	−	−	+	+	+
Fermentation of:						
Sorbitol	+	−	+	+	+	−
Xylose	+	+	+	+	−	+

[a] Biovariant 1 isolates (2336, 8025, 1P, and 129PT), biovariant 2 isolate (649), and biovariant 3 isolate (90-1574), were all from cattle; biovariant 4 isolates (L1203B, L1256-4, A8, 35P, 67P, 81P, and 93P), biovariant 5 isolates (2032, 2041, 2184, and 86-840-B), and biovariant 6 isolate (1190) were all from sheep

[b] All isolates were oxidase positive, reduced nitrate, and fermented glucose, fructose, and mannitol. None of the isolates produced urease or fermented arabinose, cellobiose, lactose, maltose, trehalose, or salicin. Isolate 1190 produced catalase and β-galactosidase and fermented sucrose while all other isolates did not produce catalase or β-galactosidase or ferment sucrose. All isolates except 1190 produced indole

[c] ± indicates a weak positive reaction for the indicated biochemical test

All isolates had previously been lyophilized or preserved in a 40% phosphate buffer (pH 7.2) plus 60% glycerol solution at −70°C. The organisms were propagated from the preserved stocks on Columbia blood agar (Difco Laboratories, Detroit, Michigan) with 5% ovine blood (CBA) or brain-heart infusion (BHI) agar (Difco) with 5% ovine or bovine blood.

BIOGROUPING AND EVALUATION OF ISOLATES FOR HEMOLYSIS

Each isolate was subsequently inoculated from CBA into a battery of 21 biochemical tests for biogrouping (Table I). Tests results for enzyme activity were recorded after 4 h and the results of tests that required growth for substrate utilization were recorded after 48 h and again after 10 d incubation.

To detect and compare hemolysis of bovine and ovine erythrocytes, isolates were streaked for isolation on BHI agar with 5% bovine and BHI agar with 5% ovine blood. Plates were incubated in a candle jar for 48 h at 37°C followed by 48 h at room temperature to allow greater development of hemolytic zones, and then evaluated for hemolytic activity.

GEL ELECTROPHORESIS AND WESTERN BLOTTING

Isolates were grown on BHI agar with 5% bovine blood prior to evaluation of cellular components. Bacteria were harvested from BHI agar in phosphate-buffered saline (PBS) and washed and resuspended in fresh PBS to produce a density of 75%T at 610 nm with a Coleman spectrophotometer. A 1 mL volume of the suspension (10^8 CFU) was centrifuged at 17,500 *g* for 4 min. The pellets were resuspended in 20 μL PBS and 20 μL sample buffer, boiled for 5 min, and loaded into the wells of a stacking gel. Electrophoresis of proteins into the 7.5% to 17.5% polyacrylamide separating gel was done at 30 amps for approximately 4 h. Gels were stained with 0.075% Coomassie brilliant blue or electrotransferred to nitrocellulose at 30V overnight, followed by 70V for 2 h for Western blotting, as described previously (20). Blotted membranes were incubated for 2 h in convalescent bovine antiserum (animals E7 and P3) at a ratio of 1:1 and a 1:1000 dilution. Blots were rinsed 3 times, and then incubated for 2 h in peroxidase conjugated antibovine IgG (H&L) diluted 1:1000 (ICN Biomedicals, Costa Mesa, California). Blots were then washed 3 times and developed in 4-chloro-1-naphthol/hydrogen peroxide substrate (Sigma, St. Louis, Missouri) for 30 min.

ISOLATION OF DNA

Bacterial isolates were grown on CBA with 5% ovine blood at 35°C in an atmosphere with 10% added CO_2 for 24 h. The bacteria were harvested from the agar using sterile glass rods

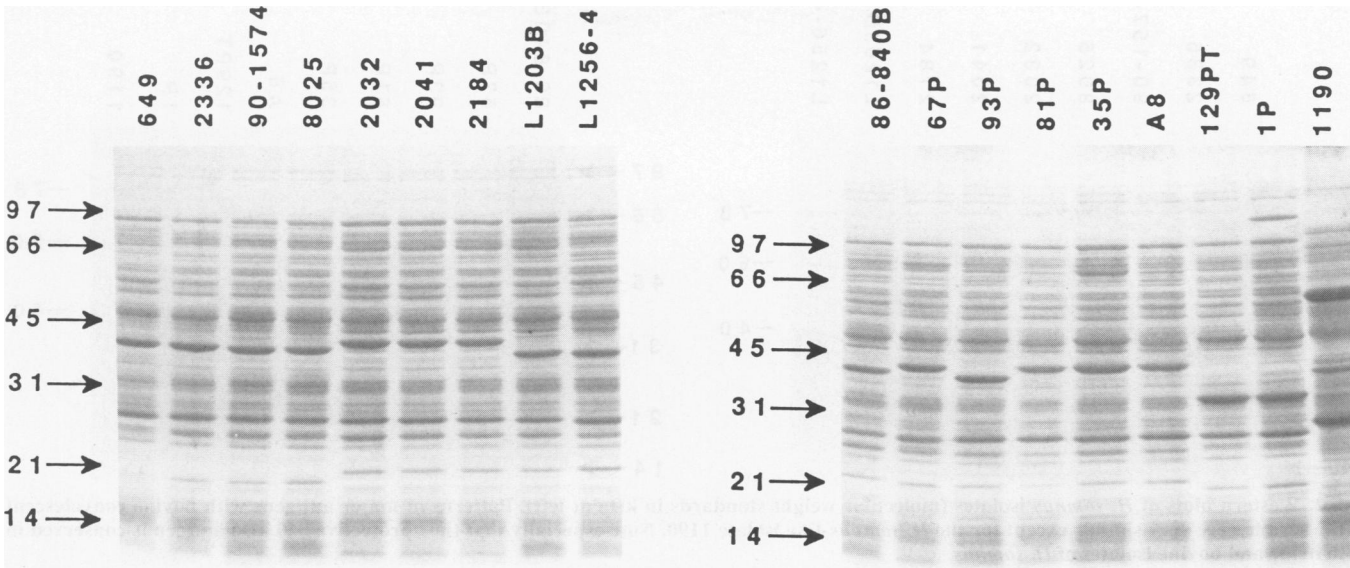

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of *H. somnus* isolates (molecular weight standards in kDa at left). Note: profiles for all isolates are similar except for the *H. somnus*-like organism 1190. However, 2 proteins vary widely. The major outer membrane proteins (MOMPs) were ~41 kDa in bovine virulent isolates and 33 kDa in bovine preputial isolates 129PT and 1P, but varied considerably between ~38 and 42 kDa in ovine isolates. Also, the protein around 21 kDa was slightly higher in ovine strains compared with bovine isolates.

and a 20 mM Tris buffer (pH 8.0) containing 20 mM ethylenediaminetetraacetate (EDTA), collected by centrifugation at 500 *g* for 20 min and then suspended in 5 mL STE-sucrose (10 mM NaCl, 50 mM Tris [pH8.0], 10 mM EDTA, and 25% sucrose). Each bacterial suspension was incubated in 1% sodium dodecyl sulfate (SDS) with 200 μg/mL of proteinase K (Promega, Madison, Wisconsin) at 45°C for 4 h for lysis. The lysates were extracted by mixing equal parts with a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol and collecting the aqueous phase following centrifugation at 500 *g* for 20 min. The DNA was precipitated from the aqueous extract by the addition of 2.5 volumes of cold ethanol and holding in an ice bath for 20 min. The DNA was spooled onto a glass rod, rinsed with cold 70% ethanol, and then dissolved overnight in 10 mM Tris, with 1 mM EDTA (pH 8.0).

RESTRICTION ENZYME ANALYSIS

The DNA from strain 8025 was treated with a battery of restriction enzymes including *Hae*III, *Bam*H1, *Pvu*II, and *Hin*fI. Digestion of the DNA with *Hin*fI gave the best REA resolution for discrimination among isolates. Therefore, each DNA extract was treated with *Hin*fI for 2 h in supplied buffers (International Biotechnology Incorporated, New Haven, Connecticut) for cutting. Digests were loaded onto 0.8% horizontal agarose gels and electrophoresed (30 V, 20 h) with TBE solution (89 mM Tris, 89 mM boric acid, 1 mM EDTA) on a Sub-Cell electrophoresis unit (Bio-Rad, Richmond, California). Gels were stained with ethidium bromide (0.5 μg/mL) for 1 h and photographed with a Polaroid MP4 instant camera system (Polaroid Corporation, Cambridge, Massachusetts) using a red filter and type 55 positive/negative film.

SOUTHERN HYBRIDIZATION AND PROBE LABELING

This procedure was conducted as previously described (32). DNA preparations were cut with *Hae*III for Southern hybridization as described above. The *Hae*III digested DNA was then transferred from agarose gels to nylon membranes in 10× SSC solution (1.5 M NaCl. 0.15 M sodium citrate) by capillary action. *Escherichia coli* 16s + 23s rRNA, labeled by the random primer method with digoxigenin-labeled dUTP, was hybridized with complementary fragments of DNA from test organisms and detected colorimetrically with antidigoxigenin alkaline phosphatase, NBT, and BCIP (Boehringer Mannheim, Indianapolis, Indiana). Blots were photographed with a Polaroid instant camera system.

RESULTS

All isolates grew well on agar with either ovine or bovine blood. Only 3 isolates, 649, 8025, and 90-1574, isolated from cases of abortion, TME, and myocarditis, respectively, produced hemolysis on BHI agar with bovine blood. None of the isolates were hemolytic on BHI agar with ovine blood.

The isolates were separated into 6 distinct biovariants (1–6) on the basis of reactions in test media (Table I). Bovine isolates from cases of pneumonia (2336) and TME (8025) produced reactions identical to 2 preputial isolates (1P and 129P) designated biovariant 1. The single bovine isolates from cases of abortion (649) and myocarditis (90-1574) were assigned to biovariants 2 and 3, respectively. Sheep isolates were also separated into 3 biovariants distinctive from those of bovine isolates. All 5 isolates from sheep preputial samples and 2 from cases of orchitis/epididymitis produced identical reactions in test media and were identified as biovariant 4 organisms. The 3 isolates from ovine septicemia and 1 from a case of orchitis/epididymitis were identified as biovariant 5. The remaining isolate, 1190, was distinctly different from all other isolates and was assigned to biovariant 6. The colony and Gram-stain morphologies of isolated 1190 were characteristic of *H. somnus* but the organism differed from the others by the lack of indole production and by catalase production.

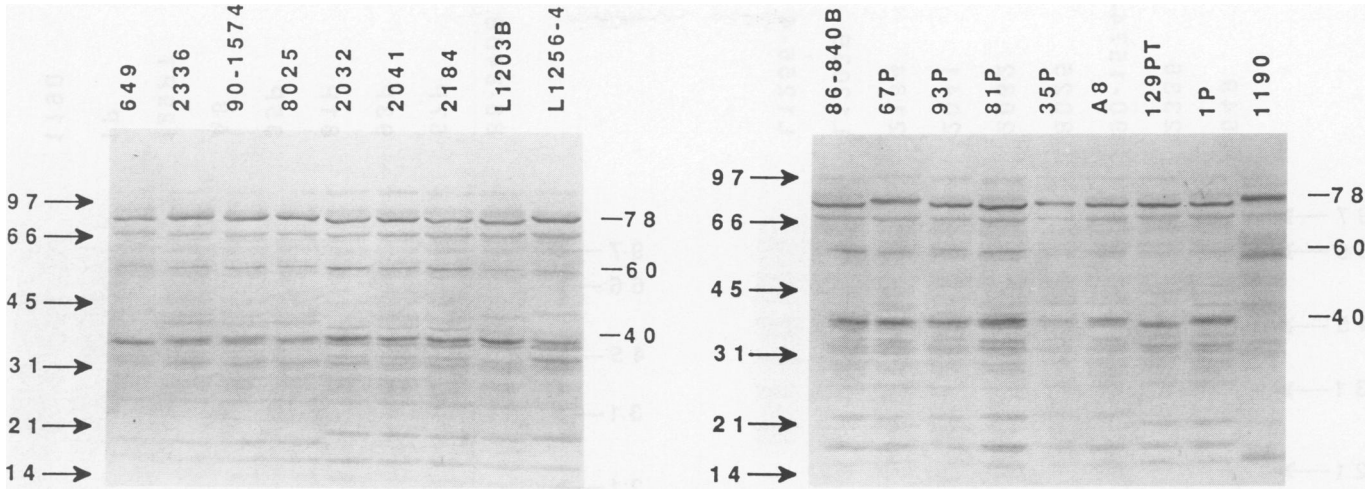

Fig. 2. Western blots of *H. somnus* isolates (molecular weight standards in kDa at left). Patterns of major antigens with bovine convalescent phase serum were very similar except for the *H. somnus*-like isolate 1190. Note especially that the "protective" 40 kDa antigen is conserved in both ovine and bovine isolates of *H. somnus*.

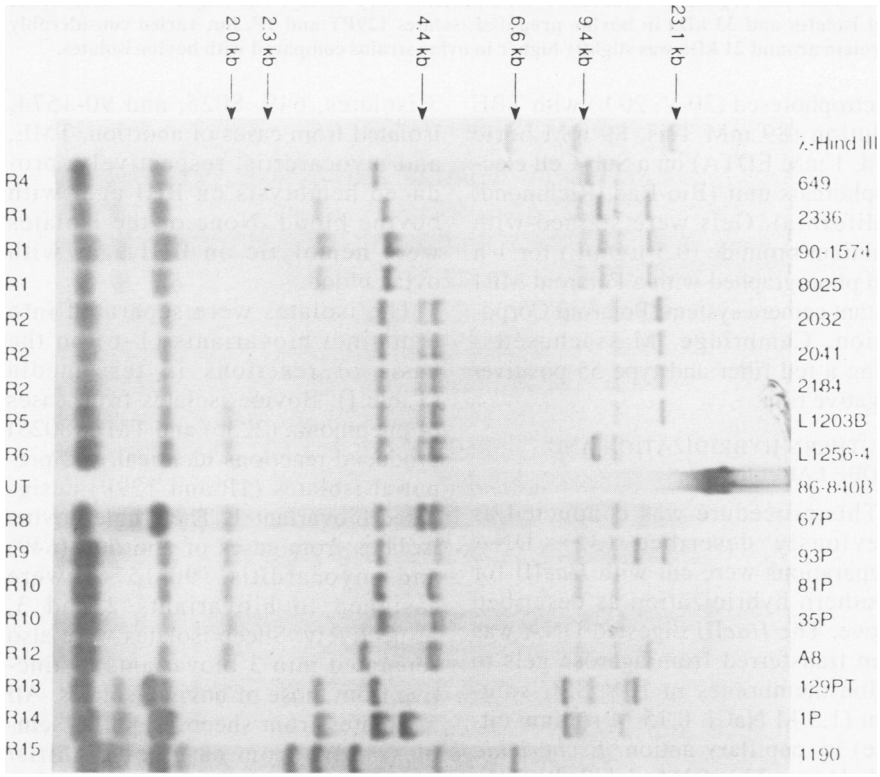

Fig. 3. Ribotypes of *H. somnus* isolates and an *H. somnus*-like isolate were produced using *Hae*III cut DNA and *Escherichia coli* 16s + 23s rRNA labeled by the random primer method with digoxigenin-labeled dUTP and detected colorimetrically with antidigoxigenin alkaline phosphatase and NBT (nitroblue tetrazolium salt in dimethylformamide) and BCIP (5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in dimethylformamide). Twelve distinct ribotypes were recognized.

Protein profiles were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for antigen analysis. All outer membrane protein (OMP) profiles were similar except for the *H. somnus*-like isolate, 1190 (Fig. 1), at least under the growth conditions used in this study. The major OMPs (MOMPs) for bovine virulent isolates had relative molecular weights (rMW) of approximately 41 kDa, while the MOMP of the 2 serum susceptible preputial isolates had an rMW near 33 kDa as has been reported previously (28). With sheep isolates from septicemia as well as isolates A8, 35P, 67P, and 81P, the MOMPs were 1 to 2 kDa higher than the 41 kDa MOMP of virulent bovine *H. somnus* isolates. The rMWs of MOMPs of other ovine isolates were 1 to 2 kDa lower than the 41 kDa MOMP of virulent bovine isolates. The major antigen profiles detected by Western blot tests using bovine convalescent phase serum were similar for both ovine and bovine isolates except isolate 1190 (Fig. 2). A potentially protective 40 kDa antigen was present in all isolates.

Twelve different ribotypes were detected by Southern hybridization (Fig. 3). Bovine isolates from TME, pneumonia, and myocarditis belonged to ribotype R1. The abortion and 2 preputial isolates each produced a distinctive pattern. The 3 isolates from cases of ovine septicemia produced profiles that were identical. Two ovine isolates, 35P and 81P, shared a common profile with each other. Each of the remaining ovine isolates produced a distinctive pattern.

A total of 15 different REA-types were detected in the 18 isolates evaluated (Fig. 4). This included 5 bovine REA-types with the isolates from the single cases of TME (8025) and pneumonia (2336) sharing REA-type H1 and each of the other isolates having a distinctive profile. The 3 ovine isolates from cases of septicemia shared a common REA-type while all of the other 9 isolates were of different REA-types. All ovine isolate REA-types were distinctively different from those of the bovine isolates.

DISCUSSION

Haemophilus somnus from cattle and from sheep had both similarities and differences in tests for both phenotypic and genotypic characteristics. The observation that ovine isolates did not hemolyze either ovine or bovine erythrocytes, whereas 3 of 4 virulent isolates of bovine *H. somnus* hemolyzed bovine, but not ovine, erythrocytes, is consistent with our earlier data that most bovine virulent and vaginal carrier isolates were hemolytic on bovine blood agar (K Blau and LB Corbeil, unpublished data). The data are also consistent with that of Stephens *et al* (5), who noted that some isolates of bovine *H. somnus* were hemolytic on bovine blood agar but *H. agni* and *H. ovis* were not. The lack of hemolysis of ovine erythrocytes by ovine *H. somnus* in our study is similar to results reported by Webb for 17 isolates of *H. agni* (33). Thus hemolysis of bovine erythrocytes by most bovine strains but not ovine strains along with lack of hemolysis of ovine erythrocytes by any of the strains may be representative.

Protein and antigenic profiles of all isolates of *H. somnus* were similar (except for the *H. somnus*-like isolate 1190). Protein profiles were variable only in the area of the MOMP. This is consistent with our previous report of differences in the rMW of MOMPs of virulent and some serum sensitive preputial isolates of bovine *H. somnus* (28). Thus the variability of rMWs of MOMPs of ovine isolates is not surprising. Antigens of bovine and ovine isolates were evaluated by Western blotting with convalescent phase bovine serum because this serum was previously shown to passively protect calves against pneumonia (34). Antigenic profiles of all isolates were remarkably similar, with the exception of the *H. somnus*-like 1190 isolate, as we previously demonstrated with monospecific antibodies (20). Since we previously showed that monospecific antibody to the 40 kDa OMP was passively protective in calves (35), the conservation of this antigen in sheep isolates is noteworthy. It may be that other antigens recognized by convalescent serum such as the high molecular weight immunoglobulin binding proteins (IgBPs), or the 76 kDa antigen that is genetically linked to IgBPs and/or the 60 kDa antigens and 78 kDa antigen, may also be protective (26,29,36). All of these antigens are conserved in sheep isolates. Thus, even if ovine isolates are not pathogenic for cattle and vice versa, it may be that bovine vaccines will protect sheep against diseases associated with *H. somnus*, as has been suggested by Bulgin *et al* (37).

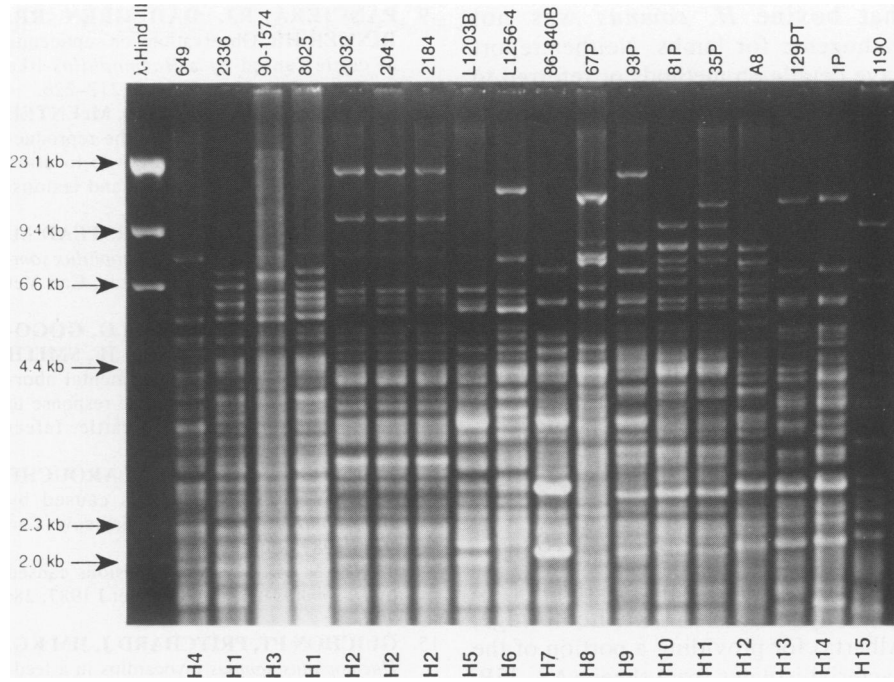

Fig. 4. *Hin*fI restriction endonuclease analysis patterns of *H. somnus* and 1 *H. somnus*-like isolate. Isolates were assigned to 15 different REA types based on similarities of DNA fragment electrophoretic patterns. Note that sheep isolates 2032, 2041, and 2184 produced identical patterns and that bovine isolates 2336 and 8025 shared a common REA pattern similar, but not identical, to 90-1574. All other isolates produced unique patterns.

Separation of the 18 isolates evaluated in this study into 6 different biovariants demonstrated phenotypic variability. Fussing and Wegener recognized 21 biotypes in 105 isolates from cattle (31). The majority of their biotype I isolates were from cases of pneumonia, although 5/66 of the biotype I isolates were recovered from semen or preputial samples. Similarly, we found that at least 2 biovariants (2 and 4) contained isolates from both disease cases and asymptomatic preputial carriers. Both studies indicate that biotyping cannot be used for predictability of virulence, but may be useful epidemiologically.

A greater correlative relationship was evident between ribotypes or REA-types and association of isolates with clinical disease. Isolates from pneumonia, TME, and myocarditis shared a single ribotype that was distinctive from that of 2 preputial isolates and from a single isolate from an abortion. Furthermore, isolate 8025, from a case of TME in Iowa over 30 y ago (36), belongs to the same ribotype as 2 more recent cases in the northwestern USA (2336 and 90-1574). Two of the isolates, 8025 and 2336, also belonged to the same REA-type, which establishes that these organisms are genetically very similar. The 3 ovine isolates (2031, 2041, and 2184) that had identical REA profiles and ribotypes were from cases of ovine septicemia, one of which was used to reproduce disease in sheep (20). These data suggest that ribotyping and REA-typing may have value in correlation of isolates with virulence, as was concluded by Fussing and Wegener for bovine isolates (31). The REA-typing also has great potential in epidemiological surveys and monitoring for transmission of unique bacterial strains, as we previously showed for *Pasteurella haemolytica* (32).

Since both ribotyping and biotyping placed ovine and bovine isolates in separate groups, the question of host specificity arises. Young and Hoerlein reported in an abstract that bovine isolates do not infect sheep (38). In a review paper (39), Biberstein indicated

that bovine *H. somnus* was not pathogenic for lambs. Neither report gave details on methods or referred to experimental studies of ovine *H. somnus* in cattle. We are unaware of other reports in the literature regarding studies of the pathogenicity of ovine *H. somnus* in cattle. We hypothesize that ovine and bovine isolates would not infect cattle and sheep, respectively, based on the ribotyping and biotyping data. This hypothesis needs to be tested by inoculating cattle with biologically relevant numbers of ovine isolates and similar inoculating sheep with bovine *H. somnus*.

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