Deoxyribonucleic Acid Relatedness Among "*Haemophilus somnus*," "*Haemophilus agni*," "*Histophilus ovis*," "*Actinobacillus seminis*," and *Haemophilus influenzae*

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The results of deoxyribonucleic acid-deoxyribonucleic acid hybridization studies demonstrated a high degree of relatedness among "*Haemophilus somnus*," "*Haemophilus agni*," "*Histophilus ovis*," strains isolated from the preputial cavities of rams, and strains isolated from clinical cases of epididymitis in rams. The degree of relatedness between "*Haemophilus somnus*" and *Actinobacillus seminis* or *Haemophilus influenzae* was very small. From the results of our hybridization studies and previous studies on morphological characteristics, serology, and biochemical activity, we conclude that "*Haemophilus somnus*," "*Haemophilus agni*," and "*Histophilus ovis*" should be considered a single species. Inclusion in the genus *Haemophilus* does not appear to be appropriate. The data also suggest that bovine and ovine isolates may constitute two separate groups within the species. More extensive hybridization studies will be required for establishment of the definitive taxonomic position of this group of organisms.

Since the mid-1950s a number of gram-negative bacteria with exacting nutritional requirements have been isolated from disease processes of ruminants in various parts of the world. The first of these organisms was "*Histophilus ovis*" isolated from mastitis in sheep (29). This bacterium was subsequently isolated from epididymitis in rams (7, 11), from synovitis and pyemia in lambs (18, 27), and from vaginal discharge of ewes (14). Another organism, "*Haemophilus agni*," was isolated from a septicemic disease of lambs (20). A similar agent was found to be responsible for a disease of cattle, described earlier as "thromboembolic meningoencephalitis," and this organism was designated simply a *Haemophilus*-like agent (1, 19); it has since been isolated from respiratory tracts of cattle (25), prepuce and semen of bulls (15, 16), uteri, cervixes, and vaginas of cows (8, 9), and aborted bovine fetuses (6), and it has been implicated as having a role in arthritis in cattle (25, 26). The names "*Haemophilus somnus*" (W. E. Bailie, Ph.D. thesis, Kansas State University, Manhattan, 1969) and "*Haemophilus somnifer*" (24) have been proposed for this bacterium. Another agent sometimes included in this group, "*Actinobacillus seminis*," was first isolated from the semen of rams by Baynes and Simmons in Australia (2); it has since been reported in the United States (22, 32) and South Africa (33).

Organisms of this kind are being isolated with increased frequency from cases of epididymitis in ram lambs (4, 10, 30), and they have been variously identified as *Haemophilus* spp., *Actinobacillus actinomycetemcomitans*, HB5, *Haemophilus suis*, *Haemophilus parasuis*, *Haemophilus parahemolyticus*, "*Haemophilus somnus*," "*A. seminis*," and "*Histophilus ovis*" (4).

It has been generally accepted for some time that these agents, whatever their relationships to each other, do not qualify for inclusion in the genus *Haemophilus* as currently defined (21). It has also been noted that some, if not all, members of this group bear a close resemblance to one another. The possibility that "*A. seminis*" and "*Histophilus ovis*" are biochemical variants of the same organism was

suggested by Webb (34). Other workers have suggested that "*Haemophilus somnus*," "*Histophilus ovis*," and "*Haemophilus agni*" belong to a single taxon and that the relationship of "*A. seminis*" to this group is unclear (31).

Although a number of investigations have addressed various laboratory and pathogenic aspects of members of this group (19, 23, 31), with particular reference to differences between cultures of bovine origin versus cultures of ovine origin, a direct comparison of nucleic acid sequence homologies, an approach enjoying much favor as an aid in the solution of taxonomic problems, has not been described previously. In this paper we report such a comparison.

MATERIALS AND METHODS

Bacterial strains. A total of 11 strains of bacteria (Table 1) were grown on chocolate agar or 5% bovine blood agar ("*A. seminis*") in 10% CO₂ at 37°C for 48 to 72 h. The bacteria were harvested in a physiological saline solution (pH 8) and centrifuged at 8,000 × g for 20 min in a refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 5°C. Pellets were stored at -70°C.

DNA extraction and purification. The deoxyribonucleic acid (DNA) extraction and purification techniques used were adapted from a previously described method (17). Briefly, bacterial pellets were suspended in 10 ml of buffer containing 20 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 100 mM NaCl, and 5 mM ethylenediaminetetraacetate. To lyse the cells, 0.25 ml of a 20% sodium dodecyl sulfate solution was added, and the preparation was heated to 60°C for 15 min. After 0.25 ml of 20% Sarkosyl (Sigma Chemical Co., St. Louis, Mo.) was added, the mixture was again heated to 60°C for 15 min. The lysate was then incubated with 1 ml of a solution containing 10 mg of pronase (Calbiochem-Behring, La Jolla, Calif.) per ml at 37°C for 2 h and extracted twice with phenol-chloroform. A 2-ml sample was removed for CsCl centrifugation, and the remaining nucleic acid was precipitated with 2 volumes of ethanol at -20°C overnight. The precipitated nucleic acid was collected by centrifugation at 6,000 × g for 20 min, and the pellet was vacuum dried. The nucleic acid was dissolved in 4.5 ml of distilled water and alkali treated at 37°C overnight

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TABLE 1. Sources of isolates used in this study

Strain	Source		Origin of isolate
	Animal	Organ	
" <i>Haemophilus somnus</i> " 805	Bovine	Lung	California ^a
" <i>Haemophilus somnus</i> " 608	Bovine	Lung	Colorado ^b
" <i>Haemophilus somnus</i> " 2078	Bovine	Brain	California ^a
" <i>Haemophilus agni</i> " 902	Ovine	Septicemia	California ^a
" <i>Histophilus ovis</i> " 9L	Ovine	Vagina	Canada ^c
3384Y	Ovine	Epididymis	Idaho ^a
5688T	Ovine	Epididymis	Idaho ^a
714	Ovine	Prepuce	California ^a
642A	Ovine	Prepuce	California ^a
<i>Haemophilus influenzae</i> R431 (biovar II)	Human	Lung	California ^a
" <i>A. seminis</i> " ATCC 15768	Ovine	Semen	Australia ^e

^a Isolated by authors.^b A. B. Hoerlein, Department of Microbiology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins.^c R. Higgins, Département de Pathologie de Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Quebec, Canada.^d B. Hansen, Department of Pathology, University of California at Davis Medical Center, Sacramento.^e American Type Culture Collection, Rockville, Md.

in 0.5 M KOH. The solution was then adjusted to pH 7 to 8 with 5 N HCl and diluted to 10 ml with distilled water, and the DNA was precipitated with 2 volumes of ethanol at -20°C overnight. The precipitated DNA was collected by centrifugation at $6,000 \times g$ for 20 min, vacuum dried, and dissolved in 1 ml of distilled water. The final concentration of DNA was determined by measuring the optical density at 260 nm with a Spectronic 2000 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.), with 1 U of optical density taken to be 50 $\mu\text{g/ml}$.

DNA to be nick translated for use as a hybridization probe was purified by centrifugation to equilibrium in CsCl gradients. "*Haemophilus somnus*" nucleic acid saved after phenol-chloroform extraction was mixed with a CsCl solution containing 2 mM ethylenediaminetetraacetate and 50 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5) that provided a final density of 1.700 g/cm^3 (refractive index, 1.399). The DNA solution was then centrifuged in 12.5-ml tubes by using a type T-865.1 Sorvall rotor at $91,200 \times g$ for 48 h at 22°C . Fractions from the gradient were monitored for the presence of DNA by agarose gel electrophoresis, and 20- μl portions of each fraction were subjected to electrophoresis in 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml for 2 h at 2.5 V/cm. The DNA was then visualized by ultraviolet transillumination. Fractions containing DNA were pooled and dialyzed against four 1-liter changes of deionized glass-distilled water over a period of 48 h.

DNA-DNA hybridization studies. "*Haemophilus somnus*" DNA purified by equilibrium CsCl bouyant density centrifugation was labeled in vitro by nick translation with [^3H]thymidine triphosphate (45 Ci/mmol; ICN, Irvine, Calif.) as described by Rigby et al. (28). The labeled DNA (10^6 cpm/ μg) was approximately 20% S1 nuclease resistant after denaturation. This S1 nuclease resistance was reduced to approximately 5% by treating undenatured labeled DNA with S1 nuclease as described by Grimont et al. (13). For hybridization reactions, 0.08 μg (80,000 cpm) of labeled "*Haemophilus somnus*" DNA was mixed with 150 μg of unlabeled bacterial DNA and 250 μg of calf thymus DNA in a 0.5-ml volume containing 0.42 M NaCl, 0.02 M

tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), and 0.005 M ethylenediaminetetraacetate. Ten samples of the reaction mixture were sealed into 50- μl micropipettes and heated at 110°C for 50 min in an ethylene glycol bath. In preliminary experiments the hybridization samples were incubated at 60°C for varying lengths of time over a 24-h period. Each sample was frozen and stored at -20°C until all were collected. In subsequent experiments, four hybridization samples were incubated at 60°C , and four were incubated at 75°C for 18 h. Two samples were immediately frozen at -20°C .

The single-strand-specific S1 nuclease was used to distinguish between double-stranded and single-stranded DNAs. Hybridization samples were diluted 1:30 in a solution containing 0.1 mM ZnCl_2 , 300 mM NaCl, 25 mM sodium acetate (pH 4.5), 20 μg of sonicated native calf thymus DNA per ml, and 20 μg of denatured calf thymus DNA per ml. The mixture was then divided into three equal portions. Two of these each received 30 U of S1 nuclease (Sigma) and were incubated for 90 min at 43°C before acid precipitation by the addition of ice-cold 5% (vol/vol) trichloroacetic acid and 50 μg of calf thymus DNA. The DNA in the other sample was acid precipitated without S1 nuclease digestion. The DNA precipitates were collected on 0.45- μm nitrocellulose filters and processed for liquid scintillation counting with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.). Under the conditions described above, S1 nuclease digested less than 5% of the native and 96% of the denatured nick-translated "*Haemophilus somnus*" DNA.

RESULTS

DNA-DNA hybridization studies were performed between "*Haemophilus somnus*" ^3H -labeled DNA and various strains (Table 2). This hybridization procedure demonstrated high degrees of homology between "*Haemophilus somnus*" 805 labeled DNA and DNAs from two other "*Haemophilus somnus*" strains isolated from cattle (strains 608 and 2078) (Table 1). There were also high degrees of relatedness

TABLE 2. Levels of homology between ^3H -labeled "*Haemophilus somnus*" DNA and unlabeled DNAs from other organisms

Source of unlabeled DNA ^a	% Homology at: ^b	
	60°C	75°C
" <i>Haemophilus somnus</i> " 805	100.0	100.0
" <i>Haemophilus somnus</i> " 608	97.0 ± 1.21	98.8 ± 4.95
" <i>Haemophilus somnus</i> " 2078	95.3 ± 1.64	100.0 ± 1.28
" <i>Haemophilus agni</i> " 906	86.8 ± 6.10	87.4 ± 10.90
" <i>Histophilus ovis</i> " 9L	83.6 ± 2.11	89.6 ± 3.11
Strain 3384Y	90.3 ± 1.74	86.7 ± 4.35
Strain 5688T	86.2 ± 2.18	87.7 ± 1.50
Strain 714	86.5 ± 0.44	84.1 ± 7.89
Strain 642A	81.0 ± 4.80	91.9 ± 4.01
<i>Haemophilus influenzae</i>	11.7 ± 1.80	4.2 ± 1.32
" <i>A. seminis</i> " ATCC 15768	12.1 ± 0.60	9.9 ± 0.28
Salmon testes	5.6 ± 0.41	2.5 ± 0.52

^a In each reaction the ^3H -labeled DNA was from "*Haemophilus somnus*" 805.^b Homology values are averages $\pm$ standard deviations of four separate determinations.

demonstrated between the DNA of bovine "*Haemophilus somnus*" strain 805 and the DNAs of "*Haemophilus agni*," "*Histophilus ovis*," and strains 3384Y, 5688T, 714, and 642A, all of which were isolated from sheep. We found levels of DNA relatedness of more than 80% for both the cattle and sheep isolates at an incubation temperature of 60°C and at a more stringent incubation temperature (75°C). However, there was a statistically significant difference, as measured by a two-sample *t*-test, between the mean level of DNA relatedness of the cattle strains and the mean level of relatedness of the sheep strains at both 60°C ($P = 0.0012$) and 75°C ($P = 0.0002$). Our data indicate that there is very little DNA relatedness between "*Haemophilus somnus*" 805 labeled DNA and the DNA of either *Haemophilus influenzae* or "*A. seminis*" ATCC 15768.

DISCUSSION

Our data point strongly to the existence of a group of closely related organisms comprising members of which are presently designated "*Haemophilus somnus*," "*Haemophilus agni*," and "*Histophilus ovis*" and certain isolates from ovine male genital tracts (strains 3384Y, 5688T, 714, and 642A). The members of this group, with levels of homology of more than 80%, appear to be related closely enough genetically to be combined in a single species (3).

Our data further show a very low level of relatedness (4.2 to 11.7%) between this group and a representative of *Haemophilus influenzae*, the type species of the genus *Haemophilus*. This observation is partially at odds with that of Gonzales and Bingham (12), who reported 39% homology at a hybridization temperature of 60°C (but only 17% homology at a more stringent temperature, 75°C). However, our data are in agreement with the data of Mutters et al. (R. Mutters, K. Piechulla, and W. Mannheim, personal communication), who found a negligible level of DNA-DNA reassociation between European and American strains of "*Haemophilus somnus*" on the one hand and members of the family *Pasteurellaceae*, including *Haemophilus* spp., on the other. These data confirm that there is little basis for inclusion of the "*Haemophilus somnus*" homology group in the genus *Haemophilus*, a conclusion arrived at long ago after consideration of phenotypic traits.

Finally, it is apparent that the degree of genetic relatedness between the "*Haemophilus somnus*" group and "*A. seminis*" is equally negligible (i.e., well below that of a common genus).

There is evidence that the strains derived from sheep and those isolated from cattle may constitute distinct populations within the species. Such evidence comes from serological, biochemical, pathological, and epidemiological observations (5, 19), as well as our DNA homology data; thus far it is based on fragmentary observations that should be further expanded. If borne out, this evidence may eventually justify the recognition of appropriate subspecies. At present there are no known phenetic traits that permit the separation of ovine isolates from bovine isolates other than the source of the organisms (31).

Therefore, we conclude that the members of the "*Haemophilus somnus*" DNA homology group, including "*Haemophilus somnus*," "*Haemophilus agni*," "*Histophilus ovis*," the epididymal isolates (strains 3384Y and 5688T), and the preputial isolates (strains 714 and 642A), should be considered members of one distinct species. This conclusion is in agreement with that arrived at by other workers who compared the morphological, biochemical, antigenic, and cytochemical relationships of representative strains of mem-

bers of this group (31). The exact taxonomic position of this group is not yet clear, and further hybridization studies exploring relationships with other gram-negative, nonenteric, fermentative bacteria will be required to determine its proper placement.

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