Reassignment of *Actinobacillus actinomycetemcomitans* to the Genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov.

T. V. POTTS,^{1,2*} J. J. ZAMBON,² AND R. J. GENCO²

Departments of Operative Dentistry¹ and Oral Biology,² School of Dentistry, State University of New York at Buffalo, and the Periodontal Disease Clinical Research Center, Buffalo, New York 14214

Serological techniques were used to assess the relationships among the following species: *Actinobacillus equuli*, *Actinobacillus lignieresii*, *Actinobacillus pleuropneumoniae*, "*Actinobacillus seminis*," *Actinobacillus suis*, *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, *Haemophilus segnis*, *Haemophilus parainfluenzae*, *Haemophilus influenzae*, *Haemophilus parahaemolyticus*, *Haemophilus aegyptius*, and *Gardnerella vaginalis*. Deoxyribonucleic acid-deoxyribonucleic acid hybridization analyses of *A. actinomycetemcomitans*, *H. segnis*, *H. aphrophilus*, and *H. paraphrophilus* showed that these four species have measurable deoxyribonucleic acid relatedness of 28% or greater, consistent with their placement in a single genus. These bacterial species also demonstrated common antigens by immunodiffusion. In view of these genetic and serological relationships and previous inclusion of X- and V-factor-independent bacterial species in the genus *Haemophilus*, reassignment of *A. actinomycetemcomitans* to the genus as *Haemophilus actinomycetemcomitans* comb. nov. is proposed.

The morphological and cultural characteristics of *Actinobacillus actinomycetemcomitans* were first described by Klinger (12) in 1912. The initial isolates of this gram-negative facultative anaerobe were from actinomycotic lesions and were given the name "*Bacterium actinomycetemcomitans*." The name of this organism was subsequently changed twice, first by Lieske (13) in 1921 to "*Bacterium comitans*" and then by Topley and Wilson (31) in 1929 to *Actinobacillus actinomycetemcomitans*.

The first detailed description of the biochemical and serological characteristics of this species was published by King and Tatum (11), who compared *A. actinomycetemcomitans* with *Haemophilus aphrophilus* and found these species to be closely related. More recently, a number of studies have delineated the phenotypic (1, 6, 8, 10, 19, 26, 29), serological (24, 27, 33, 34), and deoxyribonucleic acid (DNA)-DNA hybridization characteristics (2, 4, 20, 22, 30) of *A. actinomycetemcomitans*.

The purpose of the DNA-DNA hybridization work reported here was to further delineate the relationships among *A. actinomycetemcomitans*, *Haemophilus segnis*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus*. Serological studies were carried out to further evaluate the relationships among the following species: *Actinobacillus equuli*, *Actinobacillus lignieresii*, *Actinobacillus pleuropneumoniae*, "*Actinobacillus seminis*," *Actinobacillus suis*, *A. actinomycetemcomitans*, *H. aphrophilus*, *H. segnis*, *H. paraphrophilus*, *Haemophilus parainfluenzae*, *Haemophilus influenzae*, *Haemophilus paraphrohaemolyticus*, *Haemophilus aegyptius*, and *Gardnerella vaginalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in the production of rabbit antisera are shown in Table 1. Strains of *Actinobacillus* and *Haemophilus* species were grown to late logarithmic or early stationary phase in National Institutes of Health thioglycolate liquid medium

(Difco Laboratories, Detroit, Mich.). X- and V-factor-requiring strains were grown on chocolate agar for 72 h. All strains were grown at 37°C in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) containing 85% N₂, 10% H₂, and 5% CO₂. The bacteria grown in liquid media were harvested by centrifugation and washed three times in phosphate-buffered saline (pH 7.2). The strains grown on agar plates were harvested with a platinum loop and washed three times in phosphate-buffered saline.

Antiserum production. Whole bacterial cells to be used as immunogens were suspended in sterile saline to a concentration of 10 mg (wet weight) per ml. Eight-week-old female New Zealand white rabbits weighing 4 kg received intravenous injections of 1.0 ml of bacterial suspensions via the marginal ear vein; the protocol of McCarty and Lancefield was used (17). For each strain, antisera were produced in two or more rabbits. Trial blood samples were obtained from the central ear artery, and the antibody titers were determined by immunodiffusion. Once a satisfactory antibody titer had been obtained, the rabbits were exsanguinated by cardiac puncture. The antisera were heated to 56°C for 30 min and stored in small portions at -76°C until they were used.

Sonic extraction of bacterial cell antigens. Whole bacterial cells (100 mg, wet weight) were placed in 15 ml of phosphate-buffered saline in an ice bath and sonicated with a model 350 Sonifier cell disrupter (Bronson Sonic Power Co., Danbury, Conn.) at 25 W with a tapered microtip having an end diameter of 3 mm (model 419; Heat Systems Ultrasonics, Plainview, N.Y.) until more than 95% of the cells were disrupted, as determined by phase-contrast microscopy. The sonic extracts were centrifuged at 12,000 × g for 30 min at 4°C, and the supernatants were removed, dialyzed overnight against distilled water, and lyophilized. The sonic extracts were then suspended to a protein concentration of 25 mg/ml, as determined by the assay of Lowry et al. (14), and stored at -20°C until they were used.

Immunodiffusion. Immunodiffusion was carried out in 1.2% agarose (SeaKem ME; FMC Corp., Rockland, Maine) in 0.033 M Veronal buffer (pH 8.2) by the method of

* Corresponding author.

TABLE 1. *Actinobacillus*, *Gardnerella*, and *Haemophilus* species used in the preparation of rabbit antisera, bacterial sonic extracts, and bacterial DNA

Species	Strain	Serotype	Source ^a
<i>A. actinomycetemcomitans</i>	ATCC 29522		ATCC
	ATCC 29523		ATCC
	ATCC 29524		ATCC
	NCTC 9709		NCTC
	NCTC 9710 ^T		NCTC
	Y4	b	Socransky
<i>A. equuli</i>	75	a	SUNYaB
	67	c	SUNYaB
	ATCC 19392 ^T		ATCC
<i>A. lignieresii</i>	ATCC 19393		ATCC
<i>A. pleuropneumoniae</i> ^b	ATCC 27088 ^T		ATCC
	ATCC 27089		ATCC
	ATCC 27090		ATCC
" <i>A. seminis</i> "	ATCC 15768		ATCC
<i>A. suis</i>	ATCC 15557		ATCC
<i>G. vaginalis</i>	ATCC 14018 ^T		ATCC
<i>H. aegyptius</i>	ATCC 11116 ^T		ATCC
<i>H. aphrophilus</i>	ATCC 13252		ATCC
	ATCC 19415		ATCC
	NCTC 5906 ^T		NCTC
	NCTC 5908		NCTC
	ATCC 19418		ATCC
	ATCC 9006	a	ATCC
	ATCC 9795	b	ATCC
	ATCC 9007	c	ATCC
	ATCC 9332	d	ATCC
	ATCC 8142	e	ATCC
<i>H. parahaemolyticus</i>	ATCC 9833	f	ATCC
	ATCC 10014 ^T		ATCC
	ATCC 7901		ATCC
<i>H. parainfluenzae</i>	ATCC 9796		ATCC
<i>H. paraphrohaemolyticus</i>	ATCC 29237 ^T		ATCC
<i>H. paraphrophilus</i>	ATCC 29240		ATCC
	ATCC 29241 ^T		ATCC
	ATCC 29242		ATCC
<i>H. segnis</i>	NCTC 10977 ^T		NCTC

^a ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, United Kingdom; Socransky, S. S. Socransky, Forsyth Dental Center, Boston, Mass.; SUNYaB, State University of New York at Buffalo, Buffalo, N.Y.

^b This species was recently transferred to the genus *Actinobacillus* from the genus *Haemophilus* (21).

Ouchterlony (18). All combinations of bacterial cell sonic extracts and rabbit immune sera were examined. The negative controls included preimmune rabbit sera.

DNA purification and DNA-DNA homology. DNA was isolated and purified, and the guanine-plus-cytosine content of each DNA was measured by the melting point method (15). DNA-DNA hybridization reactions were carried out at 25°C below the melting temperature of the probe DNA. Experiments in which either *A. actinomycetemcomitans* ATCC 9710^T (T = type strain) or *H. aphrophilus* ATCC 5906^T DNA was used as the probe were carried out at 63°C; experiments in which *H. segnis* ATCC 10977^T DNA was used as the probe were conducted at 61°C. In all other respects, the hybridization reactions were carried out exactly as described previously (23); the S1 nuclease method was used for measuring DNA-DNA homoduplexes (3).

RESULTS

Table 2 shows DNA-DNA hybridization data for representative strains of *H. segnis*, *H. aphrophilus*, *H. paraphrophilus*, and *A. actinomycetemcomitans*. Three tritium-

labeled probes were used in these experiments to determine the reciprocal hybridization values for reactions in which cold *H. segnis* ATCC 10977^T DNA was used. All of the DNAs tested hybridized at a level of 28% or greater with the labeled test probes. For each reciprocal pair of labeled and unlabeled DNAs, the results were in close agreement.

The results of serological experiments are shown in Table 3. Antiserum to *A. pleuropneumoniae* (formerly *Haemophilus pleuropneumoniae*) reacted only with antigenic extracts from six *Haemophilus* species and *G. vaginalis*. Antigenic extracts from *A. pleuropneumoniae* cross-reacted only with antibodies directed against four *Haemophilus* species and *G. vaginalis*. Antiserum directed against *A. actinomycetemcomitans* reacted with at least one antigenic determinant on four species belonging to the genus *Actinobacillus*, as well as with four members of the genus *Haemophilus*. In the reciprocal experiments, antisera directed against each of these eight reactive species cross-reacted with one *A. actinomycetemcomitans* antigen. The only two other cross-reactions between the groups of haemophili and actinobacilli studied were observed with *H. aphrophilus*; antiserum directed against *H. aphrophilus* reacted with one *A. suis* antigen, whereas antiserum directed against *A. lignieresii* reacted with one *H. aphrophilus* antigen. Among *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, and *H. parainfluenzae*, all antigen preparations reacted with antisera directed against each of the other species, with the exception of the *H. parainfluenzae* antigen preparation, which did not react with antiserum directed against *H. aphrophilus*. Antiserum directed against *H. parainfluenzae* reacted with all of the *Haemophilus* strains tested, as well as with *A. actinomycetemcomitans* and *G. vaginalis*.

DISCUSSION

King and Tatum (11) recognized long ago that the species *A. actinomycetemcomitans* more closely resembled *H. aphrophilus* than *A. lignieresii*, the type species of the genus *Actinobacillus*. Several additional investigations have verified the close phenotypic relationship between *A. actinomycetemcomitans* and *H. aphrophilus* (8, 10, 26, 29), and Zambon et al. (33) and McArthur et al. (16) have shown that there are antigenic similarities between these two organisms. By the same token, a very close phenotypic relationship has been noted between *H. aphrophilus* and *H. paraphrophilus* (8, 9, 28). Common antigens have also been reported for *A. actinomycetemcomitans* and *H. para-*

TABLE 2. Results of DNA-DNA hybridization experiments

Source of unlabeled DNA	% Homology with the following labeled DNA probes:		
	<i>H. segnis</i> NCTC 10977 ^T	<i>H. aphrophilus</i> NCTC 5906 ^T	<i>A. actinomycetemcomitans</i> NCTC 9710 ^T
<i>H. segnis</i> NCTC 10977 ^T	100	34 ± 4 (4) ^a	31 ± 4 (2)
<i>H. aphrophilus</i> NCTC 5906 ^T	34 ± 7 (3)	100	35 ± 3 (3) ^b
<i>H. paraphrophilus</i> ATCC 29240	40 ± 6 (3)	73 ± 1 (2) ^b	32 ± 7 (3) ^b
<i>A. actinomycetemcomitans</i> NCTC 9710 ^T	28 ± 4 (4)	35 ± 8 (5) ^b	100

^a Mean ± standard deviation. The numbers in parentheses are the numbers of times the experiment was performed.

^b Data previously published by Potts and Berry (19).

TABLE 3. Serological relationships among *Actinobacillus*, *Haemophilus*, and *Gardnerella* species

Rabbit anti-sera to:	No. of specific anti-gens	No. of common antigens in extracts of: ^a														
		<i>A. equuli</i>	<i>A. lignieresii</i>	<i>A. pleuropneumoniae</i>	" <i>A. seminis</i> "	<i>A. suis</i>	<i>A. actinomycetemcomitans</i>	<i>H. aphrophilus</i>	<i>H. segnis</i>	<i>H. paraphrophilus</i>	<i>H. parainfluenzae</i>	<i>H. influenzae</i>	<i>H. parahaemolyticus</i>	<i>H. paraphrohaemolyticus</i>	<i>H. aegyptius</i>	<i>G. vaginalis</i>
<i>A. equuli</i>	1		1	0	1	1	1	0	0	0	0	0	0	0	1	0
<i>A. lignieresii</i>	1	1		0	1	1	1	1	0	0	0	0	0	0	0	0
<i>A. pleuropneumoniae</i>	1	0	0		0	0	0	0	1	1	1	2	2	0	1	1
" <i>A. seminis</i> "	2	1	1	0		1	1	0	0	0	0	0	0	0	0	0
<i>A. suis</i>	1	1	1	0	1		1	0	0	0	0	0	0	0	0	0
<i>A. actinomycetemcomitans</i>	2	1	1	0	1	1		1	1	1	1	0	0	0	0	0
<i>H. aphrophilus</i>	1	0	0	0	0	1	1		1	1	0	0	0	0	0	0
<i>H. segnis</i>	1	0	0	0	0	0	1	1		1	1	1	0	0	0	0
<i>H. paraphrophilus</i>	1	0	0	0	0	0	1	1	1		1	1	0	0	0	0
<i>H. parainfluenzae</i>	2	0	0	2	0	0	1	1	1	1		2	2	2	1	1
<i>H. influenzae</i>	1	0	0	1	0	0	0	0	0	1	1-2		1	1-2	1	1
<i>H. parahaemolyticus</i>	1	0	0	1	0	0	0	0	1	1	1	1		0	1	1
<i>H. paraphrohaemolyticus</i>	1	0	0	0	0	0	0	0	0	1	1	1-2	0		1	1
<i>H. aegyptius</i>	1	0	0	1	0	0	0	0	0	1	1	1	1	1		0
<i>G. vaginalis</i>	2	0	0	1	0	0	0	0	0	1	1	1	1	1	0	

^a Maximum number of bands visible by double immunodiffusion when all of the strains listed in Table 1 were used.

phrophilus (33), but data on this point have not been presented.

A taxonomic study by Sakazaki et al. (25) verified that *A. actinomycetemcomitans* is metabolically quite different from either *A. lignieresii* or *A. equuli*. Calhoon et al. (1) have shown that there are distinct differences in the soluble protein profiles of *A. actinomycetemcomitans* and the profiles of "*A. seminis*," *A. equuli*, and *A. lignieresii*. In addition, Hollander et al. (6) have shown that *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* produce only one respiratory quinone (dimethylmenaquinone), whereas most actinobacilli, including *A. lignieresii*, produce both dimethylmenaquinone and ubiquinone.

Several DNA-DNA hybridization studies have assessed the relationships among species of actinobacilli, including *A. actinomycetemcomitans* (2, 4, 20, 22, 30). All agree that *A. actinomycetemcomitans* is distant from the type species, *A. lignieresii*, as well as the other *Actinobacillus* species. Escande et al. (4) have shown that organisms presently classified as *A. lignieresii*, *A. equuli*, *Actinobacillus capsulatus*, *A. suis*, and *Pasturella ureae* are related by DNA-DNA homology values of 30% or higher, suggesting that these species belong in the same genus. Of special interest is the finding of these authors that *A. actinomycetemcomitans* shares very little homology (1 to 3%) with these species and, hence, should not be grouped with them at the genus level. Coykendall et al. (2), Potts and Berry (22), Phillips (19), and Escande et al. (4) have all suggested on the basis of DNA-DNA hybridization studies that *A. actinomycetemcomitans* should be removed from the

genus *Actinobacillus*. On the basis of phenotypic data, Kilian (8) suggested that it might be appropriate to move this organism to the genus *Haemophilus*, whereas Coykendall et al. (2) and Potts and Berry (22) have made the same suggestion based on DNA-DNA hybridization results.

The DNA-DNA hybridization data presented above demonstrate that *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* are related at a homology level of 28% or higher. These findings are consistent with the placement of these species in the same genus. Based strictly upon the serological results reported here, *A. actinomycetemcomitans* could fit into either the genus *Actinobacillus* or the genus *Haemophilus*. However, the other species of actinobacilli examined constitute a group of organisms which are antigenically distinct from the haemophili. When the serological data are considered together with the DNA-DNA hybridization data presented here and reported previously (2, 4, 20, 22, 30), it is clear that *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* should be placed in the same genus.

Table 4 shows the definitive characteristics for the genus *Haemophilus* (9), the genus *Actinobacillus* (19), and *A. actinomycetemcomitans* (2, 22, 26). As this table shows, *A. actinomycetemcomitans* does not fulfill two of the criteria for the genus *Actinobacillus* due to its inability to produce either urease or β -galactosidase. On the other hand, *A. actinomycetemcomitans* does not require either X factor (hemin) or V factor (nicotinamide adenine dinucleotide); therefore, it does not fulfill the criteria for inclusion in the genus *Haemophilus*. However, it should be noted that *H.*

TABLE 4. Definitive characteristics of the genus *Haemophilus*, the genus *Actinobacillus*, and *A. actinomycetemcomitans*^a

Taxon	Guanine-plus-cytosine content (mol %)	X- or V- factor requirement	β -Galactosidase production	Urease production	Indole production
<i>Haemophilus</i>	37–44	+	+/-	+/-	+/-
<i>Actinobacillus</i>	40–43	—	+	+	—
<i>A. actinomycetemcomitans</i>	45–49	—	—	—	—

^a In addition, the members of all three taxa are gram negative, have coccobacillary morphology, reduce nitrate to nitrite, and ferment carbohydrates.

aphrophilus has been included in the genus *Haemophilus* despite the fact that it does not require either X or V growth factor (8, 9, 28, 29). Similarly, *Haemophilus gallinarum* and *Haemophilus suis* contain strains which do not require X factor for growth. With these precedents in mind and in view of the overwhelming evidence of a close relationship between *A. actinomycetemcomitans* and four species of haemophili, it seems most logical to include *A. actinomycetemcomitans* in the genus *Haemophilus*.

Therefore, we propose that *A. actinomycetemcomitans* be reassigned to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov. This reassignment should serve to emphasize the taxonomic and antigenic relationships between *H. actinomycetemcomitans* and the other *Haemophilus* species. These relationships may be of considerable importance in clinical studies of *H. actinomycetemcomitans*, a microorganism which is thought to play an important role in the etiology of certain types of periodontal disease (32).

H. actinomycetemcomitans is defined as a small gram-negative coccobacillus which is nonmotile, fermentative, and capnophilic and has no requirement for either X or V factor. This organism reduces nitrate to nitrite, decomposes hydrogen peroxide, does not produce indole, and is oxidase negative. *H. actinomycetemcomitans* gives positive fermentation reactions with glucose, fructose, and mannose, but does not ferment lactose, sucrose, trehalose, or starch (26). The guanine-plus-cytosine content of this species is between 44.9 and 48.7 mol% (2, 22).

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