Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization Analysis of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*

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Deoxyribonucleic acids (DNAs) were isolated and purified from 20 strains of *Actinobacillus actinomycetemcomitans*, 6 strains of *Haemophilus aphrophilus*, 2 strains of *Haemophilus paraphrophilus*, 2 strains of *Haemophilus pleuropneumoniae*, 2 strains of *Haemophilus paraphrohaemolyticus*, 2 strains of *Haemophilus influenzae*, and 1 strain each of *Actinobacillus lignieresii*, *Actinobacillus suis*, *Haemophilus aegyptius*, *Haemophilus parainfluenzae*, and *Haemophilus para-haemolyticus*. The guanine-plus-cytosine contents of the DNAs were determined, and they agreed closely with previous estimates. DNA-DNA hybridization analyses revealed that all of the strains identified as *A. actinomycetemcomitans* were at least 69% homologous to DNA probes from two *A. actinomycetemcomitans* strains (strains NCTC 9710^T [T = type strain] and Y4). The *H. aphrophilus* and *H. paraphrophilus* strains were homologous to the two *A. actinomycetemcomitans* probes at levels of 25 to 37%. The DNAs of all *A. actinomycetemcomitans* strains were homologous to a DNA probe from *H. aphrophilus* strain NCTC 5906 at levels of 30 to 39%. The two strains of *H. paraphrophilus* tested were homologous to the *H. aphrophilus* probe at levels of 73 and 77%, indicating a very close relationship between these groups of organisms. *H. parainfluenzae* strain ATCC 9796 DNA seemed to be homologous to the *H. aphrophilus* and *A. actinomycetemcomitans* probes at low but significant levels (12 to 16%). All of the other strains of haemophili and actinobacilli tested gave 10% or less homology with the three test probes.

Actinobacillus actinomycetemcomitans, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus* are frequently cultured from oral cavities and make up a small but significant component of human dental plaques (15, 16, 18, 19, 27, 31). These organisms have been isolated from a variety of nonoral infections and are pathogens of some interest (6, 12, 17, 18, 30, 33). *A. actinomycetemcomitans* has been isolated from destructive periodontal lesions (27, 31) and has been shown to cause alveolar bone loss when it is implanted in gnotobiotic rats (8). These observations indicate that the characterization and classification of these organisms are of clinical importance.

The phenotypic similarity between *H. aphrophilus* and *A. actinomycetemcomitans* was first noted by King and Tatum (17), who suggested that the placement of these organisms in separate genera was "most unsatisfactory." Subsequent investigations have also indicated a close phenotypic relationship between these two species (16, 26). On the other hand, an investigation

(24) in which optical deoxyribonucleic acid (DNA) reassociation methods were used (5) suggested that *A. actinomycetemcomitans* is more closely related to *Actinobacillus lignieresii* than to *H. aphrophilus*. Soluble protein profiles have also shown distinctly different patterns for *A. actinomycetemcomitans*, *H. aphrophilus*, and other *Actinobacillus* species (2).

The nature of the relationships which *A. actinomycetemcomitans* and *H. aphrophilus* have with other species of *Haemophilus*, such as *H. paraphrophilus* and *Haemophilus parainfluenzae*, has not been completely delineated. However, a close relationship has been demonstrated between *H. aphrophilus* and *H. paraphrophilus* strains (13, 24, 29).

This investigation was undertaken to determine the genetic variation in oral isolates of *A. actinomycetemcomitans* and to assess the relationship between *A. actinomycetemcomitans* and representative strains of several species of haemophili and actinobacilli. The relationships of *H. aphrophilus* to *Haemophilus paraphrohae-*

molyticus (34) and to other species of haemophili and actinobacilli were also assessed.

MATERIALS AND METHODS

Strains and media. All of the strains used in this study, as well as their sources and sites of origin, are listed in Table 1. The criteria used to identify the *A. actinomycetemcomitans* isolates, as well as their phenotypic properties, have been described previously (26). The two strains listed as *H. pleuropneumoniae* strains (ATCC 27089 and ATCC 27090) were previously designated *Haemophilus parahaemolyticus* (14).

Liquid culture of all actinobacilli was accomplished in a medium containing 37 g of brain heart infusion broth per liter, 5 g of yeast extract per liter, and 1.0 g of NaHCO_3 per liter. All haemophili were grown in

liquid medium containing 18.5 g of brain heart infusion broth per liter, 10 g of yeast extract per liter, 10 g of tryptose per liter, 10 g of Biosate peptone (Becton Dickinson & Co., Cockeysville, Md.) per liter, 1.0 mg of vitamin K_1 per liter, 1.0 mg of hemin per liter, 1.6 g of NaHCO_3 per liter, and 10 ml of Isovitale X (Becton, Dickinson & Co.) per liter.

The bacterial cells used for DNA purification were grown in 1.5-liter quantities of the appropriate media. The cells were harvested by centrifugation during the late log phase of growth, washed with NaCl-ethylenediaminetetraacetate buffer (0.15 M NaCl, 0.01 M ethylenediaminetetraacetate, pH 8.0), and stored at -20°C until use.

Cell lysis. Cells were washed and centrifuged twice with 100 ml of NaCl-ethylenediaminetetraacetate buffer and then resuspended in 40 ml of NaCl-ethylenediaminetetraacetate buffer. Sodium dodecyl sulfate was

TABLE 1. List of strains used

Species	Strain	Source ^a	Site of isolation
<i>A. actinomycetemcomitans</i>	1	Slots	Tongue
	32	Slots	Tongue
	58	Slots	Cheek mucosa
	67	Slots	Cheek mucosa
	68	Slots	Dental plaque
	69	Slots	Dental plaque
	74	Slots	Dental plaque
	75	Slots	Dental plaque
	78	Slots	Cheek mucosa
	83	Slots	Cheek mucosa
	88	Slots	Cheek mucosa
	105	Slots	Dental plaque
	120	Slots	Dental plaque
	131	Slots	Dental plaque
	NCTC 9709	NCTC	Lumbar abscess
	NCTC 9710 ^T	NCTC	Lung abscess
	ATCC 29522	ATCC	Mandibular abscess
	ATCC 29523	ATCC	Blood
	ATCC 29524	ATCC	Chest aspirate
<i>A. lignieresii</i>	Y4	FDC	Dental plaque
	ATCC 19393	ATCC	Bovine lesion
	ATCC 15557	ATCC	Blood, adult swine
<i>A. suis</i>	ATCC 11116	ATCC	Conjunctiva
<i>H. aegyptius</i>	NCTC 5906 ^b	NCTC	Endocarditis
<i>H. aphrophilus</i>	NCTC 5907 ^b	NCTC	Endocarditis
	NCTC 5908 ^b	NCTC	Endocarditis
	ATCC 13252	ATCC	Unknown
	ATCC 19415 ^{Tb}	ATCC	Endocarditis
	ATCC 9007	ATCC	Unknown
<i>H. influenzae</i>	ATCC 9332	ATCC	Unknown
<i>H. parahaemolyticus</i>	ATCC 10014 ^T	ATCC	Pulmonary infection
<i>H. pleuropneumoniae</i>	ATCC 27089	ATCC	Swine pleuropneumonia
	ATCC 27090	ATCC	Swine abscess
<i>H. parainfluenzae</i>	ATCC 9796	ATCC	Unknown
<i>H. paraphrohaemolyticus</i>	ATCC 29237 ^T	ATCC	Sputum
	ATCC 29238	ATCC	Sputum
	ATCC 29239	ATCC	Throat swab
<i>H. paraphrophilus</i>	ATCC 29240	ATCC	Parietal abscess
	ATCC 29242	ATCC	Trachea

^a ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England; FDC, Forsyth Dental Center, Boston, Mass.; Slots, J. Slots, State University of New York at Buffalo Dental School, Buffalo, N.Y.

^b Strains isolated from the blood of one patient by Khairat (12).

added to a final concentration of 2%, and lysis was observed as a clearing of the mixture. Rapid lysis was apparent at room temperature with all strains of actinobacilli and haemophili used in this study.

DNA purification, G+C content determination, and DNA-DNA hybridization technique. DNA was purified as previously described (25) by using a modification of the technique of Marmur (21). The guanine-plus-cytosine (G+C) content of each DNA was determined by the thermal melting point method (23, 25). In our DNA-DNA hybridization procedure we used the S1 nuclease assay for detecting hybrid complexes (4), as described previously (25). The hybridization reactions were performed at 63°C; this temperature is 25°C below the melting temperature of the probe DNAs, as previously recommended (10, 22).

RESULTS

The melting temperature and G+C content of the DNA of each strain used in this study are

shown in Table 2; the species are arranged in order of decreasing melting temperature and G+C content. Strains identified as *A. actinomycetemcomitans* contained DNAs with G+C contents in the range from 44.9 to 46.6 mol%. This range is distinct from and does not overlap the G+C contents of the DNAs of the strains of *H. aphrophilus* and *H. paraphrophilus* which we examined. *H. aphrophilus* and *H. paraphrophilus* DNAs melted in the same temperature range and had G+C contents between 43.2 and 44.4 mol%. The other strains of haemophili and actinobacilli tested all had G+C contents of 42 mol% or less.

Table 3 shows the results of DNA-DNA hybridizations in which we used two tritiated *A. actinomycetemcomitans* probes (³H labeled strain NCTC 9710^T and Y4 DNAs [T = type strain]) and one tritiated *H. aphrophilus* probe

TABLE 2. G+C contents of bacterial DNAs

Species	Strain	No. of measurements	Mean melting temp (°C)	G+C content (mol%)
<i>A. actinomycetemcomitans</i>	1	2	88.2	46.1
	32	2	87.9	45.4
	58	2	88.1	45.9
	67	2	88.1	45.9
	68	2	87.9	45.4
	69	3	87.9	45.4
	74	3	87.9	45.4
	75	3	88.3	46.3
	78	2	88.4	46.6
	83	2	87.4	45.4
	88	2	88.1	45.9
	105	3	88.1	45.9
	120	2	87.7	44.9
	131	2	88.0	45.6
	NCTC 9709	2	88.0	45.6
	NCTC 9710 ^T	3	88.0	45.6
	ATCC 29522	7	88.1	45.9
<i>H. aphrophilus</i>	ATCC 29523	3	87.7	44.9
	ATCC 29524	5	88.0	45.6
	Y4	3	88.3	46.3
	NCTC 5906	4	87.1	43.4
	NCTC 5907	2	87.0	43.2
<i>H. paraphrophilus</i>	NCTC 5908	3	87.1	43.4
	ATCC 13252	2	87.0	43.2
	ATCC 19415 ^T	3	87.2	43.7
	ATCC 29240	3	87.5	44.4
	ATCC 29242	3	87.0	43.2
<i>A. lignieresii</i>	ATCC 19393	3	86.5	42.0
<i>A. suis</i>	ATCC 15557	4	86.2	41.2
<i>H. parainfluenzae</i>	ATCC 9796	3	86.0	40.7
<i>H. influenzae</i>	ATCC 9007	3	85.8	40.2
	ATCC 9332	3	85.4	39.3
<i>H. paraphrohaemolyticus</i>	ATCC 29237 ^T	3	85.7	39.9
	ATCC 29238	3	85.9	40.5
	ATCC 29239	3	85.8	40.2
<i>H. aegyptius</i>	ATCC 11116	3	85.2	38.8
<i>H. parahaemolyticus</i>	ATCC 10014 ^T	3	85.0	38.2
<i>H. pleuropneumoniae</i>	ATCC 27089	5	86.4	41.7
	ATCC 27090	3	85.2	38.8

TABLE 3. Results of DNA-DNA hybridizations

Source of unlabeled DNA		³ H-labeled strain NCTC 9710 ^T DNA		³ H-labeled strain Y4 DNA		³ H-labeled strain NCTC 5906 DNA	
Species	Strain	No. of measure- ments	% Hybridi- zation	No. of measure- ments	% Hybridi- zation	No. of measure- ments	% Hybridi- zation
<i>A. actinomycetem- comitans</i>	1	2	90 ± 5 ^a	1	99	2	31 ± 3
	32	2	84 ± 6	1	85	2	31 ± 3
	58	2	78 ± 2	1	91	2	34 ± 1
	67	2	86 ± 7	2	100 ± 7	1	32
	68	2	89 ± 11	1	85	1	30
	69	2	96 ± 2	2	93 ± 5	2	32 ± 3
	74	3	72 ± 10	4	70 ± 4	3	39 ± 8
	75	4	80 ± 7	5	86 ± 4	5	36 ± 4
	78	2	78 ± 4	2	77 ± 7	2	32 ± 1
	88	2	77 ± 5	2	90 ± 5	2	33 ± 8
	105	2	69 ± 3	2	85 ± 6	2	31 ± 3
	120	2	97 ± 5	2	90 ± 4	2	33 ± 3
	131	2	74 ± 6	1	81	2	31 ± 3
	NCTC 9709	2	86 ± 8	2	87 ± 2	2	31 ± 2
	NCTC 9710 ^T		100	6	88 ± 7	5	35 ± 8
	ATCC 29522	6	90 ± 10	3	80 ± 1	3	30 ± 3
	ATCC 29523	8	81 ± 3	5	86 ± 3	6	31 ± 3
	ATCC 29524	8	87 ± 8	8	98 ± 5	9	32 ± 4
	Y4	7	83 ± 10		100	6	32 ± 2
<i>H. aphrophilus</i>	NCTC 5906	3	35 ± 3	2	34 ± 1		100
	NCTC 5907	2	32 ± 5	2	30 ± 1	2	96 ± 8
	NCTC 5908	2	35 ± 5	2	31 ± 3	2	90 ± 9
	ATCC 13252	2	31 ± 4	2	32 ± 3	2	92 ± 7
	ATCC 19415 ^T	2	32 ± 3	1	25	2	98 ± 8
	ATCC 29240	3	32 ± 7	4	33 ± 3	2	73 ± 1
<i>H. paraphrophilus</i>	ATCC 29242	3	37 ± 1	4	36 ± 3	3	77 ± 4
	ATCC 19393	4	7 ± 5	3	9 ± 2	5	7 ± 1
<i>A. lignieresii</i>	ATCC 15557	2	5 ± 4	2	3 ± 1	1	5
<i>A. suis</i>	ATCC 9796	3	16 ± 4	2	12 ± 2	2	15 ± 1
<i>H. parainfluenzae</i>	ATCC 9007	2	9 ± 3	2	7 ± 4	2	6 ± 0
<i>H. influenzae</i>	ATCC 9332	3	6 ± 2	2	9 ± 1	2	6 ± 2
<i>H. paraphro- haemolyticus</i>	ATCC 29237 ^T	2	2 ± 5	1	4	2	6 ± 1
	ATCC 29238	1	9	2	3 ± 3	1	9
<i>H. aegyptius</i>	ATCC 11116	3	8 ± 2	2	10 ± 1	2	2 ± 2
<i>H. parahaemolyticus</i>	ATCC 10014 ^T	2	7 ± 3	2	0 ± 0	3	0 ± 0
<i>H. pleuropneumoniae</i>	ATCC 27089	1	7	2	6 ± 2	1	4 ± 3
	ATCC 27090	2	6 ± 6	2	6 ± 2	2	9 ± 2
<i>Escherichia coli</i>	B	2	3 ± 1	2	3 ± 0	2	2 ± 2

^a Mean ± standard deviation.

(^3H -labeled strain NCTC 5906 DNA). Table 3 also shows the number of hybridizations performed with each pair of DNAs and lists the means and standard deviations for these observations. All *A. actinomycetemcomitans* strains hybridized with the ^3H labeled strain NCTC 9710^T and Y4 probes at levels of 69% or higher, and all *H. aphrophilus* and *H. paraphrophilus* strains hybridized with these probes at levels of 25 to 37%. Strains of all of the other species examined except *H. parainfluenzae* hybridized with these *A. actinomycetemcomitans* probes at levels of 10% or less. *H. parainfluenzae* strain ATCC 9796 hybridized with the two *A. actinomycetemcomitans* probes at levels of 12 to 16%.

The tritiated *H. aphrophilus* probe (^3H -labeled strain NCTC 5906 DNA) hybridized with unlabeled DNA from *A. actinomycetemcomitans* strains at levels of 30 to 39%. These results are similar to those seen when the reciprocal experiment was performed (tritiated *A. actinomycetemcomitans* probes were hybridized with unlabeled *H. aphrophilus* DNA). The *H. aphrophilus* probe gave high levels of homology (90 to 98%) with the four other strains classified as *H. aphrophilus*, as well as with two reference strains classified as *H. paraphrophilus* (73 and 77%). *H. parainfluenzae* strain ATCC 9796 was homologous at the 15% level to the *H. aphrophilus* probe, whereas all of the other strains tested gave levels of homology of 9% or less.

DISCUSSION

Our determinations of G+C contents are in close agreement with previous estimates (7, 13, 19). The only exception is *A. lignieresii* DNA, which we measured at 42 mol% G+C, whereas Mannheim et al. (20) obtained a value of 43.4 to 44.2 mol% G+C. The G+C content of *H. pleuropneumoniae* strain ATCC 27089 appears to be significantly different than the G+C content of strain ATCC 27090. This result may indicate some heterogeneity in this group of organisms.

After using the filter-binding method of DNA-DNA hybridization, Johnson (9) suggested that homology values in the range from 20 to 60% indicate that organisms should be considered separate species within the same genus, whereas levels of homology greater than 60% indicate that organisms should be placed within the same species. According to this scheme, subspecies designations are appropriate for organisms with 60 to 70% levels of homology. With these guidelines in mind and with the observation that the S1 nuclease assay gives levels of homology that are equal to or slightly below those given by the filter-binding method (3, 11), we made the following interpretations with regard to our DNA-DNA hybridization data.

Our DNA-DNA hybridization results clearly indicated that the organisms isolated as *A. actinomycetemcomitans* were highly interrelated and should be considered one species. The two *A. actinomycetemcomitans* probes used were from organisms in different serogroups (28); however, the data obtained with these probes did not identify distinct subgroups within this species. Our data do suggest that the commonly used laboratory strains Y4 and ATCC 29524 are very closely related. It is also evident that *A. actinomycetemcomitans* is more closely related to *H. aphrophilus* and *H. paraphrophilus* (25 to 39% homology) than to either *A. lignieresii* or *Actinobacillus suis*. Also, a low but seemingly significant level of homology was observed between *A. actinomycetemcomitans* and *H. parainfluenzae* strain ATCC 9796 (12 to 16%). These observations move us to suggest that either *A. actinomycetemcomitans* should be placed in the genus *Haemophilus* or a new genus should be created that would include *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus* species. Placement of *A. actinomycetemcomitans* in the genus *Haemophilus* would require an alteration in the definition of this genus to include organisms which do not require either X or V factors, an idea which has met with considerable resistance in the past (1, 32).

These results are at variance with those of a previous study (24), in which it was reported that *A. actinomycetemcomitans* is related at the 20 to 40% level to all other species of haemophili and actinobacilli tested (all of the species which we used in this study were included). *A. actinomycetemcomitans* was also found to be more closely related to *A. suis* and *A. lignieresii* than to *H. aphrophilus* and *H. paraphrophilus*. We believe that these discrepancies are most likely accounted for by the different methodologies employed. Pohl (24) used optical measurements of DNA-DNA renaturation rates (5). This method employs much shorter hybridization times (30 to 40 min). In addition, the two DNAs tested are present in solution at the same relatively high concentration (75 to 80 $\mu\text{g/ml}$). It seems possible that a less specific form of hybrid formation is being measured or that the contributions of self-hybridization to the renaturation may be difficult to assess or both. Further study on these points is needed.

The data obtained by using a tritiated *H. aphrophilus* probe (^3H -labeled strain NCTC 5906 DNA) were similar to those obtained in the reciprocal experiment which used the two tritiated *A. actinomycetemcomitans* probes and reinforced the view that *H. aphrophilus* and *A. actinomycetemcomitans* should be placed in the same genus. The results obtained with the *H. aphrophilus* probe also indicated that *H. aphro-*

philus and the two strains of *H. paraphrophilus* examined are very closely related (73 and 77% homology). Perhaps a subspecies designation would be more appropriate for *H. paraphrophilus*. However, an alteration in nomenclature should await a more exhaustive hybridization analysis of a series of *H. aphrophilus* and *H. paraphrophilus* isolates in order to determine whether the two groups are genetically distinguishable. *H. parainfluenzae* strain ATCC 9796 seems measurably related to *H. aphrophilus* (15% homology), whereas all other species of haemophili and actinobacilli tested gave homology levels of 9% or less with the *H. aphrophilus* probe, indicating a very distant relationship between *H. aphrophilus* and these organisms.

We have been unable to demonstrate a relationship between *H. aphrophilus* and *H. paraphrohaemolyticus* strains ATCC 29237^T and ATCC 29238, as previously described (24). These results reinforce the view previously expressed by Kilian (13) that *H. paraphrohaemolyticus* is not closely related to either *H. aphrophilus* or *H. paraphrophilus*.

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