Deoxyribonucleic Acid Relatedness Among *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and Other *Actinobacillus* Species

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The relationship among 22 strains of *Actinobacillus actinomycetemcomitans*, 3 strains of *Haemophilus aphrophilus*, 1 reference strain of *Actinobacillus lignieresii*, and the type strain of *Actinobacillus equuli* were assessed by deoxyribonucleic acid (DNA)-DNA hybridizations on membrane filters. We found that the *A. actinomycetemcomitans* strains formed a homogeneous group that was distinct from *H. aphrophilus* and the other *Actinobacillus* species. There was about 40% hybridization between *H. aphrophilus* DNA and *A. actinomycetemcomitans* DNA, indicating some similarity, but *A. lignieresii* and *A. equuli* DNAs hybridized much less (never more than 20%) with *A. actinomycetemcomitans* DNA.

Interest in *Actinobacillus actinomycetemcomitans* has increased recently since this bacterium has been found to be associated with human periodontal disease, especially those forms of periodontal disease that seem to progress rapidly (15). This organism can also be found in the mouths of subjects without periodontal disease. In addition, *A. actinomycetemcomitans* is frequently associated with actinomycosis (15).

Oral isolates of this species show some degree of heterogeneity in serology (13) and sugar fermentation patterns (12), although the overall characteristics of 136 strains were recently shown to be quite uniform (14). The same study pointed out the characteristics by which *A. actinomycetemcomitans* can be differentiated from *Haemophilus aphrophilus*, an organism that it closely resembles. Calhoon et al. showed that soluble proteins extracted from 20 strains of *A. actinomycetemcomitans* formed similar patterns when they were separated electrophoretically on polyacrylamide gels and that the protein patterns of *H. aphrophilus* and various *Actinobacillus* species were distinct from each other and from the protein pattern of *A. actinomycetemcomitans* (1). The purposes of this study were to determine deoxyribonucleic acid (DNA) base sequence homologies among several *A. actinomycetemcomitans* strains and to compare the DNA base sequence of *A. actinomycetemcomitans* with the DNA base sequences of *H. aphrophilus*, *Actinobacillus lignieresii*, and *Actinobacillus equuli*.

A total of 27 bacterial strains from the collection of J. Slots were used in this study (Table 1). Cultures were maintained on blood agar which

contained casein and soy digests (Trypticase soy agar; BBL Microbiology Systems, Cockeysville, Md.), 0.1% yeast extract, and 10% (vol/vol) sheep blood.

We found that it was convenient to grow and maintain cultures in an anaerobic chamber with an atmosphere containing 85% N₂, 10% H₂, and 5% CO₂. Broth cultures were grown in a medium which contained 0.5% yeast extract, 1.5% casein peptone, 0.75% glucose, 0.25% NaCl, 0.075% L-cysteine, and 0.05% sodium thioglycolate. After this broth was autoclaved, it was supplemented with 4 g of filter-sterilized NaHCO₃ per liter. For production of radioactive DNA, the medium was supplemented with 1 mCi of [methyl-³H]thymidine per ml.

The biochemical reactions of *A. actinomycetemcomitans* and *H. aphrophilus* strains were determined by using commercially prepared test reagents (API-20A; Analytab Products, Plainview, N.Y.).

To prepare DNA, 1 liter of the broth described above was inoculated with 10 ml of a fresh culture and incubated for 24 h at 37°C. A sample was streaked onto blood agar to check for purity; then the culture was centrifuged, washed, and suspended in 25 ml of 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 8.0). After 1 drop of the washed cells was Gram stained, the cells were lysed with sodium dodecyl sulfate (1 ml of a 25% [wt/vol] solution), and the suspension was held at 60°C for 30 min. The material was cooled to 35°C and then treated with about 50 mg of protease (type XIV; Sigma Chemical Co., St. Louis, Mo.). The DNA was purified by phenol and chloroform extractions, treatment

TABLE 1. Extent of DNA hybridization between *A. actinomycetemcomitans* and *H. aphrophilus* strains^a

Source of unlabeled DNA	% Hybridization of index (labeled) DNA from strain:		Guanine-plus-cytosine content of DNA (mol%)
	<i>A. actinomycetemcomitans</i> ATCC 29523	<i>H. aphrophilus</i> NCTC 5908	
<i>A. actinomycetemcomitans</i> strains			
ATCC 29522	113 (104) ^b	50 (47)	47.1 ± 0.8 ^c
ATCC 29523	100 (100)	47 (53)	47.3 ± 0.4
ATCC 29524	66	38	47.4 ± 1.0
NCTC 9710 ^T	76	46	48.4 ± 0.9
NCTC 9709	95	36	47.4 ± 1.2
14	90	37	48.7 ± 0.7
20	33	46	46.9 ± 1.0
27	68	35	46.7 ± 1.0
28	75	40	47.0 ± 0.9
29	113	36	47.1
32	87	40	47.7 ± 0.1
39	113	48	48.7 ± 0.3
55	71	38	46.8 ± 0.45
60	93	21	47.6 ± 0.1
76	105	46	46.9 ± 0.35
79	100	35	48.4 ± 0.5
118	78	32	48.6 ± 0.5
120	97	28	46.9 ± 1.1
131	98	35	48.2 ± 0.6
161	81	35	48.5 ± 0.2
218	85	34	48.2 ± 0.1
Y4	75	31	48.1 ± 0.68
<i>H. aphrophilus</i> strains			
NCTC 5906 ^T	36 (30)	71 (76)	44.5 ± 0.7
NCTC 5908	39 (46)	100 (100)	46.5 ± 1.1
ATCC 19415	43	80	46.3 ± 0.8
<i>A. lignieresii</i> ATCC 19393	(14)	(20)	44.0
<i>A. equuli</i> ATCC 19392 ^T	(11)	(10)	43.2
<i>B. gingivalis</i> Slots 2561 ^T (= ATCC 33277 ^T)	3 (5)	0 (6)	47.1 ± 0.67 ^d

^a Expressed as hybrid duplex formation compared with homologous duplex formation.

^b The results of a second experiment are indicated in parentheses.

^c Mean ± standard deviation.

^d Data from reference 3.

with ribonuclease for 30 min at 60°C and then for 30 min at 35°C, and precipitation with ethanol, essentially by the method of Marmur (9).

The base compositions of the DNA preparations were determined by thermal denaturation in 1× SSC (10). The temperature of each sample was automatically raised 0.5°C/min with an elec-

trically heated cuvette holder (model 2527 thermoprogrammer; Gilford Instrument Laboratories, Oberlin, Ohio).

DNA hybridizations were carried out by using the membrane filter method of Legault-Démare et al. (8), as described elsewhere (3). The renaturation incubation temperature used was 51°C and was determined from the formula of De Ley and Tijtgat (5). Each experiment included a homologous control and a negative control. The homologous control reassociation was the reassociation of a labeled DNA with unlabeled DNA of the same bacterial strain. The degree of reassociation in the homologous control, as measured by liquid scintillation counting of the [³H]DNA, was considered to be 100%, and the degrees of interstrain reassociation were expressed as percentages of the control value. The negative control reassociation was the reassociation between a labeled DNA and the DNA of the clearly unrelated organism *Bacteroides gingivalis* Slots 2561^T (type strain).

Biochemical tests of the *A. actinomycetemcomitans* and *H. haemophilus* strains produced results that were consistent with a previous description of the phenotypic traits of these species (13).

The DNA base compositions of the various strains are shown in Table 1. The DNAs of *A. actinomycetemcomitans* strains contained between 46.7 and 48.7 mol% guanine plus cytosine. The *H. aphrophilus* strains had slightly lower guanine-plus-cytosine contents. *A. lignieresii* and *A. equuli* had clearly different guanine-plus-cytosine contents than *A. actinomycetemcomitans* (44.0 and 43.2 mol%, respectively).

Hybridization experiments indicated considerable base sequence similarities among the DNAs of 20 *A. actinomycetemcomitans* strains. DNAs from these strains formed hybrid reassociation products with strain ATCC 29523 [³H]DNA to a level greater than 65% of the homologous control reassociation.

The DNAs of 17 strains hybridized more than 74%. One strain (strain 20) appeared to be completely unrelated to *A. actinomycetemcomitans*. This strain did not differ in any other way from the other strains, and we suspect that the culture from which strain 20 DNA was extracted was contaminated or mislabeled or that the DNA had deteriorated. In contrast, *H. aphrophilus* DNA hybridized only about 40% with *A. actinomycetemcomitans* [³H]DNA. The reciprocal experiment, in which the labeled DNA was from *H. aphrophilus*, gave the expected reciprocal results (homology among *H. aphrophilus* strains and much lower degree of relatedness to *A. actinomycetemcomitans* strains).

A second pair of experiments (Table 1) confirmed the relationship between *A. actinomyce-*

temcomitans and *H. aphrophilus* and revealed that neither of these species is closely related to *A. equuli* or *A. lignieresii*.

The results of our experiments, together with the results of biochemical studies, strengthen the view that *A. actinomycetemcomitans* is a species distinct from *H. aphrophilus*. Although the reassociation values for some strains were less than 75%, these strains were even less related to *H. aphrophilus*. For example, strain ATCC 29524 DNA reassociated only 66% with strain ATCC 29523 DNA, but it reassociated only 38% with *H. aphrophilus* NCTC 5908 DNA. Interestingly, the degrees of relationship between *A. actinomycetemcomitans* and the other *Actinobacillus* species were even lower. Kilian has pointed out that *A. actinomycetemcomitans* is more similar to *H. aphrophilus* than to *A. lignieresii*, the type species of *Actinobacillus*, and suggested that *A. actinomycetemcomitans* could be placed in the genus *Haemophilus* (7). (*H. aphrophilus* itself is not a true *Haemophilus* species in some respects; for a detailed discussion of *Haemophilus* taxonomy, see reference 7.) Our results are consistent with this. *A. actinomycetemcomitans* DNA did not hybridize appreciably with either *A. lignieresii* or *A. equuli* DNA, but it did hybridize at levels of $40 \pm 10\%$ with *H. aphrophilus* DNA. On the other hand, Pohl (11), who used the renaturation rate method (4) of measuring DNA hybridization, found that *A. actinomycetemcomitans* was somewhat more related to *A. lignieresii* than to *H. aphrophilus* (40 versus 30%).

Since the method which we used compares the hybridization of a labeled DNA with several other DNAs in the same experiment and because we used labeled DNAs from both *A. actinomycetemcomitans* and *H. aphrophilus*, we believe that the consistently higher level of hybridization of *A. actinomycetemcomitans* DNA with *H. aphrophilus* DNA indicates a significantly higher degree of relationship between these two species than between *A. actinomycetemcomitans* and the other *Actinobacillus* species. It should be borne in mind that hybridization values of 40% do not necessarily indicate 40% base sequence homology since at such low levels of reassociation the DNA duplexes may contain nonspecific base pairings (2, 6).

We think that our results support the conclusion that *A. actinomycetemcomitans* is a distinct

species which is genetically somewhat related to *H. aphrophilus* and minimally related to other *Actinobacillus* species.

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