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Identification of *Haemophilus aphrophilus* and *Actinobacillus actinomycetemcomitans* by DNA-DNA Hybridization and Genetic Transformation

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DNA-DNA hybridization was used to identify clinical isolates as *Haemophilus aphrophilus* or *Actinobacillus actinomycetemcomitans*. Some of the isolates were naturally competent for genetic transformation and were also used as DNA recipients for identification of other isolates. The results obtained by hybridization were supported by interstrain-to-intrastrain transformation ratios. Distinction between the closely related species *H. aphrophilus* and *A. actinomycetemcomitans* was generally clear-cut by both methods. Distinction of *H. aphrophilus* and *A. actinomycetemcomitans* from type and reference strains of a diversity of species in the family *Neisseriaceae* and other gram-negative species was also demonstrated by both methods. This is the first description of the identification of clinical isolates of *H. aphrophilus* or *A. actinomycetemcomitans* by using them as recipients in genetic transformation. The results suggest that this is a reliable system for identification of new clinical isolates belonging to these taxonomic entities.

Haemophilus aphrophilus and *Actinobacillus actinomycetemcomitans* are part of the normal oral flora but can give rise to invasive infections (3, 11, 12, 14, 26). They are capnophilic, nonmotile, and gram-negative bacteria, often coccobacillary or coccoid in shape. *H. aphrophilus* has small colonies that are variably sized, which gives it a "contaminated" look on the agar (12, 14). It was first described by Khairat in 1940 (11), when it was isolated in a case of endocarditis. *H. aphrophilus* is predominantly found in the gingival crevices but is also found in dental plaques (13, 19). Its properties of adherence and aggregate formation seem similar to those of *A. actinomycetemcomitans* (14, 18), but *H. aphrophilus* may be regarded as more invasive in respect to causing endocarditis and brain abscesses (18, 22). *A. actinomycetemcomitans* grows well anaerobically and is a more-common cause of dental plaques (18, 25, 26).

Both *H. aphrophilus* and *A. actinomycetemcomitans* are thus potential etiologic agents of systemic disease, and exact identification of these species is therefore important in medical microbiology. This is difficult with conventional phenotypical characterization (12, 16, 21). For instance, the catalase reaction is most often negative in *H. aphrophilus*, although some strains are reported to be catalase positive; in *A. actinomycetemcomitans*, the reaction is usually positive, but not always in periodontal isolates (21). Gas chromatography of the two species does not separate them from each other or from other *Haemophilus* species (9).

Natural competence for genetic transformation occurs among a wide variety of bacterial species (1, 2, 15). This ability can conveniently be used to obtain reliable identification of clinical isolates (4, 5, 10, 24). The aim of this study is to demonstrate the identification of clinical isolates of *H. aphrophilus* and *A. actinomycetemcomitans* by DNA-DNA hybridization and genetic transformation.

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MATERIALS AND METHODS

Bacterial strains. The type strains of *H. aphrophilus*, *Haemophilus paraphrophilus*, *A. actinomycetemcomitans*, and *Haemophilus influenzae* and one reference strain of *H. aphrophilus* were included in this study (Table 1). In addition, 12 new isolates from human diseases were included. Their designations and clinical sources are listed in Table 1. All of these strains had been tentatively identified as *H. aphrophilus*, *H. paraphrophilus*, or *A. actinomycetemcomitans* on the basis of conventional characterization. Other bacterial strains included in the experiments were *Kingella kingae* ATCC 23330 (type strain), *Kingella indologenes* NCTC 10883 (type strain), *Kingella denitrificans* NCTC 10995 (type strain), *Moraxella nonliquefaciens* ATCC 19975 (type strain) (= 4663/62 = NCTC 7784 [23]), *Moraxella lacunata* ATCC 17967 (type strain), *Moraxella atlantae* A1922 (23), *Moraxella phenylpyruvica* 752/52 (23), *Moraxella osloensis* 5873 (23), *Neisseria elongata* ATCC 25295 (type strain) (= M2), *Oligella urethralis* ATCC 17960 (type strain), *Eikenella corrodens* 31745/80 (24), *Cardiobacterium hominis* ATCC 15826 (type strain), *Gardnerella vaginalis* ATCC 14018 (type strain), *Capnocytophaga canimorsus* (DF-2) D8549 (23), *Pasteurella multocida* 646 (pig), and *Pasteurella haemolytica* NVI (serotype 1). The *P. multocida* and *P. haemolytica* strains were received from the Norwegian College of Veterinary Medicine, Oslo, Norway. The strain of *C. canimorsus* (DF-2) was received from R. E. Weaver, Centers for Disease Control, Atlanta, Ga.

Conventional characterization. Examinations of colony and cell morphology (6), production of acid from carbohydrates (12), and nitrite reduction (6, 12) were performed as described previously (Table 1). The catalase reaction was investigated with fresh 3% hydrogen peroxide (6, 12). The oxidase reaction was performed with 1% dimethyl- and tetramethyl-*p*-phenylenediamine reagents (6). The test for the requirement for V factor (NAD) for growth was performed with V-factor disks (AB Biodisk) on heart infusion broth (Oxoid Ltd.) (12).

DNA-DNA hybridization. DNA-DNA hybridization was

TABLE 1. Sources and biochemical characteristics[a] of strains of *H. aphrophilus*, *H. paraphrophilus*, *A. actinomycetemcomitans*, and *H. influenzae* included in the study

Strain	Clinical source	Reaction result			Formation of acid from		Nitrite reduction	Requirement for NAD
		Catalase	Oxidase[b]					
			Di	Tetra	Lactose	Sucrose		
Type and reference strains[c]								
H. aphrophilus								
NCTC 5906^T	Endocarditis	−	−	−	+	+	−	−
NCTC 5886	Endocarditis	−	−	−	−	+	+	−
H. paraphrophilus NCTC 10557^T	Paronychia	−	−	+	−	+	−	+
A. actinomycetemcomitans NCTC 9710^T	Lung abscess	+	−	+	−	−	−	−
H. influenzae NCTC 8143^T		+	−	+	−	−	−	+
New clinical isolates[d]								
E.H./79	Endocarditis	−	−	−	w	+	−	−
IIMO A1/82	Brain abscess	−	w	+	w	+	+	+
IIMO A2/82	Brain abscess	−	w	+	w	+	+	+
3203/86	Sinusitis	−	−	−	+	+	+	−
9574/86	Sinusitis	−	−	−	+	+	−	−
46/87	Unknown	−	−	−	+	+	−	−
40503/88	Brain abscess	−	−	−	+	+	−	−
800/89	Brain abscess	−	−	+	w	−	−	+
1489/79	Endocarditis	vw	−	−	w	−	−	−
11305/79	Endocarditis	vw	−	−	w	−	−	−
47/87	Unknown	vw	−	−	w	−	−	−
5775/87	Endocarditis	+	−	−	+	−	−	−

[a] Methods used were as described previously (7, 12). +, Positive; −, negative; w, weak; vw, very weak.
[b] Di, Reaction performed with 1% dimethyl-*p*-phenylenediamine reagent; Tetra, reaction performed with 1% tetramethyl-*p*-phenylenediamine reagent.
[c] NCTC, National Collection of Type Cultures; T, type strain.
[d] The last two figures in these strain designations indicate the year of isolation.

performed as previously described (23). DNA was extracted by using a modification of the method of Marmur (17).

(i) DNA dot blot. The dot blot of single-stranded DNA on nitrocellulose paper was performed according to the method of Kafatos (12). The DNA was heat denatured at 95°C for 10 min. After the DNA was cooled on ice, 1 volume of cold 2 M ammonium acetate was added. The application of DNA to nitrocellulose filters was performed by using the Hybri-Dot System (Bethesda Research Laboratories). Eight parallel dots for each DNA and salmon sperm DNA and TE buffer (10 mM Tris hydrochloride [pH 7.6] plus 1 mM EDTA) controls were applied. The filters were washed in 6× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then soaked in 2× Denhardt solution for 1 h before they were baked in a vacuum oven at 80°C for 1 to 2 h. The filters were stored dry.

(ii) Mechanical shearing of DNA. Mechanical shearing of DNA was performed by passing the DNA solution 20 times through an 18-gauge hypodermic needle.

(iii) Labeling of probe DNA. The fragmented DNA was labeled with ^{32}P-dCTP to a specific activity of 10^7 cpm with a nick-translation kit (Bethesda Research Laboratories).

(iv) Hybridization technique. The filters were prehybridized for 4 h and then hybridized for 16 h at 65°C in a plastic bag. The sodium salt concentration of the prehybridization and hybridization fluids was 0.1 M. The posthybridization washing was carried out with the same sodium salt concentration and temperature as those used in the hybridization reaction. The hybridization results were obtained by scintillation counting (Packard Instrument Co.) of standard-cut pieces of nitrocellulose paper.

(v) Quantitation of the hybridization reaction. The mean of the counts per minute for the eight parallels minus the control counts per minute of salmon sperm DNA for each of the strains was determined. The mean counts per minute value of the autologous strain was defined to represent a DNA homology ratio or a relative binding ratio (RBR) of 100%. The mean RBR of each strain was calculated by dividing the mean counts per minute of each strain by the mean counts per minute of the autologous reaction and multiplying the quotient by 100. The 95% confidence interval of the sample mean was calculated (Table 2).

Genetic transformation. The media and reagents for genetic transformation have been described previously (24). Mutants were selected for streptomycin resistance, and DNA was extracted. DNA isolation was performed by adding lysozyme to the suspension of bacteria, freezing and thawing the solution, adding sodium dodecyl sulfate to 1%, and heating the suspension to 60°C to obtain lysis. Extraction in chloroform-isoamyl alcohol (20:1) was performed once, and the DNA in solution was subjected to ethanol precipitation. The concentration of DNA was measured by using the diphenylamine reaction and adjusted to 200 μg/ml. All of the strains in the study were tested in a competence survey, being exposed to DNA in a semiquantitative transformation procedure (24). In all transformation procedures, both buffer- and DNase-treated DNA were used as negative controls. When a quantitative transformation assay was performed, 0.5 ml of the recipient suspension was exposed to 0.05 ml of each DNA for 20 minutes (24). After DNase treatment, 0.1-ml samples of each final suspension and appropriate dilutions were inoculated on blood agar plates (which were preincubated for 7 h) before they were placed on top of an agar layer, giving a final concentration of 50 μg/ml of streptomycin after diffusion. The plates were further incubated for 3 to 5 days before the counting of colonies and calculation of interstrain-to-intrastrain transformation ratios. In the semiquantitative transformation, the addition of DNase was omitted, which increased the transformant colony yield per plate greatly (24).

TABLE 2. RBRs appearing by DNA-DNA hybridization between strains of *H. aphrophilus*, *H. paraphrophilus*, *A. actinomycetemcomitans*, and *H. influenzae* with *H. aphrophilus* NCTC 5906^T and *A. actinomycetemcomitans* NCTC 9710^T as radioactively labeled probes

Filter DNA source[a]	Mean RBR (95% confidence interval)[b] with:	
	H. aphrophilus NCTC 5906	*A. actinomycetemcomitans* NCTC 9710
Type and reference strains		
H. aphrophilus NCTC 5906^T	100 (93–107)	27 (24–30)
H. aphrophilus NCTC 5886	100 (96–104)	26 (24–28)
H. paraphrophilus NCTC 10557^T	78 (71–85)	22 (21–23)
A. actinomycetemcomitans NCTC 9710^T	17 (15–19)	100 (94–106)
H. influenzae NCTC 8143^T	3 (2–4)	6 (4–8)
New clinical isolates		
E.H./79	76 (67–85)	20 (18–22)
IIMO A1/82	75 (68–82)	22 (19–25)
IIMO A2/82	65 (62–68)	20 (16–24)
3203/86	94 (88–100)	23 (21–25)
9574/86	94 (89–99)	24 (21–27)
46/87	79 (72–86)	31 (29–33)
40503/88	92 (88–96)	24 (20–28)
800/89	84 (80–88)	23 (20–26)
1489/79	16 (13–19)	100 (95–105)
11305/79	12 (11–13)	90 (84–96)
47/87	13 (10–16)	81 (71–91)
5775/87	9 (7–11)	73 (65–81)

[a] See legend of Table 1, footnotes *c* and *d*, for strain designations.
[b] Of the sample mean of eight parallels.

RESULTS

DNA-DNA hybridization: distinction of type strains. The type strains of *H. aphrophilus* NCTC 5906 and *A. actinomycetemcomitans* NCTC 9710 were clearly distinguished from each other by DNA-DNA hybridization (Table 2). This correlates well with earlier hybridization results (8, 19, 20, 21). The *H. paraphrophilus* type strain was not clearly distinguished from *H. aphrophilus*; this was demonstrated when its DNA was used as a probe and as filter DNA. The distinction of the *H. influenzae* type strain from the other species was clear-cut.

The type strains of *H. aphrophilus* and *A. actinomycetemcomitans* were also compared with type and reference strains of a diversity of species in the family *Neisseriaceae* and other gram-negative species by using them as probes in DNA-DNA hybridization. The species investigated were *K. kingae*, *K. indologenes*, *K. denitrificans*, *M. nonliquefaciens*, *M. lacunata*, *M. atlantae*, *M. phenylpyruvica*, *M. osloensis*, *N. elongata*, *O. urethralis*, *E. corrodens*, *Cardiobacterium hominis*, *G. vaginalis*, *C. canimorsus*, *P. multocida*, and *P. haemolytica* (see Materials and Methods for the strains employed), representing a relevant spectrum of differential diagnoses. Both *H. aphrophilus* and *A. actinomycetemcomitans* were clearly distinguished from the others, revealing RBRs of less than 5% for all the other DNAs.

Investigation of new clinical isolates by DNA-DNA hybridization. When the type strains of *H. aphrophilus* and *A. actinomycetemcomitans* were used as probes, the new clinical isolates appeared in two well-separated clusters: one around *H. aphrophilus* (E.H./79, IIMOA1/82, IIMOA2/82, 3203/86, 9574/86, 46/87, 40503/88, and 800/89) and one around *A. actinomycetemcomitans* (1489/79, 11305/79, 47/87, and 5775/87) (Table 2). Results similar to those from the *A. actinomycetemcomitans* type strain probe were obtained by using the new clinical isolate 47/87 as a probe. The type strain of *H. paraphrophilus* used as a probe gave results corresponding to those from the *H. aphrophilus* type strain. New clinical isolates, tentatively identified as *H. paraphrophilus* by conventional characterization (IIMOA1/82, IIMOA2/82, and 800/89; Table 1), were not distinguished from *H. aphrophilus* by hybridization.

Genetic transformation: competence survey. Some of the clinical isolates were found to be naturally competent in genetic transformation. Competence was demonstrated in the strains 9574/86, E.H./79, 3203/86, and 40503/88 appearing in the *H. aphrophilus* cluster and the strains 47/87 and 1489/79 in the *A. actinomycetemcomitans* cluster. Autologous transformant frequencies varied between 5.1×10^{-4} and 2×10^{-3} in strain 9574/86 and between 6.2×10^{-5} and 1.3×10^{-3} in strains 47/87 and 1489/79. Transformation was demonstrated by the virtual absence of streptomycin-resistant colonies after the DNase treatment of donor DNA. The frequencies of spontaneous streptomycin-resistant mutants occurring on the plates were about 10^3 times lower than the autologous transformant yields in the quantitative transformation experiments. Since the type strains were not competent, strains 9574/86, 47/87, and 1489/79 were used as recipients in the transformation studies.

Investigation of type strains and new clinical isolates by genetic transformation. Results obtained by genetic transformation with 9574/86, representing the *H. aphrophilus* cluster, and 47/87 and 1489/79, representing the *A. actinomycetemcomitans* cluster, as recipients are shown in Table 3. The type strains revealed transformation ratios indicating genetic affinities comparable to those obtained by hybridization. Clustering of the clinical strains appeared around *H. aphrophilus* and around *A. actinomycetemcomitans* (Table 3). The type strain of *H. paraphrophilus* and new clinical isolates tentatively identified as such by conventional means (strains IIMOA1/82, IIMOA2/82, and 800/89) behaved generally like *H. aphrophilus* in the transformation experiments. The lower heterologous transformant yields with the recipient 47/87 than with 1489/79 may be explained by experimental bias, due to a lower competence of the former. This made a dense recipient population for 47/87 necessary, possibly leading to relatively inefficient penetration of the transformant colonies through the background growth in the least-diluted parallels (Table 3). The distinction of *H. aphrophilus* and *A. actinomycetemcomitans* from the panel of other gram-negative species investigated was also demonstrated by genetic transformation. Both species displayed transformation ratios of less than 10^{-3} versus all of the other species.

DISCUSSION

In this study, *H. aphrophilus* and *A. actinomycetemcomitans* were easily distinguished and identified by DNA-DNA hybridization (Table 3). DNA-DNA hybridization is a direct, chemical method for indication of DNA homology. The use of total genomic DNA from the type strains of each species makes these DNA probes most accessible, representative, and stable to minor variations in the genomes of the strains to be tested. Filter hybridization allows the processing of many samples at the same time. The stringent conditions used are necessary to obtain optimal distinction of closely related taxa. The method is meant to be used in addition to

TABLE 3. Affinities of strains of *H. aphrophilus*, *H. paraphrophilus*, *A. actinomycetemcomitans*, and *H. influenzae* as they appear by quantitative genetic transformation

Donor DNA source[a]	Frequency ratio[b] in:		
	H. aphrophilus	*A. actinomycetemcomitans*	
	9574/86	47/87	1489/79
Type and reference strains			
H. aphrophilus NCTC 5906^T	6.6×10^{-1}	$<10^{-2}$	4.1×10^{-2}
NCTC 5886	5.6×10^{-1}	$<10^{-2}$	
H. paraphrophilus NCTC 10557^T	1.5×10^{-1}	$<10^{-2}$	4.8×10^{-2}
A. actinomycetemcomitans NCTC 9710^T	2.2×10^{-2}	6.2×10^{-1}	4.1×10^{-1}
H. influenzae NCTC 8143^T	2.6×10^{-3}	$<10^{-2}$	
New clinical isolates			
E.H./79	7.4×10^{-1}	$<10^{-2}$	
IIMO A1/82	1.5×10^{-1}	$<10^{-2}$	1.5×10^{-2}
IIMO A2/82	3.6×10^{-1}	$<10^{-2}$	3.2×10^{-2}
3203/86	4.9×10^{-1}	$<10^{-2}$	2.1×10^{-2}
9574/86	1	$<10^{-2}$	3.9×10^{-2}
40503/88	4.3×10^{-1}	$<10^{-2}$	5.9×10^{-2}
46/87	2.8×10^{-1}	$<10^{-2}$	5.9×10^{-2}
800/89	4.2×10^{-1}	$<10^{-2}$	
11305/79	9.4×10^{-2}	1.4×10^{-1}	5.2×10^{-1}
1489/86	8.8×10^{-2}	5.4×10^{-1}	1
47/87	1.4×10^{-2}	1	4.9×10^{-1}
5775/87	2.3×10^{-2}	6.6×10^{-1}	4.5×10^{-1}

[a] See Table 1, footnotes *c* and *d*, for strain designations.
[b] Ratio of interstrain-to-intrastrain transformant frequency.

conventional characterization for the identification of new clinical isolates, as a means to distinguish among different species. In this study, distinction between two genetically closely related entities was demonstrated, with no overlapping RBRs. Repeating the experiments verified the reproducibility of results. There was some variability in the lower range of RBRs as compared with earlier findings (Table 2; 23), but this does not at all affect the actual discrimination between the entities.

The RBRs of the new clinical isolates were more clearly clustered around the type strains of *H. aphrophilus* and *A. actinomycetemcomitans* in DNA-DNA hybridization than the transformation ratios around the recipient strains in genetic transformation. Because of incompetent type strains, the recipient strains were chosen from the new clinical isolates after conventional characterization, DNA-DNA hybridization, and competence survey. One reason for the discrepancy may be that these strains are not as representative for the species as the type strains are. Still, when the recipient strain 47/87 was used as a probe in DNA-DNA hybridization, the RBRs were similar to those obtained when the *A. actinomycetemcomitans* type strain (NCTC 9710) was used as a probe (data not shown). Another reason may be the use of the well-conserved ribosomal marker of streptomycin resistance. Also, in this biological method, factors other than the recombination reflected by DNA homology may be of importance. Restriction and modification systems are abundantly present in strains of these species, as in other species naturally competent for DNA uptake. Strain variability in this respect may cause false-negative results in routine transformation assays. In our material, the significance of restriction and modification systems seems minor; our transformation results with the stably competent strains generally corresponded well with those obtained by DNA-DNA hybridization. But, restriction endonucleases may be at least part of the reason for the variation in interstrain transformation efficiency. Other mechanisms affecting the interstrain transformation, such as the possible existence of recognition sequences for DNA binding and uptake, are also our concern. These obstacles are avoided in hybridization.

The distinction of *H. aphrophilus* and *A. actinomycetemcomitans* by filter hybridization and transformation is much clearer than what is obtainable by conventional bacteriological identification assays (8, 21). The ratios correlate well with results obtained by hybridization in solution (19, 21) and isoenzyme electrophoresis (17). As far as we know, this is the first report of the use of genetic transformation in the primary identification of these two species. It is fully possible to obtain species identification of new isolates of these species as recipients by genetic transformation alone, by the use of donor DNA of both *H. aphrophilus* and *A. actinomycetemcomitans* type strains in parallel in a semiquantitative assay (continuous DNA exposure without DNase; 24).

H. aphrophilus and *H. paraphrophilus* do not appear as two distinct species by these molecular methods. Some strains show the conventional characteristics of a positive oxidase reaction and a requirement for V factor (NAD) for growth (7, 12, 19), but taxonomic studies using hybridization in solution (19, 21) and isoenzyme electrophoresis (17) do not support the classification of these as a separate species.

Both DNA-DNA hybridization and genetic transformation are easy to perform; although a bit time-consuming, they are precise and reliable, obviating the need for advanced skill or equipment. Even crude DNA can be used. The hybridization method may be a little more laborious than transformation, but it gives a better distinction in shorter time and is generally more applicable. The DNA homology appearing by genetic transformation may be regarded as more functional and "real" than the steric conformation complementarity measured in DNA-DNA hybridization.

The results presented here are examples of definite identification of clinical isolates of *H. aphrophilus* and *A. actinomycetemcomitans* by molecular methods. Both DNA-DNA hybridization and genetic transformation distinguish them sufficiently from each other and from other species.

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