Differentiation between *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* by multilocus enzyme electrophoresis

DOMINIQUE A. CAUGANT,¹* ROBERT K. SELANDER² and INGAR OLSEN³

¹ Department of Bacteriology, National Institute of Public Health, 0462 Oslo 4, Norway

² Department of Biology, Pennsylvania State University, University Park, Pennsylvania 16802, USA

³ Department of Microbiology, Dental Faculty, University of Oslo, 0316 Oslo 3, Norway

(Received 12 March 1990; revised 13 June 1990; accepted 27 June 1990)

Genetic relationships among isolates assigned to *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus* and *H. paraphrophilus* were determined by analysis of electrophoretically demonstrable allelic variation in 14 structural genes encoding metabolic enzymes. Among the 51 isolates analysed there were 25 electrophoretic types (ETs), among which mean genetic diversity per locus was 0.753. Cluster analysis of ETs demonstrated one well-defined group of 11 ETs representing solely the genotypes of all 17 isolates assigned to *A. actinomycetemcomitans*. The remaining 14 ETs represented the genotypes of the 34 isolates of *H. aphrophilus* and *H. paraphrophilus*. With the exception of ATCC 13252, all strains of *H. aphrophilus* were closely related, whereas strains assigned to *H. paraphrophilus* included distantly related lineages, some of which were similar to those of *H. aphrophilus* and should be assigned to this species. Thus, the study showed that there is no significant overall genetic similarity between *A. actinomycetemcomitans* and the two *Haemophilus* spp.

Introduction

Actinobacillus and *Haemophilus* species are currently attracting the attention of many clinical microbiologists and taxonomists. This particularly applies to *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*, partly because of the increasing frequency of their isolation from unusual clinical sites. *A. actinomycetemcomitans* and *H. aphrophilus* have also been isolated from healthy gingiva and from gingivitis and periodontitis (Kraut *et al.*, 1972; Kilian & Schiøtt, 1975; Tanner *et al.*, 1979). While there seems to be an association between the presence of *A. actinomycetemcomitans* in dental plaque and localized periodontitis in juveniles (Slots *et al.*, 1980), the causal role of *H. aphrophilus* in that disease is negligible (Tempo & Slots, 1986). *A. actinomycetemcomitans* has also been recovered from extra-oral diseases such as bacterial endocarditis, pericarditis, post-extraction and brain abscesses, urinary tract infections and vertebral osteomyelitis (for a review, see Kaplan *et al.*, 1989). Similarly *H. aphrophilus* has been isolated from brain abscesses, meningitis, sinusitis, pneumonia, empyema, otitis media, wound and post-operative infec-

tions, arthritis and osteomyelitis (King & Tatum, 1962; Sutter & Finegold, 1970; Ellner *et al.*, 1979). *H. paraphrophilus*, a member of the indigenous flora of the oral cavity and pharynx (Kilian & Schiøtt, 1975), may be responsible for subacute endocarditis, paronychia, brain abscess, osteomyelitis of the jaw, appendicitis (Kilian & Frederiksen, 1981) and arthritis (von Essen *et al.*, 1987). Occasionally, this organism has also been isolated from urine and the vagina (Kilian & Biberstein, 1984). The oral cavity may represent a spearhead of the infectious spread of these bacteria, as suggested by the association of endocarditis caused by *A. actinomycetemcomitans* with rapidly destructive periodontitis (Anolik *et al.*, 1981).

Biochemical tests suggest that *A. actinomycetemcomitans* and *H. aphrophilus* are very similar, and their taxonomic positions have been open to question (King & Tatum, 1962). Kilian (1976) emphasized that these two species do not require haemin and NAD for growth, and therefore do not meet the criteria for inclusion in the genus *Haemophilus*. On the other hand, Potts *et al.* (1985) suggested, on the basis of DNA–DNA reassociation experiments and previous inclusions of X and V factor independent species in the genus *Haemophilus*, that *A. actinomycetemcomitans* should be reassigned to the genus *Haemophilus* as *H. actinomycetemcomitans*. This was

*Abbreviation: ET, electrophoretic type.

Table 1. *Origins and sources of the strains of A. actinomycetemcomitans, H. aphrophilus and H. paraphrophilus investigated*

ET	Strain*	Received as	Geographical source	Site of isolation
1	FDC 511	<i>A. actinomycetemcomitans</i>	USA, Mass.	Periodontitis
	FDC N27	<i>A. actinomycetemcomitans</i>	USA, Mass.	Periodontosis
2	SUNY 75	<i>A. actinomycetemcomitans</i>	USA, New York	Dental plaque
3	HK 435	<i>A. actinomycetemcomitans</i>	Denmark	Pus
4	ATCC 29523	<i>A. actinomycetemcomitans</i>	USA, Colorado	Blood
5	FDC Y4	<i>A. actinomycetemcomitans</i>	USA, Mass.	Periodontosis
6	ATCC 29522	<i>A. actinomycetemcomitans</i>	USA, Illinois	Mandibular abscess
7	NCTC 9709	<i>A. actinomycetemcomitans</i>	Denmark	Lumbar abscess
	FDC 2043	<i>A. actinomycetemcomitans</i>	USA, Mass.	Periodontitis
	and FDC 2097†			
8	FDC M61	<i>A. actinomycetemcomitans</i>	USA, Mass.	Periodontitis
9	G1-G4‡	<i>A. actinomycetemcomitans</i>	Norway	Periodontitis
10	ATCC 33384‡	<i>A. actinomycetemcomitans</i>	Denmark	Lung abscess
11	ATCC 29524	<i>A. actinomycetemcomitans</i>	USA, Wash.	Chest aspirate
12	ATCC 29241‡	<i>H. paraphrophilus</i>	UK	Paronychia
13	HK 319	<i>H. paraphrophilus</i>	Denmark	Dental plaque
14	IMoAI	<i>H. paraphrophilus</i>	Norway	Cerebellar abscess
	and IMoAII†			
15	ATCC 29240	<i>H. paraphrophilus</i>	UK	Parietal abscess
16	HK 477	<i>H. paraphrophilus</i>	Denmark	Dental plaque
17	ATCC 19415	<i>H. aphrophilus</i>	UK	Endocarditis
	H76	<i>H. paraphrophilus</i>	UK	Oral cavity
	IIIRA43	<i>H. paraphrophilus</i>	Norway	Sputum
18	ATCC 33389‡	<i>H. aphrophilus</i>	UK	Endocarditis
19	FDC 654	<i>H. aphrophilus</i>	USA, Mass.	Periodontitis
	and FDC 655†			
20	FDC 621	<i>H. aphrophilus</i>	USA, Mass.	Periodontosis
	FDC 626	<i>H. aphrophilus</i>	USA, Mass.	Periodontosis
	1-15†	<i>H. aphrophilus</i>	Norway	Saliva
21	HK 159	<i>H. paraphrophilus</i>	Denmark	Saliva
22	ATCC 13252	<i>H. aphrophilus</i>	Unknown	Unknown
23	HK 296	<i>H. paraphrophilus</i>	Denmark	Chronic bronchitis
24	HK 321	<i>H. paraphrophilus</i>	Denmark	Dental plaque
25	ATCC 29242	<i>H. paraphrophilus</i>	Denmark	Trachea

* All the strains from Norway are recent clinical isolates. ATCC, American Type Culture Collection; FDC, Forsyth Dental Center; H, W. Sims, Royal Dental Hospital of London, UK; HK, M. Kilian, Royal Dental College, Aarhus, Denmark; NCTC, National Collection of Type Cultures (UK); SUNY, State University of New York. The 15 isolates of *H. aphrophilus* from Norway (ET 20) were collected from the saliva of one individual, using a selective medium (Tempro & Slots, 1986).

† Multiple isolates from the same individual.

‡ Type strain.

further supported by analysis of 16S rRNA sequences (Chuba *et al.*, 1988; Paster *et al.*, 1988). Tanner *et al.* (1982) found that *H. paraphrophilus* and *H. aphrophilus* strains, except for a single isolate of the former group, could not be distinguished as two separate species as there was overlap in biochemical characteristics and high degree of DNA-DNA homology. However, Brondz *et al.* (1990), using multivariate analyses of chemotaxonomic data, distinguished *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*.

In recent years, multilocus enzyme electrophoresis has been employed to assess genetic diversity and population structure in a number of bacterial species (Selander *et al.*, 1986, 1987). The method is based upon electrophoretic detection of allelic diversity by indexing variation in the amino acid sequences of many gene products, the

metabolic enzymes. Previous investigations have demonstrated that estimates of genetic relatedness based on multilocus enzyme electrophoresis are significantly correlated with measures of total genomic similarity as estimated by DNA-DNA hybridization (Ochman *et al.*, 1983; Selander *et al.*, 1985).

In the present study, we analyse allelic variation in 14 genes, to assess the genetic diversity among strains assigned to the species *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*, and to determine the relationships between isolates of the three species.

Methods

Bacterial isolates. A collection of 51 isolates was examined, comprising 17 strains of *A. actinomycetemcomitans* isolated from 13 hosts, 22 strains of *H. aphrophilus* from 7 hosts, and 12 strains of *H.*

paraphrophilus from 11 hosts. All strains had originally been recovered from humans with or without clinical disease. The sources and designations of the strains are given in Table 1. Strain identification was based on growth requirement for haemin and NAD, acid and gas production from specific sugars, and other physiological characteristics (Tanner *et al.*, 1982; Kilian & Biberstein, 1984).

Preparation of culture lysates. The isolates were cultured anaerobically (80% N₂/10% H₂/10% CO₂), usually in brain heart infusion broth (Difco), at 35 °C for 2 or 3 d. For *H. paraphrophilus*, the broth was supplemented with filter-sterilized NAD (1 mg l⁻¹) and haemin (5 mg l⁻¹). *H. paraphrophilus* strains HK 321 and HK 296 grew poorly in this medium and were therefore grown on chocolate agar plates, harvested by scraping colonies off the plates, then washed and resuspended in phosphate-buffered saline, pH 7.2. The other strains were harvested by centrifugation of broth cultures (5860 g at 4 °C for 20 min). The pellets were resuspended in 2 ml phosphate-buffered saline. Cells were lysed by sonication, using a Branson Cell Disruptor with microtip at 50 W for 60 s, with ice-cooling. After lysis and centrifugation at 25000 g at 4 °C for 30 min, aliquots of supernatants were transferred to 1 ml freeze tubes (Nunc) and stored immediately at -70 °C until electrophoresis.

Electrophoresis of enzymes. Lysates were electrophoresed on starch gels and selectively stained for 14 metabolic enzymes (Selander *et al.*, 1986). Buffer system A was used for electrophoresis of malate dehydrogenase (MDH; EC 1.1.1.37), 6-phosphogluconate dehydrogenase (6PG; EC 1.1.1.44), glucose-6-phosphate dehydrogenase (G6P; EC 1.1.1.49), indophenol oxidase (IPO; EC 1.15.1.1; superoxide dismutase), adenylate kinase (ADK; EC 2.7.4.3), alkaline phosphatase (ALP; EC 3.1.3.1), phosphoglucose isomerase (PGI; EC 5.3.1.9), glutamate dehydrogenase (GDH; EC 1.4.1.4), phosphoglucomutase (PGM; EC 5.4.2.2), leucine aminopeptidase (LAP; EC 3.4.11.1), and succinate dehydrogenase (SDH; EC 1.3.99.1). Buffer system D was used for electrophoresis of peptidase (PEP; EC 3.4.-.-), aspartate dehydrogenase (ASP; EC 1.4.3.-) and fumarase (FUM; EC 4.2.1.2).

Electromorphs (mobility variants) of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus (see Table 2). An absence of enzyme activity was attributed to a null allele, designated as 0. Each isolate was characterized by its profile of alleles at the 14 enzyme loci assayed, and distinctive combinations of alleles, representing multi-locus genotypes, were designated as electrophoretic types, ETs (Selander *et al.*, 1986).

Statistical analysis. Genetic diversity at an enzyme locus among ETs was calculated as $h = (1 - \sum x_i^2) / (n(n-1))$, where x_i is the frequency of the i th allele and n is the number of ETs. Mean genetic diversity per locus (H) is the arithmetic average of h values over all loci. Genetic distance between pairs of ETs (D) was expressed as the proportion of enzyme loci at which dissimilar alleles occurred. Null alleles were excluded from the measure of genetic distance. Clustering of ETs was performed from the matrix of genetic distances by the average-linkage method (Sneath & Sokal, 1973).

Results

Overall genetic diversity

The ET of each strain is given in Table 1, and the allele profiles at 14 enzyme loci for a representative strain from each ET are shown in Table 2.

In the collection of 51 isolates examined, all 14 enzyme

loci assayed were polymorphic for 4 (ASP) to 11 (PGM) alleles; the mean was 6.3 alleles per locus (Table 3). No ASP activity was demonstrated in the strains of *A. actinomycetemcomitans*, whereas all but two of the *Haemophilus* strains had ASP activity. The remaining 13 enzymes were expressed in at least 90% of the isolates. There were 25 ETs represented in the collection of isolates (Table 2), among which the mean genetic diversity per locus was 0.753 and the interlocus variance, s^2 , was 0.006 (Table 3). A pairwise comparison of the electromorph profiles of the 25 ETs revealed that 60% of the ET pairs differed at 11 or more of the enzyme loci assayed (mean, 10.12 loci; $s^2 = 18.45$), indicating that at least two very distinct groups of genotypes were represented among the 25 ETs (Fig. 1).

Genetic diversity within species

Eleven ETs were identified among the 17 isolates of *A. actinomycetemcomitans* examined (Table 4). The four isolates (G1 through G4) recovered from a single patient with periodontitis in Norway were identical (ET 9), as were the two isolates from a single individual with periodontitis in the USA, FDC 2043 and FDC 2097 (ET 7). Strain NCTC 9709, isolated from a patient with lumbar abscess in Denmark, was also ET 7.

Five ETs were identified among the 22 isolates of *H. aphrophilus* (Table 4). The 15 isolates recovered from the saliva of a single individual in Norway were identical (ET 20), and belonged to the same ET as strains FDC 621 and FDC 626, recovered from two cases of periodontitis in the USA (Table 1). Isolates FDC 654 and FDC 655, obtained from a single individual with periodontitis, were also genetically identical (ET 19).

The 12 isolates assigned to *H. paraphrophilus* were of 10 ETs, among which mean genetic diversity per locus was 0.686, that is 91% of the genetic diversity among the 25 ETs of the whole strain collection (Table 4). Whereas ETs of *A. actinomycetemcomitans* and *H. aphrophilus* differed at an average of 4.7 and 4.5 loci, respectively, the ETs of isolates assigned to *H. paraphrophilus* differed at an average of 8.6 loci, with 46% of the ET pairs having dissimilar alleles at 11 or more enzyme loci.

Relationships among ETs

The relationships among the 25 ETs are summarized by the dendrogram in Fig. 2. Genotypes differing solely in the occurrence of null alleles are represented by a single branch in the dendrogram, as null alleles were not included in the measure of genetic distance, D , between ETs. The smallest value of D , 0.07, corresponds to a single-locus difference between ETs.

Table 2. Allele profiles at 14 enzyme loci in ETs of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*

ET	Reference isolate	Alleles at indicated enzyme loci*													
		MDH	G6P	6PG	IPO	GDH	ADK	PGM	PGI	LAP	FUM	PEP	ALP	ASP	SDH
1	FDC N27	8	4	0	4	3	3	9	8	4	3	5	4	0	5
2	SUNY 75	8	4	0	4	3	0	9	8	4	3	5	4	0	5
3	HK 435	8	4	0	4	3	0	9	8	4	3	5	0	0	5
4	ATCC 29523	8	4	0	4	3	0	8	8	4	3	5	4	0	0
5	FDC Y4	5	4	4	4	1	3	9	2	4	3	5	3	0	0
6	ATCC 29522	5	4	4	4	1	3	9	2	4	3	5	3	0	4
7	NCTC 9709	5	4	4	4	1	3	9	2	4	3	5	1	0	4
8	FDC M61	5	4	0	4	1	3	9	2	4	3	5	1	0	4
9	G1	5	4	4	4	1	3	10	2	4	3	5	1	0	4
10	ATCC 33384	1	4	4	4	1	3	10	2	4	0	5	1	0	4
11	ATCC 29524	5	5	5	4	1	3	9	2	5	3	5	3	0	4
12	ATCC 29241	2	1	3	2	5	2	2	3	3	1	3	1	2	2
13	HK 319	2	0	3	2	5	0	1	3	0	2	3	0	0	2
14	IMoAI	6	1	2	3	5	2	5	3	3	2	4	1	3	1
15	ATCC 29240	6	1	2	3	5	2	6	3	3	2	4	1	3	1
16	HK 477	6	1	2	3	5	2	6	0	3	2	4	1	3	2
17	ATCC 19415	6	1	1	2	4	2	4	3	3	2	4	1	3	2
18	ATCC 33389	6	1	1	2	4	2	4	3	3	2	3	1	3	2
19	FDC 654	6	1	1	2	4	2	4	1	3	2	4	1	3	2
20	FDC 621	6	6	3	2	4	2	7	3	2	2	2	1	3	2
21	HK 159	3	1	4	3	5	2	2	7	2	2	3	1	3	1
22	ATCC 13252	6	6	1	2	2	0	1	7	3	4	3	0	0	2
23	HK 296	4	2	1	1	5	0	4	4	2	2	3	3	3	0
24	HK 321	5	3	1	2	0	3	8	6	2	2	4	3	3	5
25	ATCC 29242	7	2	1	0	6	1	3	5	1	6	1	2	1	3

* Enzyme abbreviations: MDH, malate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; IPO, indophenol oxidase; GDH, glutamate dehydrogenase; ADK, adenylate kinase; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; LAP, leucine aminopeptidase; FUM, fumarase; PEP, peptidase; ALP, alkaline phosphatase; ASP, aspartate dehydrogenase; SDH, succinate dehydrogenase.

Table 3. Components of genetic diversity at 14 enzyme loci in ETs of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*

Enzyme locus*	No. of alleles	Variance component†		
		Within species	Total	F_{st}
MDH	8	0.610	0.813	0.250
G6P	7	0.458	0.750	0.389
6PG	6	0.671	0.827	0.189
IPO	5	0.293	0.703	0.583
GDH	7	0.517	0.820	0.370
ADK	4	0.530	0.710	0.254
PGM	10	0.721	0.870	0.171
PGI	9	0.675	0.837	0.194
LAP	6	0.444	0.737	0.398
FUM	6	0.311	0.667	0.534
PEP	5	0.418	0.717	0.417
ALP	5	0.682	0.687	0.007
ASP	4	0.293	0.590	0.503
SDH	6	0.608	0.817	0.256
Mean	6.3	0.517	0.753	0.313

* See footnote to Table 2.

† The within-species component of genetic variance estimates the probability of a mismatch of alleles at an enzyme locus for two ETs chosen randomly from the same species; the total variance is the

comparable estimate for two ETs drawn from the pooled groups. F_{st} is the ratio of the probability of a mismatch for two ETs selected randomly from different species to the average probability in the pooled sample; it is thus a measure of the relative magnitude of genetic differentiation at an enzyme locus among groups.

Table 4. Genetic diversity in ETs of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*

Species	No. of isolates	No. of ETs	No. of polymorphic enzymes	Mean no. of alleles per locus	Genetic diversity in ETs
<i>A. actinomycetemcomitans</i>	17	11	11	2.2	0.369
<i>H. aphrophilus</i>	22	5	11	2.0	0.400
<i>H. paraphrophilus</i>	12	10	14	4.4	0.686
Total	51	25	14	6.3	0.753

Fig. 1. Frequency of numbers of enzyme loci at which unlike alleles (mismatches) occurred in pairwise comparisons of 25 ETs of 51 isolates of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*.

ETs (ET 23 to 25) represented the genotypes of three isolates assigned to *H. paraphrophilus* (HK 296, HK 321 and ATCC 29242). Cluster A joined all the other ETs at $D = 0.96$.

Discussion

DNA homology between strains of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus* has been studied by several investigators, with varying results (Tanner *et al.*, 1982; Coykendall *et al.*, 1983; Potts & Berry, 1983). The survey of Tanner *et al.* (1982) included the largest portion of isolates examined by us. When the genetic similarity ($1 - D$) assessed by multilocus enzyme electrophoresis was compared with the percentage of DNA-DNA hybridization for 30 pairs of isolates (Tanner *et al.*, 1982), there was a significant correlation ($r = 0.90$, $P < 0.001$) between relatedness obtained with the two methods. However, for all but one of the ten comparisons including one isolate of *A. actinomycetemcomitans* and one of the *H. aphrophilus/H. paraphrophilus*

Fig. 2. Genetic relationships among ETs of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of genetic distance between pairs of ETs, based on 14 enzyme loci. ■, ETs of *A. actinomycetemcomitans*; ○, ETs of *H. aphrophilus*; ●, ETs of *H. paraphrophilus*. A, B, and C indicate clusters of ETs that diverge at a genetic distance of less than 0.50.

group the similarity from multilocus enzyme electrophoresis was null. Considering only the 20 within-species pairwise comparisons, the correlation was less, but still

significant ($r = 0.71$, $P < 0.001$). The estimates of genetic distance based on the sample of 14 enzyme loci assayed should therefore be representative of the overall genomic relationships between strains.

The electrophoretic analysis of allelic variation at 14 enzyme loci confirmed previous lines of evidence derived from electrophoresis of total proteins (Jellum *et al.*, 1984; Olsen *et al.*, 1987) and DNA-DNA reassociation experiments (Pohl, 1981; Tanner *et al.*, 1982; Coykendall *et al.*, 1983; Potts & Berry, 1983) that there is no large total genetic similarity between strains of *A. actinomycetemcomitans* and *H. aphrophilus*. Although the number of strains of each species analysed was relatively small, isolates were obtained from various geographical areas. Thus, it is likely that the genetic diversity among ETs observed is representative of that in these species. It should be noted that the computation of the genetic diversity is based on the ETs and therefore is not affected by the inclusion of multiple isolates from the same host.

The diversity among genotypes of *A. actinomycetemcomitans* and *H. aphrophilus* was similar to that reported for other bacterial species (Musser *et al.*, 1986). In contrast, the 10 ETs of the 12 isolates of *H. paraphrophilus* we studied were more diverse than the 331 ETs of 688 isolates of *Neisseria meningitidis*, which is the most variable bacterial species analysed by multilocus enzyme electrophoresis thus far (Caugant *et al.*, 1987). This suggests that the isolates assigned to *H. paraphrophilus* in fact belong to several species.

Of the three most distinctive isolates in this study, HK 296 and HK 321 have been tentatively assigned to *H. paraphrophilus* (Kilian, 1976), although they differed from other strains of this species in several biochemical and physiological properties. This was also indicated by our inability to grow these isolates in broth cultures. Electrophoretic mobilities are not influenced by growth conditions (Musser *et al.*, 1986; Selander *et al.*, 1986); thus the differences observed between HK 296 and HK 321 and other *H. paraphrophilus* strains are not likely to be artefacts but reflect genetic dissimilarity. ATCC 29242 has been reported atypical of *H. paraphrophilus* in its protein pattern in two-dimensional electrophoresis (Olsen *et al.*, 1987), biochemical characteristics, G+C content, and homology to other strains of the species as measured by DNA-DNA reassociation (Tanner *et al.*, 1982). Multilocus enzyme electrophoresis clearly confirmed that ATCC 29242 is not a member of the species *H. paraphrophilus*.

When these three atypical isolates (HK 296, HK 321 and ATCC 29242) were excluded from data evaluation, strains of *H. aphrophilus* and *H. paraphrophilus* formed a genetically homogeneous group of 11 ETs among which genetic diversity per locus was 0.526. There were then no significant differences in allele frequencies between the

two species for 8 of the 14 enzymes assayed, and the magnitude of genetic differentiation between ETs of isolates of *H. aphrophilus* and those of *H. paraphrophilus* was only 0.042, as measured by F_{st} (see Table 3). This similarity supports evidence from DNA-DNA hybridization experiments (Tanner *et al.*, 1982; Potts *et al.*, 1986) and two-dimensional electrophoresis of total proteins (Olsen *et al.*, 1987). However, there are differences between *H. aphrophilus* and *H. paraphrophilus* in NAD dependence, biochemical reactions (Kilian & Biberstein, 1984) and cellular sugars (Brondz *et al.*, 1990).

The recovery of ET 7 of *A. actinomycetemcomitans* in Denmark and the USA, and the occurrence of the same ETs of *H. aphrophilus* in the UK and Norway (ET 17) and in Norway and the USA (ET 20), suggests that populations of *A. actinomycetemcomitans* and *H. aphrophilus* are clonal in structure. Like most bacterial species so far analysed by multilocus enzyme electrophoresis (Selander *et al.*, 1987), clones of *H. aphrophilus* are temporally stable, as demonstrated by the representation of ET 17 by ATCC 19415 isolated in 1938 in the UK, and by IIRA43 isolated in 1986 in Norway.

The closely related clones of *A. actinomycetemcomitans*, ET 1 to ET 4, were represented by isolates recovered from blood, pus, dental plaques and periodontal pockets, and ET 7 was recovered from patients with periodontitis and lumbar abscess. Similarly, ET 17 of *H. aphrophilus* was represented by an isolate from the oral cavity and another from blood. It is thus likely that these organisms had a common source, probably dental plaque, to the indigenous flora of which *A. actinomycetemcomitans* and *H. aphrophilus* belong. Dissemination of oral bacteria to the bloodstream is not a rare event and both accumulation of dental plaque and periodontal inflammation increase the frequency of bacteraemic conditions.

We thank Dr L. O. Frøholm for valuable discussions, and Drs K. Bøvre, J. Eng, M. Kilian and H. R. Preus for providing bacterial strains. We acknowledge the technical assistance of S. Dynna and C. Fleming. This project was supported by Public Health Service grant AI 24631 from the National Institutes of Health to R.K.S. and grant 13.48.07-026 from the Norwegian Council for Science and Humanities to D.A.C.

References

- ANOLIK, R., BERKOWITZ, R. J., CAMPOS, J. M. & FRIEDMAN, A. D. (1981). *Actinobacillus* endocarditis associated with periodontal disease. *Clinical Pediatrics* **20**, 653-655.
- BRONDZ, I., OLSEN, I. & SJÖSTRÖM, M. (1990). Multivariate analysis of quantitative chemical and enzymic characterization data in classification of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. *Journal of General Microbiology* **136**, 507-513.

- CAUGANT, D. A., MOCCA, L. F., FRASCH, C. E., FRØHOLM, L. O., ZOLLINGER, W. D. & SELANDER, R. K. (1987). Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. *Journal of Bacteriology* **169**, 2781–2792.
- CHUBA, P. J., BOCK, R., GRAF, G., ADAM, T. & GÖBEL, U. (1988). Comparison of 16S rRNA sequences from the family Pasteurellaceae: phylogenetic relatedness by cluster analysis. *Journal of General Microbiology* **134**, 1923–1930.
- COYKENDALL, A. L., SETTERFIELD, J. & SLOTS, J. (1983). Deoxyribonucleic acid relatedness among *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus* and other *Actinobacillus* species. *International Journal of Systematic Bacteriology* **33**, 422–424.
- ELLNER, J. J., ROSENTHAL, M. S., LERNER, P. I. & MCHENRY, M. C. (1979). Infective endocarditis caused by slow-growing, fastidious, gram-negative bacteria. *Medicine* **58**, 145–158.
- VON ESSEN, R., KOSTIALA, A. A. I., ANTTOLAINEN, I., HAAFASAARI, J. & SIVONEN, A. (1987). Arthritis caused by *Haemophilus paraphrophilus*: isolation of the organism by using an improved culture protocol. *Journal of Clinical Microbiology* **25**, 2447–2448.
- JELLUM, E., TINGELSTAD, V. & OLSEN, I. (1984). Differentiation between *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by high-resolution, two-dimensional protein electrophoresis. *International Journal of Systematic Bacteriology* **34**, 478–483.
- KAPLAN, A. H., WEBER, D. J., ODDONE, B. Z. & PERFECT, J. R. (1989). Infection due to *Actinobacillus actinomycetemcomitans*: 15 cases and review. *Reviews of Infectious Diseases* **11**, 46–63.
- KILIAN, M. (1976). A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. *Journal of General Microbiology* **93**, 9–62.
- KILIAN, M. & BIBERSTEIN, E. L. (1984). Genus II. *Haemophilus* Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 561 AL. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 558–560. Edited by N. R. Krieg & J. G. Holt. Baltimore & London: Williams & Wilkins.
- KILIAN, M. & FREDERIKSEN, W. (1981). Ecology of *Haemophilus*, *Pasteurella* and *Actinobacillus*. In *Haemophilus, Pasteurella and Actinobacillus*, pp. 11–38. Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.
- KILIAN, M. & SCHIÖTT, C. R. (1975). Haemophili and related bacteria in the human oral cavity. *Archives of Oral Biology* **20**, 791–796.
- KING, E. O. & TATUM, H. W. (1962). *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *Journal of Infectious Diseases* **111**, 85–94.
- KRAUT, M. S., ATTEBERY, H. R., FINEGOLD, S. M. & SUTTER, V. L. (1972). Detection of *Haemophilus aphrophilus* in the human oral flora with selective medium. *Journal of Infectious Diseases* **126**, 189–192.
- MUSSEY, J. M., HEWLETT, E. L., PEPLER, M. S. & SELANDER, R. K. (1986). Genetic diversity and relationships in populations of *Bordetella* spp. *Journal of Bacteriology* **166**, 230–237.
- OCHMAN, H., WHITTAM, T. S., CAUGANT, D. A. & SELANDER, R. K. (1983). Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. *Journal of General Microbiology* **129**, 2715–2726.
- OLSEN, I., ROSSELAND, S. K., THORSRUD, A. K. & JELLUM, E. (1987). Differentiation between *Haemophilus paraphrophilus*, *H. aphrophilus*, *H. influenzae*, *Actinobacillus actinomycetemcomitans*, *Pasteurella multocida*, *P. haemolytica*, and *P. ureae* by high resolution two-dimensional protein electrophoresis. *Electrophoresis* **8**, 532–535.
- PASTER, B. J., DEWHIRST, F. E. & OLSEN, I. (1988). The phylogeny of *Actinobacillus*, *Haemophilus* and *Pasteurella* by its 16S rRNA sequencing. *Journal of Dental Research* **67**, special issue, abstract no. 2259, p. 395.
- POHL, S. (1981). DNA relatedness among members of *Haemophilus*, *Pasteurella* and *Actinobacillus*. In *Haemophilus, Pasteurella and Actinobacillus*, pp. 245–253. Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.
- POTTS, T. V. & BERRY, E. M. (1983). Deoxyribonucleic acid-deoxyribonucleic acid hybridization analysis of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *International Journal of Systematic Bacteriology* **33**, 765–771.
- POTTS, T. V., ZAMBON, J. J. & GENCO, R. J. (1985). Reassignment of *Actinobacillus actinomycetemcomitans* to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov. *International Journal of Systematic Bacteriology* **35**, 337–341.
- POTTS, T. V., MITRA, T., O'KEEFE, T., ZAMBON, J. J. & GENCO, R. J. (1986). Relationships among isolates of oral haemophili as determined by DNA–DNA hybridization. *Archives of Microbiology* **145**, 136–141.
- SELANDER, R. K., MCKINNEY, R. M., WHITTAM, T. S., BIBB, W. F., BRENNER, D. J., NOLTE, F. S. & PATTISON, P. E. (1985). Genetic structure of populations of *Legionella pneumophila*. *Journal of Bacteriology* **163**, 1021–1037.
- SELANDER, R. K., CAUGANT, D. A., OCHMAN, H., MUSSEY, J. M., GILMOUR, M. N. & WHITTAM, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Applied and Environmental Microbiology* **51**, 873–884.
- SELANDER, R. K., MUSSEY, J. M., CAUGANT, D. A., GILMOUR, M. N. & WHITTAM, T. S. (1987). Population genetics of pathogenic bacteria. *Microbial Pathogenesis* **3**, 1–7.
- SLOTS, J., REYNOLDS, H. S. & GENCO, R. J. (1980). *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infection and Immunity* **29**, 1013–1020.
- SNEATH, P. H. A. & SOKAL, R. R. (1973). *Numerical Taxonomy*. San Francisco: Freeman.
- SUTTER, V. L. & FINEGOLD, S. M. (1970). *Haemophilus aphrophilus* infections: clinical and bacteriological studies. *Annals of the New York Academy of Sciences* **174**, 468–487.
- TANNER, A. C. R., HAFFER, C., BRATTHALL, G. T., VISCONTI, R. A. & SOCRANSKY, S. S. (1979). A study of the bacteria associated with advancing periodontitis in man. *Journal of Clinical Periodontology* **6**, 278–307.
- TANNER, A. C. R., VISCONTI, R. A., SOCRANSKY, S. S. & HOLT, S. C. (1982). Classification and identification of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by cluster analysis and deoxyribonucleic acid hybridizations. *Journal of Periodontal Research* **17**, 585–596.
- TEMPRO, P. J. & SLOTS, J. (1986). Selective medium for the isolation of *Haemophilus aphrophilus* from the human periodontium and other oral sites and the low proportion of the organism in the oral flora. *Journal of Clinical Microbiology* **23**, 777–782.