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DIFFERENTIATION BETWEEN MAJOR SPECIES OF THE *ACTINOBACILLUS—HAEMOPHILUS—PASTEURELLA* GROUP BY GAS CHROMATOGRAPHY OF TRIFLUOROACETIC ACID ANHYDRIDE DERIVATIVES FROM WHOLE-CELL METHANOLYSATES

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SUMMARY

A method based on whole-cell methanolysis and trifluoroacetic acid anhydride derivatization was developed for routine laboratory differentiation between isolates from the *Actinobacillus—Haemophilus—Pasteurella* group. All species, except *Haemophilus aphrophilus*, contained D-glycero-D-mannoheptose, although in varying concentrations. The distribution of this sugar could be used to distinguish *H. aphrophilus* from *Actinobacillus actinomycetemcomitans*, *H. paraphrophilus*, *H influenzae* type b, *Pasteurella haemolytica*, *P multocida* and *P ureae*, and also *H. influenzae* type b from *Pasteurellae* The pattern of major sugars in *P ureae* and *P. haemolytica* resembled that of *A. actinomycetemcomitans*. Major fatty acids of the whole-cell methanolysates provided no basis of interspecies differentiation.

INTRODUCTION

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* have been isolated from plaque taken from healthy gingiva and also from clinical lesions designated as gingivitis and periodontitis [1—8]. While there seems to be an association between *A. actinomycetemcomitans* and periodontal destruction

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in juveniles, the role of *H. aphrophilus* in periodontal disease has not yet been clarified. *A. actinomycetemcomitans* has also been involved in extraoral diseases such as bacterial endocarditis [9, 10], post-extraction abscess [11, 12], brain abscess [13], urinary tract infection [14] and vertebral osteomyelitis [15]. *H. aphrophilus* has been considered an etiologic agent in endocarditis [9, 10], brain abscess, meningitis, sinusitis, pneumonia, empyema, otitis media, wound and post-operative infection, arthritis and osteomyelitis [16].

H. paraphrophilus is found as a member of the normal flora of the oral cavity and pharynx [17]. It may cause subacute endocarditis, paronychia and brain abscess, and has been isolated from osteomyelitis of the jaw, inflamed appendix, urine and vagina [18].

H. influenzae type b is a leading cause of bacterial meningitis, but may also cause cellulitis, pneumonia, epiglottidis, septic arthritis and bacteriaemia, particularly in children younger than 5 years (for a review, see ref. 19).

Pasteurellae are parasitic on the mucous membranes of the upper respiratory and digestive tracts of mammals (rarely man) and birds [20]. There is an increased frequency of isolation of *Pasteurellae* and related organisms in the clinic [21], and human pasteurellosis is more common than previously realized [22]. Thus, *P. multocida* has recently been shown to cause a variety of infections in human beings, such as meningitis, encephalitis, otitis, septicaemia, sinusitis, peritonitis, bronchiectasis, and articular and skeletal infections [23–25]. *P. ureae* has been associated with human bronchiectasis, bronchitis, pneumonia, meningitis, septicaemia, sinusitis and ozaena [23], and *P. haemolytica* with a few infections in man, such as lacerations of hands, endocarditis, bacteriaemia and microabscesses of the brain (for review, see ref. 22).

Determination of a possible association between a specific bacterial agent and a clinical disease cannot be made adequately without proper diagnosis. Kilian [26], in an extensive study on the taxonomy of *Haemophilus*, concluded that the relationship between *A. actinomycetemcomitans* and *H. aphrophilus* is unclear and needs further examination. Even if *A. actinomycetemcomitans* and *H. aphrophilus*, as a result of recent research, now seem to have become established as distinct species [27], relatively few biochemical tests are available for differentiation between these closely related bacteria in the routine laboratory [28]. To avoid confusion, establishment of more differentiating tests would be helpful. Recently, we have suggested additional criteria for chemotaxonomic distinction between *A. actinomycetemcomitans* and *H. aphrophilus*, such as free fatty acids in whole cells [29–31], sugars in lipopolysaccharide (LPS), sugars in whole defatted cells, sugars in whole-cell methanolysates [32–35], cellular proteins [36, 37] and bacteriolysis by means of EDTA and lysozyme [38].

H. aphrophilus and *H. paraphrophilus* are genetically closely related [39] and otherwise have very few differential characters [40]. Sneath and Johnson [41] reported that *H. aphrophilus* and *H. paraphrophilus* strains formed a single cluster in their taxonomic studies on *Actinobacillus*, *Haemophilus* and *Pasteurella* strains, and Tanner et al. [42] found no clear separation of *H. aphrophilus* and *H. paraphrophilus* by cluster analysis of phenotypic features and DNA/DNA homology.

Differentiation between *Pasteurella* and *Actinobacillus* sometimes creates problems, and it has been claimed that the current classification of recognized actinobacilli and pasteurellas does not allow differentiation of the two genera [43].

The main purpose of this study was to examine the relationship between major species of the family *Pasteurellaceae*, which includes *Actinobacillus*, *Haemophilus* and *Pasteurella* [40], using predominant sugars and fatty acids in whole cells as test parameters. Sugar fingerprints of bacteria are susceptible to environmental factors such as carbohydrate of a growth medium, and a number

TABLE I

BACTERIA EXAMINED

Organism	Strain	Source	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)*	ATCC** (NCTC)***	Lung abscess
	29524	ATCC	Chest aspirate
	29523	ATCC	Blood
	29522	ATCC	Mandibular abscess
	2112	FDC§	Periodontitis
	2097	FDC	Periodontitis
	2043	FDC	Periodontitis
	511	FDC	Periodontitis
	HK435	Kilian	Abscess
	N27	FDC	Periodontosis
	Y4	FDC	Periodontosis
Haemophilus aphrophilus	33389 (5906)*	ATCC (NCTC)	Endocarditis
	19415 (5886)*	ATCC (NCTC)	Endocarditis
	13252	ATCC	
	655	FDC	Periodontitis
	654	FDC	Periodontitis
	626	FDC	Periodontitis
	621	FDC	Periodontitis
Haemophilus paraphrophilus	29242 (10558)	ATCC (NCTC)	Trachea
	29241 (10557)*	ATCC (NCTC)	Paronychia
	29240 (10556)	ATCC (NCTC)	Parietal abscess
Haemophilus influenzae type b	33533	ATCC	Blood
	31441	ATCC	Clinical isolate
	B51	Omland (NDML)§§	
Pasteurella ureae	10219*	NCTC	Ozaena
Pasteurella haemolytica	9380*	NCTC	
Pasteurella multocida	10322*	NCTC	

*Type strain of species.
**American Type Culture Collection (Rockville, MD, U.S.A).
***National Collection of Type Cultures (London, U.K.).
§ Forsyth Dental Center (Boston, MA, U.S.A.).
§§ Norwegian Defence Microbial Laboratory (Oslo, Norway).

of factors may affect microbial lipid composition (for a review, see ref. 44). Therefore, only major sugars and fatty acids were used for distinction of bacteria. In addition to the increased clinical significance of *Pasteurellaceae*, there is renewed interest in the classification of species within this family [43, 45–48]. Another purpose of this study was to introduce whole-cell methanolysis and simplified trifluoroacetic acid anhydride derivatization as a taxonomic method for the routine clinical laboratory.

EXPERIMENTAL

Bacteria

The strains of *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, *H. influenzae* type b, *P. ureae*, *P. haemolytica* and *P. multocida* tested are listed in Table I. Strain HK 435 was obtained from Professor M. Kilian, Aarhus, Denmark, and strain B 51 from Dr. T. Omland, Oslo, Norway (Dr. Omland had previously received this strain from Dr. H.C. Engbæk, Copenhagen, Denmark). The other strains were delivered from the American Type Culture Collection, the National Collection of Type Cultures, U.K., or from Forsyth Dental Center, Boston, MA, U.S.A. Stock cultures were kept in liquid nitrogen after reconstitution from the lyophilized state. The organisms were maintained anaerobically (80% N_2–10% H_2–10% CO_2) on blood or chocolate agar plates at 35°C, and transferred weekly. For chemotaxonomic analyses the organisms were cultivated in Brain Heart Infusion (Difco Labs., Detroit, MN, U.S.A.) broth in air plus 10% CO_2 for five days at 37°C, harvested by centrifugation, washed three times in deionized, distilled water and lyophilized over diphosphorus pentoxide (Merck, Darmstadt, F.R.G.). For *H. paraphrophilus* and *H. influenzae* type b the broth was supplemented with filter sterilized NAD (1 mg/ml) and haemin (5 mg/ml). All cultivations were made in duplicate on different days.

Methanolysis and derivatization

Whole lyophilized cells were methanolysed with 2 *M* hydrochloric acid in anhydrous methanol for 24 h at 85°C [34]. The methanolysate was dried by a stream of nitrogen and derivatized in a mixture of acetonitrile and trifluoroacetic acid anhydride (33%, v/v) (Fluka, Buchs, Switzerland) at 90°C for 3 min. Pieces of capillary tubes were included for optimal mixing of the suspension.

Reference compounds

The reference compounds used have been detailed previously [34].

Gas chromatography

A Type 5040A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.), furnished with an electronic integrator, was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 (polydimethylsiloxane) glass capillary column used was 25 m × 0.22 mm I.D. with a film thickness of 0.14 μm and height equivalent to a theoretical plate 0.25 mm. Helium served as the carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa. The temperature of the injector and flame-ionization detector was 200°C. The

following temperature programme was used: 2 min at 90°C, then increased from 90 to 190°C at 4°C/min with the attenuator set at 4. The chart paper speed was 10 mm/min. The sample (0.2 μl) was delivered by splitless injection. The identities of the methanolyzed and derivatized sugars and fatty acids were confirmed by co-chromatography of authentic standards and determined tentatively during gas chromatography. We had previously established the identities of these derivatives by gas chromatography—mass spectrometry [30, 32].

Quantitation of sugars and acids

Duplicate bacterial cultures were methanolysed separately. Each methanolysate was divided into two parts and derivatized. From each derivative three injections were made into the gas chromatograph. Accordingly, a total of twelve injections was made from each bacterial strain. The amount of substance was calculated from the area under each peak and was corrected according to its relative molar response. For sugars this was given in ref. 32. The relative molar responses for fatty acids relative to glucose were as follows: $C_{14:0}$, 2.27; 3-OH-$C_{14:0}$, 2.27; $C_{16:0}$, 2.55; and $C_{16:1}$, 2.50.

RESULTS

The major sugars and fatty acids recovered from the whole-cell methanolysates are listed in Table II. The outstanding feature of this experiment was the consistent lack of D-glycero-D-mannoheptose in *H. aphrophilus*. All the other species of *Pasteurellaceae* contained this sugar. A typical gas chromatogram provided by these other species is shown in Fig. 1. The sugar and fatty acid profiles on gas chromatograms of *H. aphrophilus* corresponded to those previously published [34]. The amounts of D-glycero-D-mannoheptose were comparable in *A. actinomycetemcomitans* and *H. paraphrophilus*, but was lower in *H. influenzae*. In *Pasteurellae* the amount of D-glycero-D-mannoheptose was highly variable, *P. haemolytica* being the organism with the highest content and *P. multocida* that with the lowest. The abundance of L-glycero-D-mannoheptose was fairly even in *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*. *H. influenzae* type b and *Pasteurellae* displayed great variability in their content of this aldoheptose, *H. influenzae* type b, strain B 51 having the lowest content and *P. multocida* the highest.

The glucose content tended to be higher in *H. aphrophilus* than in *A. actinomycetemcomitans*, strain FDC 2097 of *A. actinomycetemcomitans* showing the lowest content. The glucose content of *H. paraphrophilus*, *H. influenzae* type b and *Pasteurellae* was variable, with the highest amount in strain ATCC 29241 of *H. paraphrophilus*.

The galactose content was also higher in *H. aphrophilus* than in *A. actinomycetemcomitans*. *H. paraphrophilus*, *H. influenzae* type b and *Pasteurellae* showed varying amounts of galactose, with the highest level in *H. paraphrophilus* strain ATCC 29240 and the lowest in *P. ureae*.

The abundance of $C_{14:0}$ was fairly even in *A. actinomycetemcomitans*, *H. aphrophilus* and *Pasteurellae*. It showed great variation in *H. paraphrophilus* and *H. influenzae* type b, *H. paraphrophilus* strain ATCC 29242 exhibiting the

TABLE II

PERCENTUAL DISTRIBUTION OF SUGARS AND FATTY ACIDS IN WHOLE-CELL METHANOLYSATES (S.D. ≤ 5%, $n = 12$)

Gal = galactose, Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose.

Organism	Gal	Glc	DD-Hep	LD-Hep	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$
Actinobacillus actinomycetemcomitans								
ATCC 33384	4.9	10.7	4.5	5.8	8.5	6.1	5.4	7.9
ATCC 29524	4.6	9.2	3.9	5.6	6.6	6.0	5.1	8.0
ATCC 29523	2.7	7.7	2.7	4.6	6.8	9.6	9.1	11.4
ATCC 29522	2.8	9.2	3.8	6.0	6.8	6.4	5.6	8.0
FDC 2112	2.9	11 2	4.0	6.3	7.4	8.6	5.9	10.4
FDC 2097	1.9	6.0	2.2	3.7	6.0	5 7	7.5	8.6
FDC 2043	1.5	7.0	2.9	4.8	6.5	6.5	7.3	7.1
FDC 511	3 9	8.3	3.6	4.8	7.8	11.3	10.4	10.4
HK435	3.6	9.0	3.6	5 0	10.0	12.2	10.0	13.6
FDC N27	2.8	7.2	2.7	4.4	11.1	11.1	11.7	13.9
FDC Y4	2.1	8.0	3.2	4.7	9.0	5.9	7 7	8.0
Haemophilus aphrophilus								
ATCC 33389	9.9	21.6	—	5.4	9.5	6.9	7.2	8.7
ATCC 19415	7.2	17.3	—	5.4	11.5	9.4	10.8	14.8
ATCC 13252	7.5	21.6	—	6.4	8.5	9.5	9.5	11.0
FDC 655	5.0	9.0	—	5.0	7.6	5.9	7.3	9.2
FDC 654	9.6	17.2	—	6.8	6 8	4.8	5.2	5.6
FDC 626	7.7	14.2	—	4.7	6.9	5.7	7.8	9.7
FDC 621	9.0	14.8	—	4.6	6.8	4.8	5.0	6.8
Haemophilus paraphrophilus								
ATCC 29242	7.7	10.7	3.0	5.0	21.0	11.7	5.3	20.0
ATCC 29241	13.3	34.2	2.4	3.0	7.1	5.9	5 0	7.4
ATCC 29240	15.0	15.5	2.6	4.0	10.0	8.0	4.0	12.5
Haemophilus influenzae type b								
ATCC 33533	4.2	12.3	0.3	2.0	8.7	3.4	3.1	4.5
ATCC 31441	6 7	14.5	0.6	6.1	17.8	7.8	6.7	8.9
B51	3 9	8.6	0.7	1.8	9.7	4.3	6.1	7.2
Pasteurella ureae								
NCTC 10219	1.1	9.6	1.2	3.8	6.6	3.3	3.0	2.6
Pasteurella haemolytica								
NCTC 9380	2 7	10.9	7.3	9.1	6.7	7.3	6 4	6.5
Pasteurella multocida								
NCTC 10322	5.8	18.4	tr*	13.4	6 3	9.5	5.8	10.0

*tr. = trace amount (< 0.1%).

highest amount. The content of 3-OH-$C_{14:0}$ acid varied within all the species examined. The highest level was found in *A. actinomycetemcomitans* strain HK 435 and, the lowest in *P. ureae*.

The concentration of $C_{16:1}$ was fairly constant in *H. paraphrophilus*, but

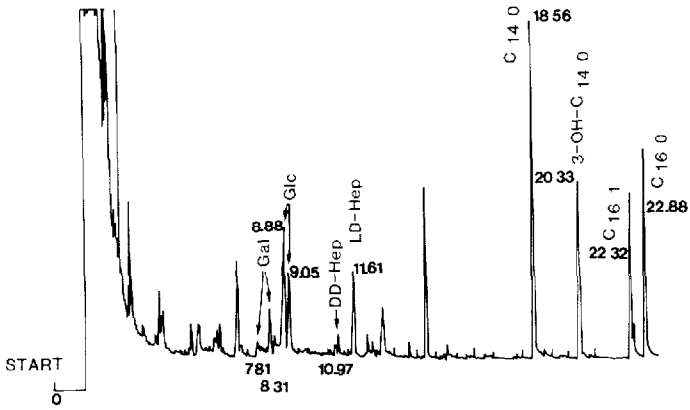

Fig. 1. Gas chromatogram of whole-cell methanolysates from *H. influenzae* type b, strain ATCC 33533. Abbreviations: Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-manno-heptose; LD-Hep = L-glycero-D-mannoheptose; $C_{14:0}$ = myristic acid; 3-OH-$C_{14.0}$ = β-hydroxy-myristic acid, $C_{16:1}$ = palmitoleic acid; $C_{16:0}$ = palmitic acid. Figures are retention times (min) of major sugars and fatty acids.

varied among the other species. Again, the largest amount was detected in *A. actinomycetemcomitans* (strain FDC N 27) and the lowest in *P. ureae.*

The concentration of $C_{16\cdot0}$ varied considerably among the species examined. The highest amount was found in *H. paraphrophilus* strain ATCC 29242 and the lowest in *P. ureae.*

DISCUSSION

Fastidious Gram-negative, facultative anaerobic rods such as those studied here are now attracting increasing interest in microbiology. Not only are they isolated more frequently than in the past, they are also recovered from sources hitherto regarded as unusual [48]. The disease spectrum of these organisms is wide, but we shall particularly emphasize endocarditis, where the share of Gram-negative bacteria involved has risen from 1—3% in the past to 5–10% on natural and 17% on prosthetic valves [49]. Bacteria belonging to the fastidious group account for 57% of the Gram-negative agents of endocarditis, among which *H. aphrophilus*, other *Haemophilus* species and *A. actinomycetemcomitans* are most prominent. One of the major habitats of these organisms is the oral cavity. It has been shown that *A. actinomycetemcomitans* is able to invade gingival connective tissue [50, 51]. In such cases the oral cavity may represent a source of infectious spread to other parts of the organism.

The increased frequency of isolation of fastidious Gram-negative, facultative anaerobic rods has created a demand for accurate identification and classification in the routine laboratory. Unfortunately, differentiation between these bacteria often poses problems, and several so far unnamed taxa exist among them. In this study such differentiation has been made easier through the demonstration of a consistent lack of D-glycero-D-mannoheptose in *H. aphrophilus.* All the other examined species of *Pasteurellaceae* contained this aldoheptose. LPS [32], whole defatted cells [33] and whole-cell

methanolysates [34] have previously indicated that this sugar can be used as a taxonomic marker for differentiation between the closely related *A. actinomycetemcomitans* and *H. aphrophilus*. The present study suggested that D-glycero-D-mannoheptose also can be used to distinguish *H. aphrophilus* from other species within *Pasteurellaceae*, such as *H. paraphrophilus*, *H. influenzae* type b, *P. ureae*, *P. haemolytica* and *P. multocida*. Mannheim [40] recently claimed that the genera *Actinobacillus*, *Haemophilus* and *Pasteurella* are heterogeneous and therefore should be redetermined in terms of genetic relatedness. One of the candidates for separate genera is the *H. aphrophilus—H. paraphrophilus—H. paraphrohaemolyticus* group. The present results suggested that *H. aphrophilus* could be a candidate for a separate genus.

D-Glycero-D-mannoheptose in *A. actinomycetemcomitans* has previusly been suggested to be located to the LPS of the cell [33]. In *Salmonella*, D-glycero-D-mannoheptose is the precursor of L-glycero-D-mannoheptose (for a review, see ref. 52). If the synthesis of epimerase is blocked because of a mutational defect, the precursor D-heptose accumulates and is partly incorporated into LPS. However, this LPS cannot serve as the acceptor for the sugar due to be incorporated next. Thus, the core remains incomplete. It is not known whether the pathway of LPS biosynthesis in the present organisms with D-glycero-D-mannoheptose follows the same patterns as in *Enterobacteriaceae*, but it should be investigated. Interestingly, *Pasteurellaceae* has been described as a group of organisms that have lost much of their genom information during phylogenetic adaptation to parasitic life [53].

Previous studies based on cluster analyses of phenotypic features and DNA/DNA homology [41, 42] have questioned the distinction between *H. aphrophilus* and *H. paraphrophilus*. Kilian [26] was able to distinguish between these species, even if the number of differentiating biochemical characters was limited. The present chemotaxonomic study suggested that distinction between *H. aphrophilus* and *H. paraphrophilus* can easily be performed through D-glycero-D-mannoheptose. Using the sugar composition as a basis, it can be suggested that *H. paraphrophilus* is more related to *A. actinomycetemcomitans* than to *H. aphrophilus*.

Whereas *P. multocida* showed only trace amounts of D-glycero-D-mannoheptose, *P. ureae* and particularly *P. haemolytica* contained significant amounts of this taxonomic marker. LPS prepared from various *P. multocida* strains either contained [54, 55] or lacked [56] D-glycero-D-mannoheptose. *P. multocida* also differed from the other species of *Pasteurellaceae* through its high content of L-glycero-D-mannoheptose, which is more universally distributed in bacteria than D-glycero-D-mannoheptose [57]. Further, *P. ureae* and *P. haemolytica* were low in galactose. This was also found in *A. actinomycetemcomitans*. Differentiation between *Pasteurella* and *A. actinomycetemcomitans* is often problematic. Sneath and Johnson [41] found a similarity index of 75% between *Actinobacillus* and *Pasteurella* in one of their clusters and held this as strong evidence against separation of the two as distinct genera. It has been suggested that *P. ureae* and *P. haemolytica* biovar A should be included in the genus *Actinobacillus* [58]. The distribution of major sugars in these organisms supported such a rearrangement.

H. influenzae may be confused with *P. multocida* [59]. In the present study,

H. influenzae type b could be distinguished from *P. multocida* through its higher content of D-glycero-D-mannoheptose. *H. influenzae* type b also differed from *P. haemolytica* and *P. ureae*, here through a lower content of D-glycero-D-mannoheptose than in these organisms. LPS of *H. influenzae* type b showed only trace amounts of D-glycero-D-mannoheptose [60].

The major fatty acids of all the examined species included $C_{14\ 0}$, 3-OH-$C_{14:0}$, $C_{16:1}$ and $C_{16\ 0}$, which was in agreement with our previous whole-cell methanolysis study on *A. actinomycetemcomitans* and *H. aphrophilus* [34]. Only quantitative differences existed in the fatty acid content of the currently examined bacteria. These differences did not allow interspecies differentiation. Jantzen et al. [61] found that *P. multocida* generally could be distinguished from *Haemophilus* by a higher level of C_{18} acids in the former. However, the differences were rather marginal. *A. actinomycetemcomitans* could not be distinguished from *H. aphrophilus* or *H. influenzae* type b.

Strain ATCC 29242 of *H. paraphrophilus* differed from the other reference strains of this species through a high content of cellular fatty acids. Also, Tanner et al. [42] found that this strain varied from other reference strains by not showing significant DNA homology with *A. actinomycetemcomitans*, *H. aphrophilus* or *H. paraphrophilus* isolates, and by not belonging to major clusters when phenetic features were analysed.

Methanolysis and trifluoroacetylation are well established procedures in bacteriology (for a discussion, see ref. 33). We have previously used these methods for differentiation between the type specific strains of *A. actinomycetemcomitans* and *H. aphrophilus* [34]. In the present study, our previous procedures of derivatization and instrumentation were simplified to meet the facilities of the routine clinical laboratory. Reproducible results were achieved, and the recovery of major sugars and fatty acids (97—102%) was comparable to that of our original procedure [34]. The derivatives were stable during the first 24 h at 4°C. Storage of derivatives for one week at 4°C and derivatization temperatures higher than 90°C tended to deteriorate lipids, particularly $C_{16\ 1}$ and 3-OH-$C_{14:0}$ acid. In our previous study with whole-cell methanolysates minor sugars and fatty acids were also identified and quantitated [34]. Such components were also assessed in this work. However, they provided no help with regard to differentiation of the organisms and have been excluded for the sake of simplification.

The present differentiation between the closely related *A. actinomycetemcomitans* and *H. aphrophilus* was supported by recent studies in our laboratories with EDTA and lysozyme [38]. With these bacteriolytic agents it was possible to divide *A. actinomycetemcomitans* into two groups of strains. The group I strains were the organisms most rapidly lysed by EDTA of the *Actinobacillus—Haemophilus—Pasteurella* group. *H. paraphrophilus* was least sensitive. *P. ureae* and the group II strains of *A. actinomycetemcomitans* were most sensitive to lysozyme, the group I strains of *A. actinomycetemcomitans*, *H. paraphrophilus* and *P. multocida* least sensitive.

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REFERENCES

1 M. Kilian and C.R. Schiött, Arch. Oral Biol., 20 (1975) 791.
2 M.S. Kraut, H.R. Attebery, S.M. Finegold and V.L. Sutter, J. Infect. Dis., 126 (1972) 189.
3 R.L. Mandell and S.S. Socransky, J. Periodontol., 52 (1981) 593.
4 M.G. Newman and S.S. Socransky, J. Periodont. Res., 12 (1977) 120.
5 J. Slots, J. Clin. Periodontol., 6 (1979) 351.
6 J. Slots, H.S. Reynolds and R.J. Genco, Infect. Immun., 29 (1980) 1013.
7 A.C.R. Tanner, C. Haffer, G.T. Bratthall, R.A. Visconti and S.S. Socransky, J. Clin. Periodontol., 6 (1979) 278.
8 J.J. Zambon, L.A. Christersson and J. Slots, J. Periodontol., 54 (1983) 707.
9 J.J. Ellner, M.S. Rosenthal, P.I. Lerner and M.C. McHenry, Medicine, 58 (1979) 145.
10 M.I. Page and E.O. King, N. Engl. J. Med., 275 (1966) 181.
11 M.M. Peel, A.M. Rich and P.C. Reade, Oral Surg., 52 (1981) 591.
12 T. Thjötta and S. Sydnes, Acta Pathol. Microbiol. Scand., 28 (1951) 27.
13 B.F. Martin, B.M. Derby, G.N. Budzilovich and J. Ransohoff, Neurology, 17 (1967) 833.
14 T.R. Townsend and J.Y. Gillenwater, J. Amer. Med. Assoc., 210 (1969) 558.
15 I. Muhle, J. Rau and J. Ruskin, J. Amer. Med. Assoc., 241 (1979) 1824.
16 V.L. Sutter and S.M. Finegold, Ann. N.Y. Acad. Sci., 174 (1970) 468.
17 M. Kilian and W. Frederiksen, in M. Kilian, W. Frederiksen and E.L. Biberstein (Editors), *Haemophilus, Pasteurella* and *Actinobacillus*, Academic Press, London, 1981, pp. 11–38.
18 M. Kilian and E.L. Biberstein, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 558–569.
19 J.D. Band, D.W. Fraser and G. Ajello, J. Amer. Med. Assoc., 251 (1984) 2381.
20 G.R. Carter, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 552–557.
21 R. Sakazaki, E. Yoshizaki, K. Tamura and S. Kuramochi, Eur. J. Clin. Microbiol., 3 (1984) 244.
22 J.K. Miller, N.Y. State J. Med., 66 (1966) 2527.
23 G.R. Carter, in M.P. Starr, H. Stolp, H.G. Trüper, A. Balows and H.G. Schlegel (Editors), The Prokaryotes. A Handbook on Habitats, Isolation and Identification of Bacteria, Springer-Verlag, Berlin, 1981, pp. 1383–1391.
24 R. Ewing, V. Fainstein, D.M. Musher, M. Lidsky and J. Clarridge, South. Med. J., 73 (1980) 1349.
25 M.S. Arons, L. Fernando and I.M. Polayes, J. Hand Surg., 7 (1982) 47.
26 M. Kilian, J. Gen. Microbiol., 93 (1976) 9.
27 J.E. Phillips, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 570–576.
28 J. Slots, Arch. Microbiol., 131 (1982) 60.
29 I. Brondz and I. Olsen, J. Chromatogr , 278 (1983) 13.
30 I. Brondz, I. Olsen and T. Greibrokk, J. Chromatogr., 274 (1983) 299.
31 I. Olsen and I. Brondz, Abstr. 16th FEBS Meet., 1984, XXII-082, p. 453.
32 I. Brondz and I. Olsen, J. Chromatogr., 310 (1984) 261.
33 I. Brondz and I. Olsen, J. Chromatogr., 311 (1984) 31.

34 I. Brondz and I. Olsen, J. Chromatogr., 311 (1984) 347.
35 I. Brondz and I. Olsen, Abstr. 16th FEBS Meet. (1984), XXII-071.
36 E. Jellum, V. Tingelstad and I. Olsen, Int. J. System. Bacteriol., 34 (1984) 478.
37 I. Olsen, V. Tingelstad and E. Jellum, Abstr. Annu. Meet. Scand. Assoc. Dent. Res., 1984, 95, p. 54.
38 I. Olsen and I. Brondz, J. Clin. Microbiol., (1985) submitted for publication.
39 S. Pohl, in Reklassifizierung der Gattung *Actinobacillus* Brumpt 1910, *Haemophilus* Winslow et el. 1971 und *Pasteurella* Trevisan 1887 anhand phänotypischer und molekular Daten, insbesondere der DNS-Verwandtschaften bei DNS:DNS-Hybridisierung in vitro und Vorschlag einer neuen Familie, *Pasteurellaceae*, Inaug.-Diss. Phillips-Universität Marburg/Lahn, Mauersberger, Marburg, 1979.
40 W. Mannheim, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 550—552.
41 P.H.A. Sneath and R. Johnson, Int. J. System. Bacteriol., 23 (1973) 405.
42 A.C.R. Tanner, R.A. Visconti, S.S. Socransky and S.C. Holt, J. Periodont. Res., 17 (1982) 585.
43 R. Mutters, K. Piechulla and W. Mannheim, Eur. J. Clin. Microbiol., 3 (1984) 225.
44 D.B. Drucker (Editor), Microbiological Applications of Gas Chromatography, Cambridge University Press, Cambridge, London, 1981, pp. 251 and 285.
45 M. Kilian, W. Frederiksen and E.L. Biberstein (Editors), *Haemophilus*, *Pasteurella* and *Actinobacillus*, Academic Press, London, 1981.
46 H. Leclerc (Editor), Les Bacilles â Gram Négatif d'Interet Médical et en Santé Publique: Taxonomie—Identification—Applications, INSERM, Paris, 1983.
47 E. Bottone (Editor), Unusual Microorganisms: Gram-negative Fastidious Species, Marcel Dekker, New York, 1983.
48 A. von Graevenitz, Eur. J. Clin. Microbiol., 3 (1984) 223.
49 J.E. Geraci and W.R. Wilson, Mayo Clin. Proc., 57 (1982) 145.
50 F.R. Saglie, F.A. Carranza, Jr., M.G. Newman, L. Cheng and K.J. Levin, J. Periodont. Res., 17 (1982) 452.
51 L.A. Christersson, B. Albini, J.J. Zambon, J. Slots and R.J. Genco, AADR Progr. Abstr., 62 (1983) 255.
52 C. Galanos, O. Lüderitz, E.T. Rietschel and O. Westphal, in T.W. Goodwin (Editor), Biochemistry of Lipids II, Vol. 14, University Park Press, Baltimore, 1977, p. 256.
53 A. Lwoff, in L'Évolution Physiologique. Étude des Pertes de Fonctions chez les Microorganismes, Actualitets Scientifiques et Industrielles, Herman, Paris, 1944.
54 W. Erler, H. Feist, K.D. Flossmann and B. Jacob, Arch. Exp. Veterinaermed., 31 (1977) 139.
55 W. Erler, H. Feist, K.D. Flossman, B. Jacob and J. Schlegel, Z. Allg. Mikrobiol., 21 (1981) 191.
56 W. Erler, H. Feist, K.D. Flossman, B. Jacob and P. Pilarski, Z. Allg. Mikrobiol., 21 (1981) 507.
57 G.A. Adams, C. Quadling and M.B. Perry, Can. J. Microbiol., 13 (1967) 1605.
58 S. Pohl, in M. Kilian, W. Frederiksen and E.L. Biberstein (Editors), *Haemophilus*, *Pasteurella* and *Actinobacillus*, Academic Press, London, 1981, pp. 246—253.
59 G.L. Lucas and D.H. Bartlett, Plast. Reconstr. Surg., 67 (1981) 49
60 J.L. Brice, T.G. Tornabene and F.M. LaForce, J. Infect. Dis., 140 (1979) 443.
61 E. Jantzen, K. Bryn, N. Hagen, T. Bergan and K. Bøvre, NIPH Ann., 1 (1978) 59.