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DETERMINATION OF ACIDS IN WHOLE LIPOPOLYSACCHARIDE AND IN FREE LIPID A FROM *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

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SUMMARY

Acids from whole lipopolysaccharide and free lipid A of the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* were determined by gas chromatography and gas chromatography—mass spectrometry. In whole lipopolysaccharide, 3-hydroxymyristic acid was most abundant, followed by myristic and 3-deoxy-D-manno-2-octulosonic acid. In the lipid A moiety, myristic acid dominated, followed by 3-hydroxymyristic acid. The acid composition of whole lipopolysaccharide and free lipid A from *A. actinomycetemcomitans* and *H. aphrophilus* was not so specific as to allow taxonomic differentiation between these bacteria. If fatty acids of lipopolysaccharide are essential for expression of endotoxicity, the present results suggested no marked difference in the endotoxic activities of *A. actinomycetemcomitans* and *H. aphrophilus*.

INTRODUCTION

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are morphologically very similar Gram-negative rods [1] indigenous to dental plaque [2]. Taxonomic differentiation between these bacterial species is difficult and usually based only on a few physiological characters [3]. Both *A. actinomycetemcomitans* and *H. aphrophilus* have been associated with endocarditis, dental plaque often being the source of extraoral infections [4].

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A. actinomycetemcomitans has recently attracted much attention due to its suspected role as a major pathogen in juvenile periodontitis (periodontosis) [5]. *H. aphrophilus* has not been implicated to the same extent, haemophili traditionally being considered to have a low pathogenic potential in the periodontal pocket [6].

Lipopolysaccharide (LPS) located in the outer membrane of Gram-negative bacteria is considered an essential factor in chronic inflammatory periodontal disease where it may elicit damage by its ability to stimulate macrophages, activate complement, act as a thymus-independent antigen and B lymphocyte mitogen, stimulate bone resorption, and by its cytotoxicity (see ref. 7 for review). Chemically, LPS is made up of a polysaccharide portion, the O-antigenicity determining chains and the core, and a covalently bound lipid, lipid A [8]. The serological properties of LPS are related to the O-specific chains, the endotoxic activity to lipid A. The latter, which is a highly biologically active molecule with a great variety of endotoxic activities [9], also promotes the functional and structural integrity of the bacterial membrane. Knowledge on what specific components of the LPS molecule are responsible for the endotoxic activity is sparse. What is known though, is that chemical [10] or enzymatic [11] removal of fatty acids results in detoxification of endotoxins. Therefore, the presence of fatty acids seems to be essential for the expression of endotoxicity. If *A. actinomycetemcomitans* and *H. aphrophilus* really have a different periopathogenic potential, this might be reflected in the fatty acid composition of their endotoxins. In the present study, the nature and quantity of acids, mainly fatty acids, present in the entire LPS molecule or in free lipid A of *A. actinomycetemcomitans* and *H. aphrophilus* isolated from periodontitis, were compared by means of gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS). Analysis of the fatty acid composition of lipid A may provide important information as to the taxonomic position of a bacterial strain [12, 13]. It was therefore hoped that differences, if present, would help to distinguish between *A. actinomycetemcomitans* and *H. aphrophilus*.

Fatty acids of the LPS macromolecule are generally removed by acidic or alkaline hydrolysis and then derivatized before GC analysis. In this report, hydrolysis and derivatization were performed simultaneously by transesterification with hydrochloric acid in anhydrous methanol. This method also enabled direct GC determination of 3-deoxy-D-manno-2-octulosonic acid (KDO), to which lipid A is covalently linked.

MATERIAL AND METHOD

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated and the sources from which they were obtained, are shown in Table I. Strains ATCC 33389, 33384, and 19415 were obtained directly from the American Type Culture Collection, strain HK 435 from M. Kilian, Aarhus, Denmark, and the remaining strains through Forsyth Dental Center. The organisms were maintained and mass cultivated as described previously [14].

TABLE I

LIST OF BACTERIA INVESTIGATED

Organism	Strain	Source*	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)	ATCC (NCTC)	Lung abscess
	29524	ATCC	Chest aspirate
	29522	ATCC	Mandibular abscess
	511	FDC	Periodontitis
	HK435	Kilian	Pus
	N27	FDC	Periodontosis
	Y4	FDC	Periodontosis
Haemophilus aphrophilus	33389 (5906)	ATCC (NCTC)	Endocarditis
	19415 (5886)	ATCC (NCTC)	Endocarditis
	655	FDC	Periodontitis
	654	FDC	Periodontitis
	626	FDC	Periodontitis
	621	FDC	Periodontitis

*ATCC = American Type Culture Collection (Rockville, MD, U.S.A.); NCTC = National Collection of Type Cultures (London, U.K.); FDC = Forsyth Dental Center (Boston, MA, U.S.A.).

Preparation of lipopolysaccharide

LPSs from *A. actinomycetemcomitans/H. aphrophilus* strain pairs were isolated by the phenol—water procedure [15]. Lyophilized bacterial cells (1 g) were suspended in 35 ml of deionized distilled water preheated to 68°C. The suspension was mixed vigorously for 20 min at 68°C with 35 ml of preheated 90% phenol (E. Merck, Darmstadt, F.R.G.). After cooling (—20°C, 1 h), the phases were separated by centrifugation (900 *g*, 20 min, 4°C). The aqueous phase was pipetted off and the phenol portion extracted with 30 ml of prewarmed deionized distilled water for another 20 min at 68°C. The combined aqueous phases were dialysed against tap water (24 h) and several changes of deionized distilled water at 4°C. They were concentrated to 6—8 ml and precipitated with 10 vols. of absolute ethanol in the presence of 2—3 drops of saturated sodium acetate. After cooling (—20°C, 1 h), the suspension was centrifuged (900 *g*, 20 min, 4°C) and the precipitate dried. It was then dissolved in deionized distilled water under sonication in the presence of ice and centrifuged (12,100 *g*, 15 min, 4°C). The supernatant was purified further by ultracentrifugation (100,000 *g*, 1 h, 4°C) twice. These procedures virtually eliminated the absorbance top at 260 nm. The resulting pellet was dissolved in deionized distilled water by sonication and lyophilized.

Preparation of free lipid A

Lipid A was prepared from *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389 by treating LPS with 1% glacial acetic acid (Merck) at 100°C for 90 min [16]. Crude lipid A was first homogenized in an agate mortar and then sonicated slowly for 1—2 min under ice cooling in the presence of 3 ml of 1% glacial acetic acid. The suspension was heated to 70°C

for 25 min in a PTFE-sealed tube with screw cap, cooled to room temperature and centrifuged (900 *g*, 10 min, 4° C). The pellet was washed with warm (50° C) glacial acetic acid and then with warm (50° C) deionized distilled water, and lyophilized.

Methanolysis

Fatty acids were liberated from samples of whole LPS (0.5—1.5 mg) and lipid A (0.2—0.3 mg) by methanolysis (2 *M* hydrochloric acid in anhydrous methanol, 2 ml, 24 h, 85° C). After cooling, the methanolysate was concentrated, while kept on ice, to 0.1—0.2 ml by a stream of nitrogen. Chloroform (Fluka, Buchs, Switzerland) (2 ml) was added and the mixture transferred to a 20-ml separatory funnel, followed by two 1-ml batches of chloroform used to wash the methanolysis tube. Distilled water (4 ml) was added to the organic phase and the mixture shaken carefully. After separation of the organic phase from the water phase, the former was washed twice, each time with 4 ml of distilled water, and the water phase twice, with 4-ml batches of chloroform. The organic phases, like the water phases, were pooled. The water phases were lyophilized and their sugar content will be published later. Anhydrous magnesium sulphate (Merck) (400 mg) was added to the organic phases, which after 30 min were filtered through a paper filter. Filter and storage flasks for the organic phases were washed three times, each time with 3 ml of chloroform. The chloroform phase, kept on ice, was dried with nitrogen. It was dissolved in *n*-hexane (Merck) previously dried by filtration through alkaline aluminium oxide grade 1 (Woelm Pharmacia, F.R.G.) and analysed by GC and GC–MS.

Reference compounds

LPSs, prepared by the phenolic extraction procedure, from *Escherichia coli* serotype 055:B5, *Salmonella typhimurium* and *Serratia marcescens* were obtained from Sigma, St. Louis, MO, U.S.A. The methyl ester of KDO was provided by incubating KDO (Sigma) in a PTFE-sealed tube with screw cap in the presence of 2 *M* hydrochloric acid in anhydrous methanol at 85° C for 24 h. Methyl esters of lauric, tridecanoic, myristic, 2-hydroxymyristic, pentadecanoic, and palmitic acid were purchased from Supelco, Bellefonte, PA, U.S.A. 3-Hydroxymyristic acid was synthesised as described previously [17], and N-glucosaminemyristate was a gift from O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G. We obtained *n*-triacontane from Fluka, and *n*-triacontanol from Fluka and from *H. perforatum* L. [18].

Recovery of acids from LPS

To determine the recovery of acids from LPS, three separate methanolysis experiments were performed, using N-glucosaminemyristate as reference. The recovery was determined by weighing the product and by means of GC with two internal standards: methyltridecanoate and methylpentadecanoate. The product consisted of methyltetradecanoate, the recovery of which was 95%. Traces of tetradecanoic acid were not detected with methods previously described [19].

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 CB (polydimethylsiloxane) capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate (HETP) 0.25 mm. Helium served as 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 220° C. The gas chromatograph was programmed from 120° C to 260° C at 5° C or 10° C/min with the attenuator set at 8, and the attenuator of the Sigma 10 data system at −1. The paper speed was 10 mm/min.

The identity of the fatty acids (methyl esters) of whole LPS and free lipid A was established by direct cochromatography and by GC—MS. The acids were identified tentatively by comparing their retention times with those of authentic standards. KDO peaks were recognized tentatively by cochromatography of KDO-methyl derivatives and by chromatography of methanolysates of LPS from *E. coli*, *S. typhimurium* and *S. marcescens*. Their identity was verified by GC–MS.

Quantification of fatty acids (methyl esters) was made by correlating the percentual area on the chromatograms constituted by each acid derivative with it percentual content in three standard solutions composed of methyl esters of myristic, 3-hydroxymyristic and palmitic acid, using *n*-triacontane and *n*-triacontanol as internal standards. KDO and 2-hydroxymyristic acid were calculated in the same manner with *n*-triacontane and *n*-triacontanol as internal standards, and the $C_{17:cycl}$ substance as percentage of the total area on the chromatograms.

Irreversible retention of substances with free hydroxy groups by the capillary column was measured by comparing molar response rates between equimolar solutions of *n*-triacontane and *n*-triacontanol.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a Carlo Erba 4200 gas chromatograph, a Micromass 7072F (Vg Micromass, Cheshire, U.K.) mass spectrometer, and a Vg data system 2200. The gas chromatograph was equipped with a glass capillary OV-1 methylsilicone column (20 m × 0.3 mm I.D.). Helium was used as carrier gas. The column temperature was programmed from 120°C to 250°C at 5°C/min. Electron impact ionizing spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 200 μA, ion-source temperature 240°C, and accelerating voltage 4 kV. High-resolution mass spectra were obtained at 70 eV from an MS902 double-focus spectrometer connected to an AEI computer (Scientific Apparatus, Manchester, U.K.).

RESULTS

Gas chromatography

A typical gas chromatogram of the acids (methyl esters) recovered from whole LPS of *A. actinomycetemcomitans* and *H. aphrophilus* is given in Fig. 1.

TABLE II

ACIDS RECOVERED FROM WHOLE LIPOPOLYSACCHARIDE

Values (means) are expressed as relative percentage (w/w) of the total in the bacterial strain (S.D. = 5%).

Strain	KDO*	$C_{12:0}$	$C_{14:0}$	2-OH-$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:0}$	$C_{14:0}$/3-OH-$C_{14:0}$	C_{17}.cycl
Actinobacillus actinomycetemcomitans								
33384	9.8	tr**	37.7		52.0	0.2	0.725	0.3
29524	8.2	tr	34.6		57 5	0.1	0.602	0.3
29522	9 4	tr	39 9		50.3	0.1	0.793	0.3
511	6.7	tr	37.5		55.1	0 3	0.681	0.4
HK435	8 4	tr	25.0		66.3	0.1	0.377	0.2
N27	9 5	tr	30.3		59.9	0.1	0.506	0.2
Y4	9 7	tr	34.7		55.0	0 2	0.631	0.4
Haemophilus aphrophilus								
33389	7.7	tr	36.3		55 4	0.1	0.655	0.5
19415	6.5	tr	36.4		56.7	0.1	0.642	0.3
655	6.5	tr	37.7		55.1	0 3	0.684	0.4
654	6.8	tr	36.4		56.3	0.1	0.647	0.4
626	6.9	tr	36.9		54 7	0.7	0.675	0.8
621	6 8	tr	36 6		55 1	0.7	0.664	0.8
Escherichia coli								
055:B5	17 4	8 8	14.5		58 3	0.4	0.250	0.6
Salmonella typhimurium								
	8 9	7 3	15.1	4.8	52.3	8.6	0.290	3.0

*3-Deoxy-D-manno-2-octulosonic acid and/or its degradation products

**tr = trace amount

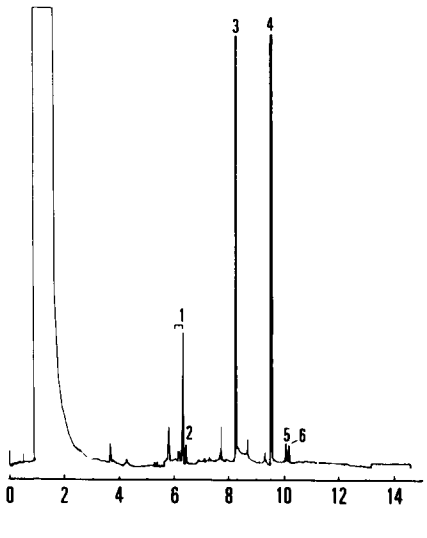

Fig. 1. Typical gas chromatogram of fatty acids (methyl esters) in whole LPS from *A. actinomycetemcomitans* and *H. aphrophilus* represented by *H. aphrophilus* strain ATCC 19415. Programme: 120°C to 260°C at 10°C/min. Temperature of the injector and flame ionization detector, 220°C. Attenuator of the Sigma 3 gas chromatograph, 8; of the Sigma 10 data system, —1. Paper speed, 10 mm/min. 1 = KDO, 2 = $C_{12:0}$, 3 = $C_{14:0}$, 4 = 3-OH-$C_{14:0}$, 5 = $C_{16:0}$, and 6 = $C_{17:cycl}$.

The composition of the major fatty acids recovered from all the LPS preparations investigated is presented in Table II. In *A. actinomycetemcomitans* and *H. aphrophilus*, the most abundant acid was 3-OH-$C_{14:0}$, followed by $C_{14:0}$. Contrary to what was observed among *A. actinomycetemcomitans* strains, the $C_{14:0}$/3-OH-$C_{14:0}$ ratio of *H. aphrophilus* strains varied little. The quantity of KDO and/or its degradation products tended to be higher in LPS from *A. actinomycetemcomitans* than from *H. aphrophilus*, and the amount of the $C_{17:cycl}$ substance higher in *H. aphrophilus* than in *A. actinomycetemcomitans*. The content of fatty acids in LPS from *E. coli* and *S. typhimurium* differed markedly from that of *A. actinomycetemcomitans* and *H. aphrophilus*, particularly with respect to $C_{12:0}$, $C_{14:0}$, and the $C_{14:0}$/3-OH-$C_{14:0}$ ratio. LPS of *S. typhimurium* also contained 2-OH-$C_{14:0}$ and was considerably higher in the $C_{17:cycl}$ substance than the other bacterial strains.

Only two major fatty acids were detected in free lipid A of *A. actinomycetemcomitans* and *H. aphrophilus*: $C_{14:0}$ and 3-OH-$C_{14:0}$ (Fig. 2), of which $C_{14:0}$ was the most abundant (Table III). It should be recognized that with flame ionization detection the detector response, expressed as molar response, was 0.33 for KDO, 1.00 for $C_{14:0}$ and 3-OH-$C_{14:0}$. By using a thermal conductivity detector, the molar response for all the detected components was 1.00.

Gas chromatography—mass spectrometry

The fragmentation pattern of the major fatty acids (methyl esters) of whole LPS and lipid A from all the strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated were in agreement with those described by Moss and

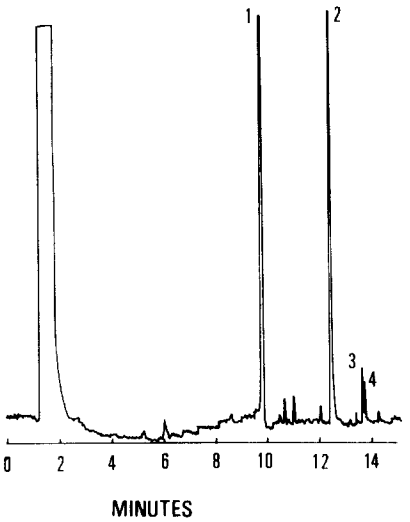

Fig. 2 Typical gas chromatogram of fatty acids (methyl esters) in free lipid A from *A actinomycetemcomitans* and *H aphrophilus*, represented by *A actinomycetemcomitans* strain ATCC 33384. Programme and setting as in Fig. 1, except 5°C/min. 1 = $C_{14:0}$, 2 = 3-OH-$C_{14:0}$, 3 = $C_{16:0}$, and 4 = $C_{17\ cycl}$.

TABLE III

ACIDS RECOVERED FROM FREE LIPID A

Values (means) are expressed as the relative percentage (w/w) of the total in the strain (S D. = 5%).

Strain	$C_{14\ 0}$	3-OH-$C_{14\ 0}$	$C_{16\ 0}$	$C_{17\ cycl}$
Actinobacillus actinomycetemcomitans 33384	57 5	38.8	2.0	1.9
Haemophilus aphrophilus 33389	58.8	36 6	2.3	2.3

Dees [20]. The fragmentation pattern of KDO (methyl ester) revealed characteristic fragments with M − 59 (loss of $COOCH_3$) and M − 61 (loss of HO—CH—CH_2—OH). The mass spectrum of the $C_{17:cycl}$ substance is presented in Fig. 3.

DISCUSSION

A number of extraction methods for the isolation of LPS from Gram-negative bacteria have been described, but the method of choice is usually the phenol—water procedure which yields a water-soluble extract subsequently purified by ultracentrifugation [21]. These LPS preparations usually have little contamination, mostly protein (about 1%). The LPSs prepared as presently described, were biologically active, as assessed from the ability to release the local Shwartzman reaction in rabbits and to stimulate bone resorption in cultured explants of mouse calvaria [22].

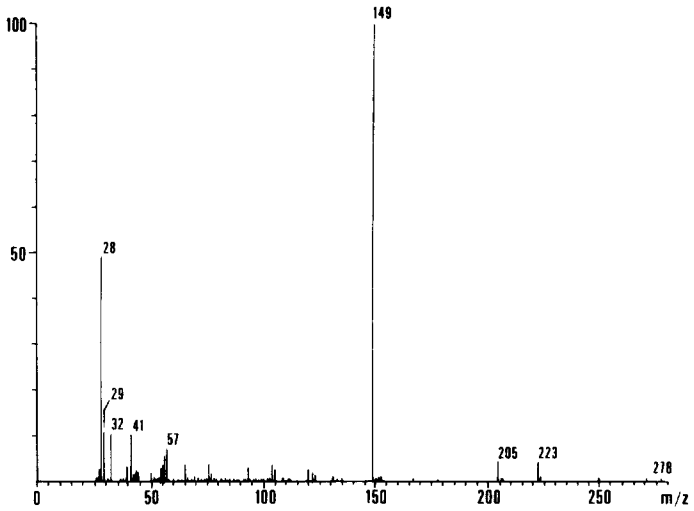

Fig. 3. Mass spectrum of unknown substance tentatively identified as $C_{17:cycl}$ acid.

Our estimates for fatty acids present in LPS from *E. coli* and *S. typhimurium*, which were used as controls, agreed well with those given by previous authors [23]. The acid composition of LPS from *A. actinomycetemcomitans* has not previously been studied systematically, and, as far as we know, the acid content of LPS from *H. aphrophilus* has not been examined at all. In this study, 3-hydroxymyristic acid was the dominant fatty acid in whole LPS from both *A. actinomycetemcomitans* and *H. aphrophilus*. Bryn and Jantzen [24], who examined one strain of *A. actinomycetemcomitans*, also found 3-hydroxymyristic acid most abundant. Since this acid is rarely found in other lipids of Gram-negative bacteria, its isolation from the gingival fluid may be taken as an indication that endotoxin or lipid A is present in the periodontal pocket. Hydroxy acids released down to picomolar amounts from lipid A of LPS sediments have been used successfully to provide an estimate of Gram-negative bacteria [25]. Our estimates for $C_{14:0}$, 3-OH-$C_{14:0}$, and $C_{16:0}$ in free lipid A from LPS of *A. actinomycetemcomitans* agreed with those of Kiley and Holt [26].

KDO appears to be a unqiue substance of Gram-negative bacteria. In LPS, KDO residues are situated at the reducing ends of the polysaccharide domains, linking them by ketosidic bonds to lipid A [27]. No entirely satisfactory method exists for the quantitative determination of KDO in polysaccharides of unknown structure and substitution pattern [27]. The thiobarbiturate assay is most frequently used, but it is subject to interference by materials in the LPS as well as other cellular components [28]. Determination of the KDO content by gas–liquid chromatography (GLC) has infrequently been reported in the literature, mostly due to lack of generally applicable, quantitative derivatization procedures. Like Kochetkov et al. [29], who studied GLC derivatives from whole LPS by methanolysis and pertrimethylsilylation, we found three peaks in the chromatograms which were due to KDO and/or its degradation products.

An unidentified substance of chain length C_{17} was detected in whole LPS

and lipid A from all the examined strains of *A. actinomycetemcomitans* and *H. aphrophilus* as well as from LPS of *E. coli*, *S. typhimurium*, and *S. marcescens*. We found a similar substance among the bound cellular acids of *A actinomycetemcomitans* and *H. aphrophilus* [17]. The base peak of this substance at *m/e* 149 and its chemical brutto formula provided by high-resolution mass spectrometry, suggested the presence of a cyclopropane fatty acid [30]. Cyclopropane fatty acids are widely distributed among Gram-negative bacteria, including *E. coli* [31], *S. typhimurium* [32], and *S. marcescens* [33]. They have occasionally been detected in lipid A from various bacteria [34], but, to our knowledge, never in *A. actinomycetemcomitans* and *H. aphrophilus*. Most bacteria convert a major fraction of their membrane phospholipids to cyclopropane derivates (for review see ref. 35), particularly when they enter stationary phase. It should be noted that phospholipid is not present in phenol—water-extracted LPS [36]. Nitrogen limitation is another factor which may promote cyclopropane fatty acid formation [37].

The similarity in the qualitative distribution of fatty acids in whole LPS and free lipid A from *A. actinomycetemcomitans* and *H. aphrophilus* indicated that these parameters are of little value as additional tools for distinction between these bacteria. The quantitative variation in fatty acids of whole LPS was larger in the strains of *A. actinomycetemcomitans* than in those of *H. aphrophilus*. We observed a similar relationship when whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* were examined for free fatty acids [14].

The great similarity in the acid composition of the LPS and lipid A preparations of *A. actinomycetemcomitans* and *H. aphrophilus* questioned the validity of considering *A. actinomycetemcomitans* as more periopathogenic than *H. aphrophilus*. If these parameters are valid measures for the relative potency of *A. actinomycetemcomitans* and *H. aphrophilus* as periopathogens, our results suggested that the ability of *H. aphrophilus* to cause periodontal diseases could be underestimated. It cannot be excluded that strains isolated from periodontal lesions may occasionally have been designated as *A. actinomycetemcomitans* when they in fact were *H. aphrophilus* due to problems with differentiation. It is also possible that different biological activities of LPS preparations cannot be predicted on the basis of their structural features. Differences in the serological behaviour of free lipid A and the parent LPS may thus indicate a masking of the determinants of the LPS bound lipid A by the O chains or by the polar head groups [21].

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