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DIFFERENTIATION BETWEEN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS* BASED ON CARBOHYDRATES IN LIPOPOLYSACCHARIDE

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SUMMARY

In the present study, the closely related facultative, Gram-negative rods, *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*, were distinguished taxonomically by means of their carbohydrate composition in phenol-extracted lipopolysaccharide. Both *A. actinomycetemcomitans* and *H. aphrophilus* lipopolysaccharide contained rhamnose, fucose, galactose, glucose, L-glycero-D-mannoheptose, galactosamine, and glucosamine. The content of galactose was approximately twice as high in lipopolysaccharide from *H. aphrophilus* as in lipopolysaccharide from *A. actinomycetemcomitans*. D-Glycero-D-mannoheptose was detected exclusively in lipopolysaccharide from *A. actinomycetemcomitans* where it constituted 11.8—16.7% of the sugar content. This aldoheptose may therefore serve as a marker for chemotaxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. The present study also describes fragmentation of methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose) from *A. actinomycetemcomitans* as suggested by mass spectrometry.

INTRODUCTION

Lipopolysaccharide (LPS), located in the outer membrane of Gram-negative bacterial cells, represents a unique class of macromolecules. It consists of a lipid portion, lipid A, and a long covalently linked heteropolysaccharide, the core and the O-specific chain [1]. O-Specific polysaccharides, comprising a series of different sugar residues in many combinations and glycosidic linkages, may display great variability between bacterial strains. In the common core,

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where a number of closely related oligosaccharides are found, the degree of structural freedom is lower. The O chain carries the immune determinant structure. Through the core, serologic R specificity is expressed [2]. LPS has been classified into chemotypes based on the content of sugar constituents [3]. In *Salmonella* this chemical classification correlated well with the serological classification of the Kauffman—White scheme [4]. In the present study we have used the sugar composition of LPS for taxonomic differentiation between the closely related dental plaque bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Identification of these organisms in the routine laboratory is generally done by biochemical tests, but only a few of these have been found useful for differentiation [5]. The present study suggested that LPS may be a useful parameter for accurate distinction between *A. actinomycetemcomitans* and *H. aphrophilus* when conventional methods are insufficient. Accurate identification is urgent when it comes to clarification of a possible association between distinct bacterial species and clinical forms of disease. So far, an association has been suggested between *A. actinomycetemcomitans* and a special form of periodontal disease affecting juveniles (juvenile periodontitis) [6—8]. The relationship between *H. aphrophilus* and periodontal destruction, however, has not yet been determined. It should also be realized that both *A. actinomycetemcomitans* and *H. aphrophilus* may be implicated in a series of extraoral infections, e.g. bacterial endocarditis and miscellaneous abscesses (for review, see ref. 9), where similar problems of differentiation may occur.

The present study also describes fragmentation of trifluoroacetylated (TFA-derivatized) methylglycosides of D-glycero- and L-glycero-D-manno-heptose from *A. actinomycetemcomitans*, as suggested by mass spectrometry. The purpose of this aspect of the study was to make identification of these sugars easier when reference bacteria containing them are not at hand.

MATERIAL AND METHODS

Bacteria

The thirteen strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, the sources from which they were obtained, and the procedures for maintenance and cultivation have already been described [10, 11].

Preparation of lipopolysaccharide

LPS from *A. actinomycetemcomitans/H. aphrophilus* strain pairs was isolated by the phenol—water procedure [11]. The preparations from *A. actinomycetemcomitans* strain ATCC 33384 and from *H. aphrophilus* strain ATCC 33389 were made in duplicate from batches cultured at different time periods. All LPS preparations were divided into two parts, each of which was methanolysed and derivatized separately.

Methanolysis

Samples (0.5—1.5 mg) of LPS were methanolysed (2 *M* hydrochloric acid in anhydrous methanol, 2 ml, 24 h, 85°C) [11]. 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 organic phase was washed twice, each time with 4 ml of distilled water, and the water phase twice, with 4-ml batches of chloroform. The water phases were pooled and lyophilized before derivatization for sugar analysis. Fatty acids have already been determined from the pooled organic phases [11].

Derivatization

The lyophilized water phase (0.1—0.2 mg) from each part of the LPS preparations was suspended in 100 or 200 μl of acetonitrile (Rathburn, U.K.). Acetonitrile and trifluoroacetic acid anhydride (Fluka), 1 or 2 ml of each, were then added, and the mixture was transferred to a PTFE-sealed tube with screw cap, which was kept in a water bath for 3 min at 90°C. After cooling the tube to room temperature, its content was diluted 1:1 with acetonitrile. Also synthetic sugars and reference LPS were methanolysed and derivatized as described above.

Reference compounds

Sigma (St. Louis, MO, U.S.A.) provided α-D(+)-fucose, D(+)-galactose, α-D(+)-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-mannose, and α-L-rhamnose. Natural galactose, glucosamine, L-glycero-D-mannoheptose, mannose, and rhamnose were identified from LPS (Sigma) of *Escherichia coli* [12] and *Salmonella typhimurium* [13]. D-Glycero-D-mannoheptose was determined from LPS of *Chromobacterium violaceum* [14]. *Ch. violaceum* and N-glucosaminemyristate were generously provided by Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.

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 (polydimethylsiloxane) glass capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate 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 200°C. Programme: hold 2 min at 90°C, then 90°C to 260°C at 9°C/min with the attenuator of the Sigma 10 data system at −1. The paper speed was 10 mm/min. The sample (0.2 μl) was delivered as a splitless injection. The identity of the sugars in LPS, prepared by trifluoroacetic acid anhydride derivatization of LPS methanolysates, was established by direct cochromatography and by gas chromatography—mass spectrometry (GC—MS). They were identified tentatively by comparing their retention times with those of authentic standards. From each portion of methanolysed and derivatized LPS, three runs were made on the gas chromatograph. Accordingly, the quantitative values given in Table I are based on means from twelve runs with LPS from ATCC

TABLE I

PERCENTAGE SUGAR COMPOSITION OF PHENOL-EXTRACTED LIPOPOLYSACCHARIDE (S.D. = 5%)

Strain*	Rhamnose	Fucose	Galactose	Glucose	D-Glycero-D-mannoheptose	L-Glycero-D-mannoheptose	Galactosamine + glucosamine
Actinobacillus actinomycetemcomitans							
ATCC 33384 (NCTC 9710)	9.8	5.7	10.1	36.5	14.4	17.8	5.6
ATCC 29524	13.0	10.7	9.3	31.8	11.8	17.1	6.2
ATCC 29522	11.8	6.9	7.1	30.9	12.3	23.0	8.0
FDC 511	6.8	7.0	8.6	29.9	16.7	26.3	4.7
Kilian HK 435	8.3	10.0	9.6	30.1	16.2	21.6	4.1
FDC N 27	8.2	9.2	9.7	32.2	12.3	20.5	7.8
FDC Y 4	13.0	8.8	10.2	31.4	12.5	18.8	5.3
Haemophilus aphrophilus							
ATCC 33389 (NCTC 5906)	7.2	4.6	20.2	40.9		23.0	4.1
ATCC 19415 (NCTC 5886)	8.1	4.8	20.3	40.1		22.9	3.8
FDC 655	8.5	6.0	16.5	34.2		29.5	5.4
FDC 654	6.2	4.5	20.8	33.6		30.0	4.7
FDC 626	10.7	6.7	18.9	33.4		24.4	5.8
FDC 621	6.9	5.0	18.4	38.6		26.4	4.7

* 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.

33384 and ATCC 33389, and from six runs with LPS from each of the remaining strains.

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 an OV-1 (methyl silicone) glass capillary column (20 m × 0.3 mm I.D.). Helium was used as carrier gas. The column temperature was programmed with a 2-min hold at 90°C, then from 90°C to 250°C at 9°C/min. Electron-impact ionization 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. Chemical ionization mass spectra were recorded with isobutane as a reactant gas and with an ionizing energy of 50 eV. 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

The content of carbohydrates in all the LPS preparations from *A. actinomycetemcomitans* and *H. aphrophilus* is given in Table I. The sugar composition of LPS from these two species was so different that *A. actinomycetemcomitans* could easily be distinguished from *H. aphrophilus*. For taxonomic differentiation, D-glycero-D-mannoheptose, which was detected exclusively in LPS from *A. actinomycetemcomitans*, served as a marker. The usefulness of this substance in chemotaxonomy was substantiated by the fact that it constituted a considerable portion of the carbohydrates in LPS from *A. actinomycetemcomitans*. There was also a marked difference in the amount of galactose in LPS from *A. actinomycetemcomitans* and *H. aphrophilus*, galactose being approximately twice as abundant in LPS from *H. aphrophilus* as in LPS from *A. actinomycetemcomitans*. The relationship between galactosamine and glucosamine was approximately 4:1, as measured in LPS from *A. actinomycetemcomitans* strain ATCC 33384.

TABLE II

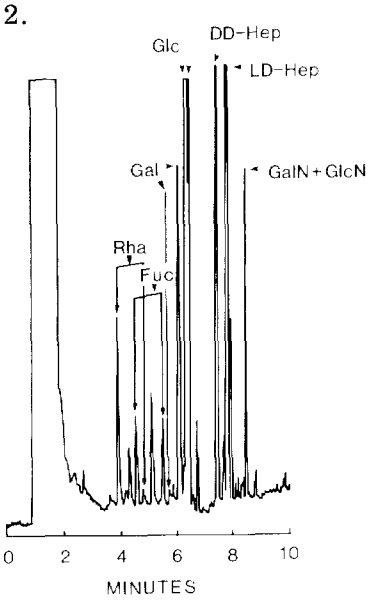
MOLAR RESPONSE RELATIVE TO TRIFLUOROACETYL DERIVATIVES OF GLUCOSE AND GALACTOSAMINE

Trifluoroacetyl derivatives	Relative molar response
Glucose	1.00
Galactosamine	1.00
Galactose	0.90
Fucose	0.86
Rhamnose	0.86
D-Glycero-D-mannoheptose*	1.25
L-Glycero-D-mannoheptose*	1.25

*Prepared by preparative gas chromatography.

Molar response factors of trifluoroacetyl derivatives of the detected sugars have been compared in Table II, using derivatives of glucose and galactosamine as basis.

Typical gas chromatograms of the methanolysed and derivatized sugars from LPS of *A. actinomycetemcomitans* and *H. aphrophilus* are given in Figs. 1 and 2.



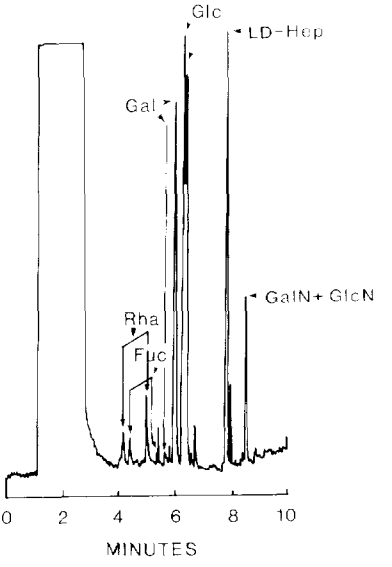


Fig. 1. Typical gas chromatogram of the sugar composition in LPS from *A. actinomycetemcomitans*, as represented by strain ATCC 33384. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN; galactosamine; GlcN, glucosamine.

Fig. 2. Typical gas chromatogram of the sugar composition in LPS from *H. aphrophilus*, as represented by strain ATCC 33389. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine.

In Fig. 3 the mass spectrum of D-glycero-D-mannoheptose is shown. Fig. 4 gives three possible routes of fragmentation (Schemes 1—3) for methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose), as suggested by electron-impact mass spectrometry. The following ions and fragments were determined through high-resolution mass spectrometry: *m/e* 69 (CF_3), 97 ($COCF_3$), and 113 ($OCOCF_3$), and the remaining ions (*m/e* 139, 157, 170, 193, 196, 223, 252, 253, 265, 278, 295, 305, 309, 337, 451, 531, and 591) were in accordance with Fig. 4. M^+ was not recorded. The first way of fragmentation (Scheme 1) involves splitting off a heterocyclic ion with *m/e* 451, which eliminates TFA with formation of an *m/e* 337 ion. This ion splits off methanol, forming an ion with *m/e* 305, or TFA, with formation of the 223 ion. Also OCH_3 and $OCOCF_3$ may split off, producing an ion with *m/e* 193. Another possibility (Scheme 2) is the ring opening and migration of $OCOCF_3$ from C-3 to C-1, and cleavage of the C-1—O binding with the formation of an intense ion with *m/e* 157, approximately 25% of the base ion, or cleavage of the C-4—C-5 binding with production of an ion with *m/e* 265.

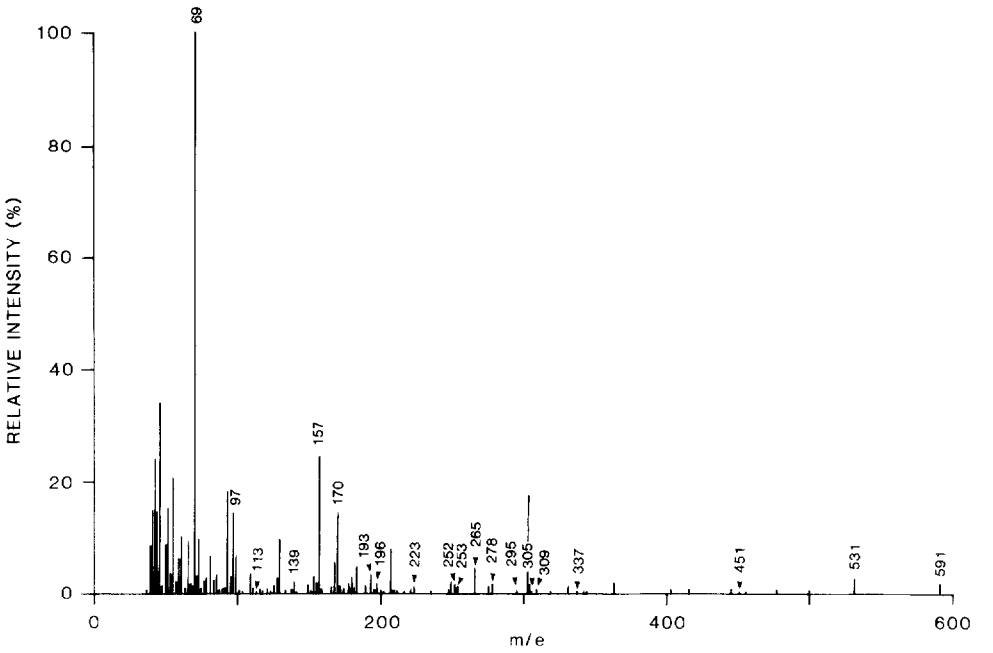


Fig. 3. Mass spectrum of D-glycero-D-mannoheptose.

The cleavage of the C-5—O binding leads to formation of an ion with *m/e* 531, which is fragmented into an ion with *m/e* 253 and 278. The third way of fragmentation (Scheme 3) involves cleavage of the C-1—O and C-2—C-3 bindings with formation of an ion with *m/e* 170 and cleavage of the C-1—C-2 and C-3—C-4 bindings with formation of an ion with *m/e* 252. Formation of the ions with *m/e* 309, 295, and 196 supported the proposals made on fragmentation of D-glycero- and L-glycero-D-mannoheptose.

DISCUSSION

Our results were in accordance with those of Kiley and Holt [15] who detected rhamnose, fucose, glucose, galactose, galactosamine, glucosamine, and heptose in LPS from *A. actinomycetemcomitans* strain FDC N 27 and Y 4. Bryn and Jantzen [16] were able to differentiate between D-glycero- and L-glycero-D-mannoheptose in one strain of *A. actinomycetemcomitans.* As far as we know, the sugars in LPS from *H. aphrophilus* have not previously been examined. Whereas the fatty acids of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* did not provide any source of differentiation between these species [11], the sugar content of their LPS did. The outstanding feature of the present study was the demonstration of D-glycero-D-mannoheptose in phenol—water-extracted LPS from all reference and laboratory strains classified as *A. actinomycetemcomitans.* LPS from none of the examined strains of *H. aphrophilus* contained this aldoheptose. D-Glycero-D-mannoheptose, constituting a considerable portion of the sugar content in LPS from *A. actinomycetemcomitans*, may therefore serve as a marker in the chemotaxonomic differentiation between *A. actinomycetemcomitans* and *H. aphro-*

philus. D-Glycero-D-mannoheptose is a rare [1] but not unique constituent of the LPS core where the predominant heptose is L-glycero-D-mannoheptose [17]. Further, D-glycero-D-mannoheptose is an intermediate in the formation of L-glycero-D-mannoheptose [18], and both sugars are interconverted in the form of their nucleotide diphosphate derivatives by the action of a specific epimerase [19]. D-Glycero-D-mannoheptose has also been detected in LPS from *Haemophilus influenzae* type b [20]. This organism has $C_{15:0}$ as a bound cellular fatty acid [20]. Previously, we have found iso-$C_{15:0}$ acid as a free cellular acid in *A. actinomycetemcomitans* strain ATCC 29522 and FDC Y 4 [10]. The distribution of biological markers such as D-glycero-D-mannoheptose and C_{15} acids in specific strains of *A. actinomycetemcomitans* and type b of *H. influenzae* suggested some relationship between them. It is noteworthy that these strains seem to be more pathogenic in human disease than other strains of *A. actinomycetemcomitans* and *H. influenzae* [21, 22].

SCHEME 1

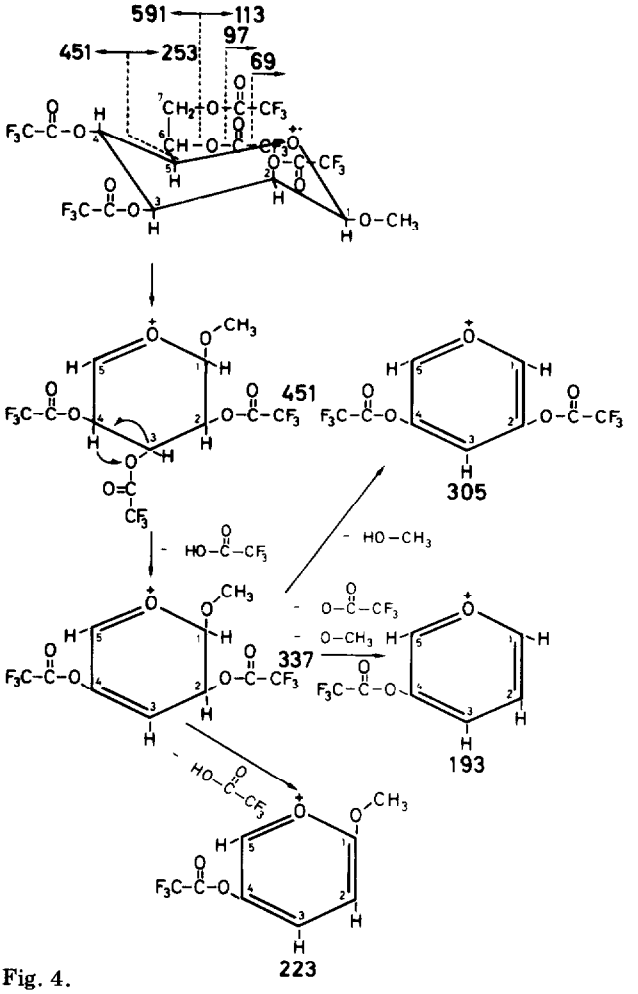
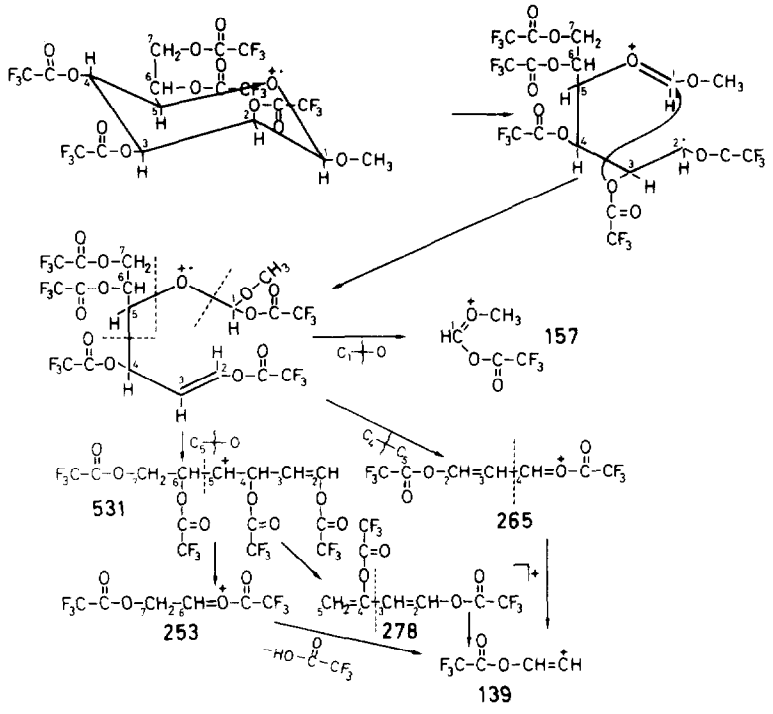


Fig. 4.

SCHEME 2



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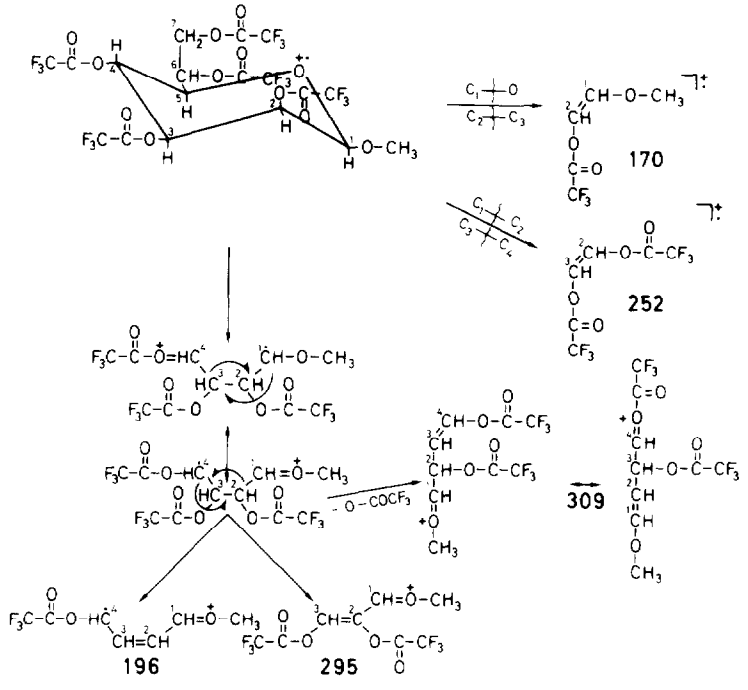

Fig. 4. Three main routes of fragmentation (Schemes 1–3) for methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose), as suggested by electron-impact mass spectrometry.

LPS from *H. aphrophilus* contained twice as much galactose as LPS from *A. actinomycetemcomitans*. This may reflect interspecies differences in the chemical composition of the O chain, and/or of the core, where galactose is a common component [1].

Previous taxonomic work has suggested that *A. actinomycetemcomitans*, being closely related to *H. aphrophilus*, should be included in the genus *Haemophilus* [23]. Until recently, *A. actinomycetemcomitans* was listed in Bergey's Manual of Determinative Bacteriology [24] as a species incertae sedis, uncertainty especially existing in the relationship to *H. aphrophilus*. The present study, based on the carbohydrate composition of LPS, provided clear evidence that *A. actinomycetemcomitans* and *H. aphrophilus* are distinct species and therefore should be maintained as such in current taxonomy. Even in our first study with free fatty acids in whole cells it was possible to distinguish between *A. actinomycetemcomitans* and *H. aphrophilus* [10]. Other reports based on biochemical characters [5], deoxyribonucleic acid relatedness [9, 25] and cellular proteins [26, 27] support this differentiation, and in a recent edition of Bergey's Manual of Systematic Bacteriology [28] *A. actinomycetemcomitans* was listed as an established species. Even if *Actinobacillus* and *Haemophilus* can be differentiated on the species level, this does not exclude relatedness at genus or family level. Actually, creation of a new family based on *Actinobacillus*, *Haemophilus* and *Pasteurella* has been suggested [9, 29].

The present study demonstrated that LPS is a preparation well fitted for taxonomic differentiation between closely related bacteria. As well as in *Enterobacteriaceae* [30], LPS has been found useful as a taxonomic marker in *Bacteroidaceae* [31].

The sugar content of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* did not vary so much as to allow establishment of different chemotypes within these species. Usually, there is a correlation between the sugar composition and serological specificities of O antigens in LPS. King and Tatum [32] and Zambon et al. [21] were able to divide *A. actinomycetemcomitans* into serogroups which shared a common antigen with *H. aphrophilus*. It cannot be excluded that capsular polysaccharides of *A. actinomycetemcomitans* [33] represent the serotype specific antigens of this species, preventing anti-O or anti-R antibodies from reacting with the LPS antigens. LPS in the aqueous phase would be freed of possible contaminating capsular polysaccharides by our repeated ultracentrifugation [3, 11].

The difference in the sugar composition of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* may have implications as to the virulence of these organisms. For *Fusobacterium* it has been demonstrated that the polysaccharide fraction of LPS stimulates bone resorption in vitro even more than lipid A [34]. Differences in the sugar composition of LPS may therefore confer different potential for induction of bone resorption to *A. actinomycetemcomitans* and *H. aphrophilus*.

Our experiments confirmed that conversion of carbohydrates into their polytrifluoroacetates, which are extremely volatile and polar, is a rapid and dependable analytical method for carbohydrates in a complex mixture [35, 36]. This method has been found superior to previously reported gas–liquid

chromatographic methods based on derivatization to methyl esters, polyacetates, or polytrimethylsilyl ethers (for review, see ref. 37). Methanolysis can in principle yield four different glycosides for each sugar, i.e. α- and β-anomers of both the methyl pyranosides and the methyl furanosides [38]. The relative portion of the different isomers for a given sugar is a function of the methanolysis conditions and usually is not altered by subsequent derivatization of the methyl glycosides. Quantitation of the sugars in the present chromatograms were usually based on multiple peaks, which were reproducible. It can also be made from selected peaks because the ratio between the peaks was stable under the experimental conditions used.

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