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CARBOHYDRATES OF WHOLE DEFATTED CELLS AS A BASIS FOR DIFFERENTIATION BETWEEN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

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SUMMARY

Using gas chromatography and gas chromatography—mass spectrometry, the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* were distinguished by means of the sugar content in whole defatted cells. Both species contained rhamnose, fucose, galactose, glucose, galactosamine, glucosamine, and L-glycero-D-manno-heptose. Contrary to *H. aphrophilus*, whole cells of *A. actinomycetemcomitans* contained D-glycero-D-mannoheptose. This sugar may thus serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

INTRODUCTION

In Gram-negative bacteria polysaccharides are found as integral parts of the cell wall, in the extracellular capsule, or in both these structures. The cell wall polysaccharides of Gram-negative bacteria are bound to lipid, constituting the somatic lipopolysaccharide (LPS), which represents the endotoxic principle of these organisms. Polysaccharides residing on the bacterial cell surface are involved in phenomena such as cell—cell recognition, binding of bacteriophages, and antigenic expression. Since most microbial polysaccharides cannot be digested by mammalian enzymes, they remain in circulation for a considerable time [1], acting as potent antigens. The fine structure of surface polysaccharides in bacteria may therefore serve as a basis for their serological classification.

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TABLE I

PERCENTAGE CELLULAR SUGAR* COMPOSITION OF METHANOLYSED WHOLE DEFATTED CELLS (S.D. 5%)

Bacteria**	Rhamnose	Fucose	Galactose	Glucose	DD-Heptose	LD-Heptose	Galactosamine + glucosamine
Actinobacillus actinomycetemcomitans							
ATCC 33384 (NCTC 9710***)	5.0	5.7	4.0	10.9	4.1	5.3	2.6
ATCC 29523	6.1	9.3	5.6	13.9	5.9	8.1	4.6
ATCC 29522	11.5	8.8	3.5	10.2	3.6	6.2	6.2
FDC 2112	4.1	7.1	5.7	15.0	5.8	8.1	4.0
FDC 2097	10.9	16.1	7.2	17.9	4.5	6.0	6.6
FDC 2043	10.1	13.3	7.2	22.6	7.1	10.5	4.9
FDC 511	5.0	6.8	5.1	15.9	6.9	10.7	4.4
FDC N 27	4.9	9.3	10.0	10.3	4.4	7.2	3.9
Haemophilus aphrophilus							
ATCC 33389 (NCTC 5906***)	5.2	8.5	8.6	19.0		4.7	2.6
ATCC 19415 (NCTC 5886)	5.3	6.0	6.3	17.8		4.5	2.6

*DD-Heptose = D-glycero-D-mannoheptose; LD-Heptose = L-glycero-D-mannoheptose.
**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.
***Type strain of the species.

LPS has also been used as a chemical basis for such classification [2]. This fact prompted us to use carbohydrates of LPS as tools for taxonomic differentiation between the closely related Gram-negative, facultative rods *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* [3]. Since preparation of LPS is a rather time-consuming procedure, more convenient diagnostic procedures need to be established for the routine laboratory. In the present study, the sugar content of whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* has been used to distinguish between these bacteria.

MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* examined, the sources from which they were obtained, and the procedures for maintenance and cultivation have been described elsewhere [4, 5].

Methanolysis and derivatization

After removal of the free fatty acids with hexane [4, 5], whole lyophilized cells were methanolyzed (2 *M* hydrochloric acid in anhydrous methanol, 24 h, 85°C) [6]. After separating with chloroform (Fluka, Buchs, Switzerland), the organic phase of the methanolysate, which contained the bound fatty acids, from the water phase, the latter was lyophilized and derivatized with acetonitrile (Rathburn, U.K.) and trifluoroacetic anhydride (Fluka) (1:1) [3].

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* [7] and *Salmonella typhimurium* [8]. D-Glycero-D-mannoheptose was determined from *Chromobacterium violaceum* [9]. *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 CB (polydimethylsiloxane) glass capillary column used was 25 m × 0.22 mm I.D. with a film thickness of 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 gas chromatograph set at 8, and the attenuator of the Sigma data 10 system at −1. The paper speed was 10 mm/min. Splitless injection was used. The identity of the methanolysed and derivatized sugars was established by direct cochromatography and by gas chromatography–mass

spectrometry (GC—MS). The sugars were identified tentatively by comparing their retention times with those of authentic standards.

Gas chromatography—mass spectrometry

GC—MS was performed as described previously [3].

RESULTS

The distribution of sugars in whole defatted cells of *A. actinomycetemcomitans* and *H. aphrophilus* is shown in Table I. Both species contained rhamnose, fucose, galactose, glucose, L-glycero-D-mannoheptose, galactosamine, and glucosamine. The most striking feature of the present results was that D-glycero-D-mannoheptose was present in all the strains of *A. actinomycetemcomitans* examined, but in none of the *H. aphrophilus* strains. Ratios between essential sugar markers such as D-glycero- and L-glycero-D-mannoheptose and between glucose and L-glycero-D-mannoheptose are compared in Table II with those of LPS from *A. actinomycetemcomitans* and *H. aphrophilus*. The ratio between D-glycero- and L-glycero-D-mannoheptose was rather constant and similar in whole cells and LPS from *A. actinomycetemcomitans*. The glucose/L-

TABLE II

RATIOS BETWEEN SELECTED SUGARS* IN WHOLE DEFATTED CELLS AND LIPOPOLYSACCHARIDE FROM BACTERIA

	Whole defatted cells		Lipopolysaccharide**	
	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep
Actinobacillus actinomycetemcomitans				
33384	0.8	2.1	0.8	2.1
29524			0.7	1.9
29523	0.7	1.7		
29522	0.6	1.7	0.6	1.3
2112	0.7	1.9		
2097	0.8	3.0		
2043	0.7	2.2		
511	0.6	1.5	0.6	1.1
HK 435			0.8	1.4
N 27	0.6	1.4	0.6	1.6
Haemophilus aphrophilus				
33389		4.0		1.8
19415		4.0		1.8
655				1.2
654				1.1
626				1.4
621				1.5

*Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose.
**Data from ref. 3.

glycero-D-mannoheptose ratio varied somewhat between the strains of *A. actinomycetemcomitans* but was rather constant within each strain when whole cells and LPS were compared. In whole cells of the *H. aphrophilus* strains examined the glucose/L-glycero-D-mannoheptose ratio was approximately twice as high as in LPS.

Typical gas chromatograms of the sugars in whole defatted cells of the *A. actinomycetemcomitans* and *H. aphrophilus* strains are presented in Figs. 1 and 2.

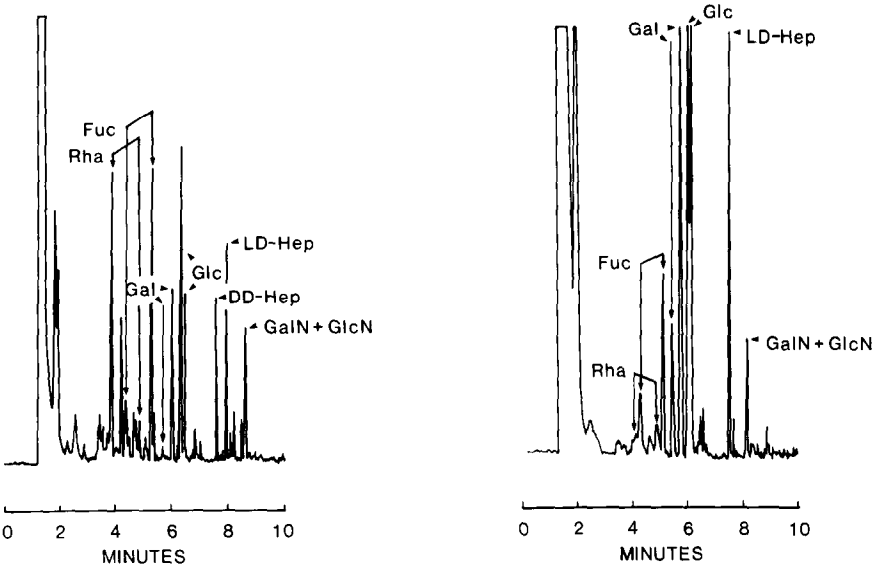

Fig. 1. Typical gas chromatogram of the sugar composition of whole defatted cells of *A. actinomycetemcomitans*, as represented by strain ATCC 33384. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine;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

Fig. 2. Typical gas chromatogram of the sugar composition of whole defatted cells of *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.

The molar response factors of trifluoroacetylated derivatives of the detected sugars were as described previously [3].

The mass spectra of D-glycero- and L-glycero-D-mannoheptose were also consistent with our previous description [3].

DISCUSSION

In the present study, the sugar composition of whole cells was used to differentiate between the closely related bacteria *A. actinomycetemcomitans* and *H. aphrophilus*. Whole cells yielded more complicated gas chromatograms than purified cell wall preparations such as LPS [3]. Trifluoroacetyl derivatized

whole-cell methanolysates from *Neisseriae* and *Moraxellae* also showed complex gas—liquid chromatographic (GLC) profiles with peaks of similar retention in different "finger prints" which were not easily distinguished and identified [10]. Therefore, selective extractions and various other identification techniques had to be performed. For easier interpretation of the present gas chromatograms, defatted cells were analysed, i.e. cells pre-extracted for free and bound fatty acids [4—6].

The most outstanding feature of the present study was that D-glycero-D-mannoheptose could be demonstrated in whole cells of *A. actinomycetemcomitans* but not of *H. aphrophilus*. A similar difference was observed in phenol-extracted LPS from a series of strains, including type strains, other reference strains and laboratory strains of *A. actinomycetemcomitans* and *H. aphrophilus* [3]. Seen together, these observations strongly suggested that D-glycero-D-mannoheptose can serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. They also supported our previous differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* based on free cellular fatty acids [5], as well as the establishment of *A. actinomycetemcomitans* as a separate species distinct from *H. aphrophilus* in a recent edition of Bergey's Manual of Systematic Bacteriology [11]. The present method of gas chromatography of derivatized methanolysates from whole defatted cells may represent a valuable step towards more accurate identification and classification of *A. actinomycetemcomitans* and *H. aphrophilus*, complementing the few biochemical tests recommended for distinction between these bacteria [12].

The ratio between the percentage of D-glycero- and L-glycero-D-mannoheptose was rather constant and similar to that detected in whole LPS from *A. actinomycetemcomitans*. This suggested that LPS is the principal source of these aldoheptoses in *A. actinomycetemcomitans*. Some bacteria, e.g. *Azotobacter indicum*, have D-glycero-D-mannoheptose in their capsule [13]. Also the glucose/L-glycero-D-mannoheptose ratio was rather constant and similar in whole cells and LPS from *A. actinomycetemcomitans*. This may indicate no other major reservoirs for glucose in *A. actinomycetemcomitans* than LPS. The examined *H. aphrophilus* strains revealed an increase in the glucose/L-glycero-D-mannoheptose ratio in whole cells compared to LPS. This suggested other sources for glucose in these bacteria than LPS. One likely source is their surface exopolymers, i.e. microcapsule, fibrils or slime layer [14], which may be eliminated during purification of LPS with ultracentrifugation [15]. The fact that glucose is a common capsular sugar in bacteria [16, 17] supported this idea.

King and Tatum [18] and Zambon et al. [19] were able to divide *A. actinomycetemcomitans* into serogroups which shared a common antigen with *H. aphrophilus*. The present study on whole defatted cells did not provide any clear chemical basis for creation of groups of strains corresponding to the serogroups suggested in *A. actinomycetemcomitans*. There are many suggestions in the literature (e.g. ref. 20) that exopolysaccharides are highly branched structures, and if so, identical chemotypes may occur within different serogroups. Alternatively, even if the carbohydrate structure is constant, alterations

due to acyl constituents, which are prevalent in exopolysaccharides, are frequently noted [17].

Depolymerization of polysaccharides through methanolysis is preferable to aqueous acid [21]. Methanolysis was used already in 1966 by Ishizuka et al. [22], and has later been modified by a number of authors (for review, see ref. 23). It does not cause significant destruction of sugars [24]. Most glycosidic linkages are quantitatively broken [25] and the reduced monosaccharides stabilized as methyl glycosides during this procedure [10]. After Vilkas et al. [26] reported GLC separation of a number of carbohydrates in 1966, including methyl glycosides of their trifluoroacetates, Tamura and co-workers [27, 28] carried out comprehensive investigations of the conditions necessary for preparation and chromatography of trifluoroacetyl derivatives of glucose, galactose, mannose, glucosaminitol, galactosaminitol, and mannitol, and subsequently adopted GLC of trifluoroacetates of alditols [29] as their standard method for estimation of mono- and disaccharides in biological fluid [30]. The advantage of trifluoroacetates is that they are more volatile than either acetates or trimethylsilyl ethers and therefore chromatographic separation may be carried out more rapidly and at lower temperatures [31, 32]. Trifluoroacetates have thus proved valuable additions to the range of carbohydrate derivatives suitable for GLC, and capillary columns permit the analysis of submicrogram quantities (for review, see ref. 33). It has also been reported that methyl glycosides are stabilised under trifluoroacetylosis and are quantitatively recovered after O-detrifluoroacetylation [34]. 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 [32]. 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 was usually based on multiple peaks, but can also be made from selected peaks because the ratio between the peaks was stable under the experimental conditions used, which was consistent with the findings of other workers in this field [24, 25, 32]. In some cases the proportion of an isomer of a sugar may be so low that it is negligible. Often, however, two or more isomers may not be resolved under a particular set of chromatographic conditions and yield only a single peak [32]. Thus, galactose yielded only two peaks when analysed as trifluoroacetylated methyl glycoside [32], whereas three peaks were observed when galactose was chromatographed as trimethylsilylated methyl glycoside [35].

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