Journal of Chromatography, 311 (1984) 347—353
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2269

Note

Whole-cell methanolysis as a rapid method for differentiation between *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*

ILIA BRONDZ*

Department of Chemistry, University of Oslo, Blindern, Oslo 3 (Norway)

and

INGAR OLSEN

Department of Microbiology, Dental Faculty, University of Oslo, Oslo 3 (Norway)

(First received April 12th, 1984; revised manuscript received July 16th, 1984)

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are Gram-negative, capnophilic, coccobacillary rods indigenous to dental plaque [1]. They have been associated with a number of extraoral infections, e.g. bacterial endocarditis, miscellaneous abscesses, and osteomyelitis (for review, see ref. 2), for which dental plaque often is the source of infection. There are many indications that *A. actinomycetemcomitans* also is implicated as a major pathogen in periodontitis among juveniles (for review, see refs. 2 and 3). The role of *H. aphrophilus* in periodontal disease has not yet been clarified. Unfortunately, the elucidation of this problem is hampered by the fact that there are relatively few tests available in the routine laboratory for distinction between these organisms [4]. To establish more criteria of differentiation, we have previously examined bound cellular fatty acids [5] and fatty acids in whole lipopolysaccharide (LPS) and free lipid A [6] in *A. actinomycetemcomitans* and *H. aphrophilus*, but no marked differences in the fatty acid profiles could be established. Free fatty acids from whole cells differed for some strains [7]. The sugar content of methanolysed and derivatized LPS [8] and whole defatted cells [9] from these species provided more consistent differentiation. Unfortunately, preparation of LPS and whole defatted cells involves rather time-consuming laboratory procedures. The present study, which is based on whole-cell methanolysates, describes a simple and rapid

0378-4347/84/$03.00

TABLE I

PERCENTAGE SUGAR* AND FATTY ACID COMPOSITION OF DERIVATIZED WHOLE-CELL METHANOLYSATES

	Rha	Fuc	Gal	Glc	DD-Hep	LD-Hep	GalN + GlcN	KDO	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$
Actinobacillus actinomycetemcomitans ATCC 33384 (NCTC 9710)**	0.9	5.0	5.0	11.2	4.7	6.0	2.1	0.4	7.9	5.6	4.9	7.2
Haemophilus aphrophilus ATCC 33389 (NCTC 5906)	1.2	5.1	10.4	23.5	—	5.9	1.8	0.2	10.2	7.0	6.7	7.9

*Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine; KDO, 3-deoxy-D-manno-2-octulosonic acid and/or its methanolysis products.
**ATCC, American Type Culture Collection, Rockville, MD, U.S.A.; NCTC, National Collection of Type Cultures, London U.K.

TABLE II

COMPARISON OF RATIOS BETWEEN SELECTED SUGARS* IN EXAMINED BACTERIAL PREPARATIONS

	Whole-cell methanolysates		Whole defatted cells [9]		Lipopolysaccharide [8]	
	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep
Actinobacillus actinomycetemcomitans ATCC 33384	1.9	0.8	2.1	0.8	2.1	0.8
Haemophilus aphrophilus ATCC 33389	4.0		4.0		1.8	

*For abbreviations see Table I footnote.

procedure for accurate distinction between *A. actinomycetemcomitans* and *H. aphrophilus* that is well fitted for the routine laboratory.

MATERIALS AND METHODS

Bacteria

The type specific strains of *A. actinomycetemcomitans* and *H. aphrophilus* were analysed. Sources of isolation and procedures for maintenance and cultivation have been described elsewhere [7].

Methanolysis and derivatization

Whole lyophilized cells were methanolysed by 2 *M* hydrochloric acid in anhydrous methanol for 24 h at 85°C [6] and derivatized with trifluoroacetic anhydride (Fluka) 1:1 in acetonitrile (Rathburn Chemicals, U.K.) [8].

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 of *Escherichia coli* [10] and *Salmonella typhimurium* [11] (Sigma). D-Glycero-D-mannoheptose was determined from *Chromobacterium violaceum* [12], provided together with N-glucosamine myristate by Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G. The ammonium salt of 3-deoxy-D-manno-2-octulosonic acid (KDO) (Sigma) was methylated in 2 *M* hydrochloric acid in anhydrous methanol at 85°C for 24 h [6]. Methyl esters of lauric, myristic, palmitic, and palmitoleic acid were obtained from Supelco (Bellefonte, PA, U.S.A.), and 13-methyltetradecanoic acid from Larodan Fine Chemicals (Malmö, Sweden). The methyl ester of racemic 3-hydroxymyristic acid was synthesized [5].

Gas chromatography

This was performed as described previously [8]. The molar response of the trifluoroacetyl derivatives of the detected monosaccharides has previously been given [8].

RESULTS

The distribution of sugars and fatty acids in trifluoroacetyl-derivatized whole-cell methanolysates of *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389 is shown in Table I. D-Glycero-D-mannoheptose was detected exclusively in *A. actinomycetemcomitans*. The concentration of galactose and glucose was approximately twice as high in *H. aphrophilus* as in *A. actinomycetemcomitans*, and the amount of KDO and/or its methanolysis products twice as high in *A. actinomycetemcomitans* as in *H. aphrophilus*. In *A. actinomycetemcomitans* the ratio between glucose and L-glycero-D-mannoheptose was 1.9, and between D-glycero- and L-glycero-D-mannoheptose 0.8 (Table II), which agreed fairly well with corresponding

data from whole defatted cells and LPS from this species. The ratio between glucose and L-glycero-D-mannoheptose was 4.0 in both whole-cell methanolysates and in whole defatted cells from *H. aphrophilus*, and 1.8 in LPS from this species.

A. actinomycetemcomitans and *H. aphrophilus* contained the same major fatty acids (Table I). Only small differences were recorded in their quantitative distribution.

The separation of sugars and fatty acids in derivatized whole-cell methanolysates of *A. actinomycetemcomitans* and *H. aphrophilus* is demonstrated on the same chromatogram in Figs. 1 and 2.

Fragmentation of fatty acids and sugars were in agreement with previously published results obtained using gas chromatography—mass spectrometry [7, 8].

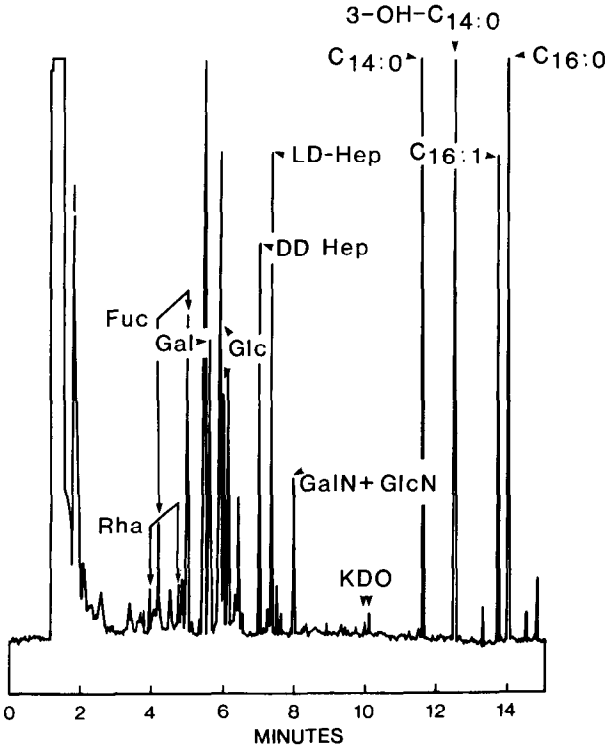

Fig. 1. Gas chromatogram of sugars and fatty acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates of *A. actinomycetemcomitans* strain ATCC 33384 (NCTC 9710). Abbreviations as in Table I footnote.

DISCUSSION

Whole-cell methanolysis, which represents a relatively new approach [13—16], has previously been found useful in taxonomic studies on *Moraxella* and *Neisseria* where fatty acids served as the best differentiating criteria [17—21]. In the whole-cell methanolysates from *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389, the composition

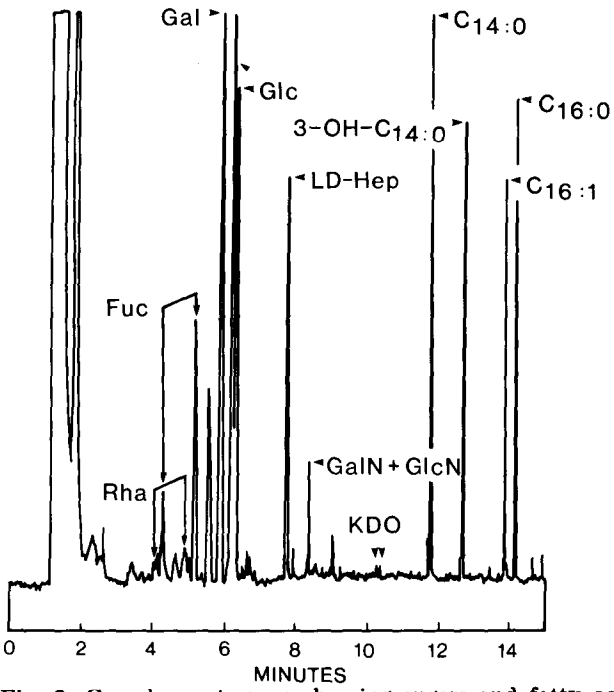

Fig. 2. Gas chromatogram showing sugars and fatty acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates of *H. aphrophilus* strain ATCC 33389 (NCTC 5906). Abbreviations as in Table I footnote.

of cellular fatty acids did not differ markedly. This agreed with our previous observations on bound cellular acids [5], and fatty acids in whole LPS and free lipid A from the same strains [6]. Trifluoroacetyl derivatives of methyl glycosides, on the other hand, provided excellent criteria of differentiation. Whereas *A. actinomycetemcomitans* contained both D-glycero- and L-glycero-D-manno-heptose, *H. aphrophilus* contained exclusively L-glycero-D-mannoheptose. This was in agreement with our previous findings on the sugar composition of LPS [8] and of whole defatted cells [9] from a series of reference as well as laboratory strains of *A. actinomycetemcomitans* and *H. aphrophilus*. D-Glycero-D-mannoheptose may therefore serve as a marker for taxonomic differentiation between these bacteria. Our findings on the distribution of sugars in LPS [8], in whole defatted cells [9], and in whole-cell methanolysates, as well as free cellular fatty acids [7] support the establishment of *A. actinomycetemcomitans* as a species distinct from *H. aphrophilus* in the 1984 edition of Bergey's Manual of Systematic Bacteriology [22].

Methanolic hydrochloric acid is assumed to be a mild and effective agent for cleaving oligosaccharides (for review, see refs. 23 and 24). However, trifluoroacetic acid may attack the methylene group between double bonds causing loss of polyunsaturated components [25]. This may affect cyclopropane fatty acid, which was detected in whole cells [5] and whole LPS and free lipid A [6] of *A. actinomycetemcomitans* and *H. aphrophilus* but not in their whole-cell methanolysates.

The ratio between glucose and L-glycero-D-mannoheptose was approximately the same in whole-cell methanolysates, whole defatted cells, and whole LPS

prepared from *A. actinomycetemcomitans* and in LPS made from *H. aphrophilus* [8, 9]. This suggested that LPS is the primary source of this aldoheptose in *A. actinomycetemcomitans* and *H. aphrophilus*. The higher concentration of galactose and glucose in *H. aphrophilus* than in *A. actinomycetemcomitans* agreed with previous results obtained with whole LPS and whole defatted cells [8, 9].

LPS and whole defatted cells, enabling differentiation by means of D-glycero-D-mannoheptose, seem to be excellent preparations for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*, but they involve rather time-consuming laboratory procedures. Whole-cell methanolysis would therefore be preferable in the routine laboratory. With our experimental set up, little material, i.e. less than 1 mg of lyophilized cells, was needed and both methanolysis and derivatization could be performed rapidly. The peak ratio reproducibility and the stability of the derivatives were good [8, 9].

CONCLUSIONS

(1) D-Glycero-D-mannoheptose may serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

(2) Gas chromatography of trifluoroacetylated whole-cell methanolysates may serve as a rapid method for differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

(3) Establishment of *A. actinomycetemcomitans* in current taxonomy as a species distinct from *H. aphrophilus* is supported.

ACKNOWLEDGEMENTS

This work was supported in part by the Anders Jahres Fond til Vitenskapens Fremme and by the Proprietær Christopher Øverland and Hustru Ingeborg Øverlands Legat. The authors are also indebted to Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., for generous supply of *Ch. violaceum* and N-glucosamine myristate.

REFERENCES

1 M. Kilian and C.R. Schiött, Arch. Oral Biol., 20 (1975) 791.
2 A.C.R. Tanner, R.A. Visconti, S.S. Socransky and S.C. Holt, J. Periodontol. Res., 17 (1982) 585.
3 J.J. Zambon, L.A. Christersson and J. Slots, J. Periodontol., 54 (1983) 707.
4 J. Slots, Arch. Microbiol., 131 (1982) 60.
5 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 282.
6 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 19.
7 I. Brondz and I. Olsen, J. Chromatogr., 278 (1983) 13.
8 I. Brondz and I. Olsen, J. Chromatogr., 310 (1984) 261.
9 I. Brondz and I. Olsen, J. Chromatogr., 311 (1984) 31.
10 W. Weidel, Z. Physiol. Chem., 299 (1955) 253.
11 M.L. Osborn, S.M. Rosen, L. Tothfield and B.L. Horecker, Proc. Nat. Acad. Sci. U.S., 48 (1962) 1831.
12 D.A.L. Davies, Nature, 180 (1957) 1129.

13 I. Ishizuka, N. Ueta and T. Yamakawa, Jap. J. Exp. Med., 36 (1966) 73.
14 E. Kondo and N. Ueta, J. Bacteriol., 110 (1972) 459.
15 Y. Okami, M. Hamada and N. Ueta, in H. Iizuka and T. Hasegowa (Editors), Culture Collections of Microorganisms. Proceedings of the International Conference on Culture Collections, Tokyo 1968, University Park Press, Tokyo, 1970, pp. 457—475.
16 T. Yamakawa and N. Ueta, Jap. J. Exp. Med., 34 (1964) 361.
17 K. Bøvre, R. Hytta, E. Jantzen and L.O. Frøholm, Acta Pathol. Microbiol. Scand. Sect. B, 80 (1972) 683.
18 L.O. Frøholm, E. Jantzen, R. Hytta and K. Bøvre, Acta Pathol. Microbiol. Scand. Sect. B, 80 (1972) 672.
19 E. Jantzen, L.O. Frøholm, R. Hytta and K. Bøvre, Acta Pathol. Microbiol. Scand. Sect. B, 80 (1972) 660.
20 E. Jantzen, K. Bryn and K. Bøvre, Acta Pathol. Microbiol. Scand. Sect. B, 82 (1974) 753.
21 E. Jantzen, K. Bryn, N. Hagen and K. Bøvre, NIPH Ann., 1 (1978) 59.
22 J.E. Phillips, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 570—576.
23 S.C. Churms, in E. Heftmann (Editor), Chromatography — Fundamentals and Applications of Chromatographic and Electrophoretic Methods, Part B: Applications, [Journal of Chromatography Library, Vol. 22B], Elsevier, Amsterdam, 1983, p. B262.
24 K. Bryn and E. Jantzen, J. Chromatogr., 240 (1982) 405.
25 R. Wood and F. Snyder, Lipids, 1 (1966) 62.