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REVIEW

CHEMOTAXONOMY OF SELECTED SPECIES OF THE *ACTINOBACILLUS—HAEMOPHILUS—PASTEURELLA* GROUP BY MEANS OF GAS CHROMATOGRAPHY, GAS CHROMATOGRAPHY—MASS SPECTROMETRY AND BIOENZYMATIC METHODS*

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CONTENTS

1. Introduction 2
   1.1. Bacterial taxonomy 2
   1.2. Fatty acids 2
   1.3. Sugars 3
   1.4. Proteins and enzymes 4
   1.5. *Pasteurellaceae* 4
2. Experimental 6
   2.1. Methanolysis and trifluoroacetic acid anhydride derivatization 6
   2.2. Sample size 6
   2.3. Lipopolysaccharide 6
   2.4. Whole defatted cells 6
   2.5. Whole cells 8
   2.6. Single bacterial colonies 8
   2.7. Gas chromatography 9
   2.8. Mass spectrometry 10

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3. Results and discussion 10
3.1. Differentiation by means of cellular acids. 10
3.2. Differentiation by means of cellular sugars 10
3.3. Chemotaxonomy of a single bacterial colony 12
3.4. Differentiation by means of cellular proteins 12
3.5. Differentiation by means of cellular hydrogenase. 12
3.6. Differentiation by means of lysozyme and EDTA 13
3.7. Biomedical applications 14
4. Conclusions 14
5. Acknowledgements 15
6. Summary 15
References 15

1. INTRODUCTION

1.1. Bacterial taxonomy

Taxonomy, which applies numeric, genetic and chemical methods, as well as serology and analysis of nucleic acids as tools, includes classification, nomenclature and identification of microorganisms. Despite the rapid development of bacterial taxonomy during the last decades, it still has certain shortcomings, and existing classification schemes should be considered as only interim, not final arrangements. Current taxonomy is based on a variety of methods with large differences in resolution [1]. This makes it difficult to compare results. Many efforts have been made to classify specific bacteria, whereas other bacteria have been studied less intensively, and may therefore have been given more random assignments to groups. Classification of microorganisms has been hampered by the fact that several bacteria do not seem to grow in liquid media, and cannot, therefore, be subjected to conventional studies. There is no "official" classification of bacteria among bacteriologists [2]. Probably, Bergey's manual comes closest, but even this publication is not unanimously accepted by the community of microbiologists. Bergey's manual has also tended to become obsolete in parts because new information has accumulated more rapidly than new editions have appeared.

Chemotaxonomy is concerned with the elucidation of the chemical composition of whole bacterial cells or parts of them. Techniques such as analysis of fermentation products and enzyme systems and their regulations are also included in efforts made to demonstrate compounds that are specific, characteristic or unique for a particular group or species of bacteria.

1.2. Fatty acids

Fatty acids are among the components most commonly subjected to chemotaxonomic examination. They are included in the group of substances designated as lipids, which are rather heterogeneous in bacteria. In some cases, the fatty acid pattern may characterize a particular taxon (for a review, see ref. 3). Several external factors may influence the fatty acid composition of bacteria, such as medium, temperature, age of the culture and analytical techniques [4]. Therefore, fatty acid analyses require careful standardization, and

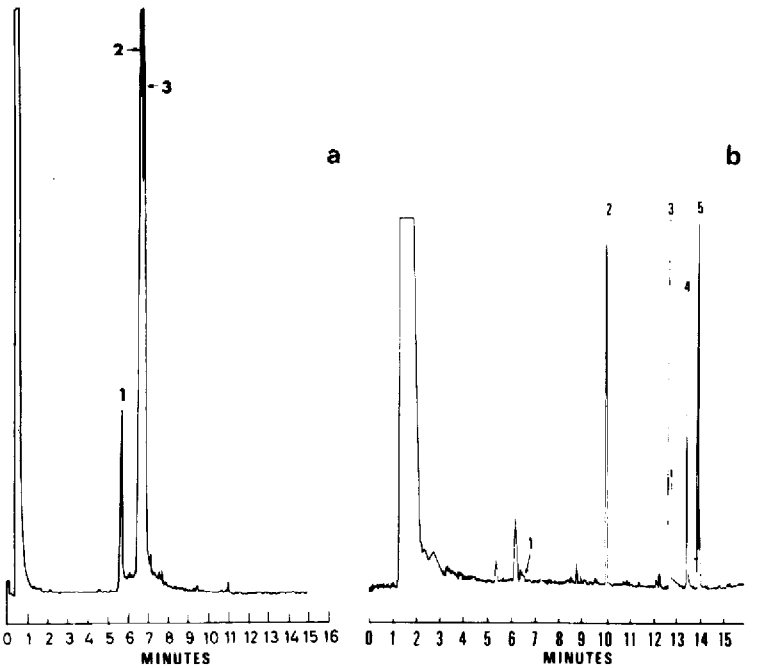

Fig. 1. (a) Gas chromatogram of strain FDC 511, characteristic of free fatty acids in group I of *A. actinomycetemcomitans* and *H. aphrophilus* (from ref. 8). Peaks: 1 = $C_{14:0}$; 2 = $C_{16:1}$; 3 = $C_{16:0}$. (b) Gas chromatogram of bound fatty acids (methyl esters) from *A. actinomycetemcomitans* and *H. aphrophilus*, as represented by *A. actinomycetemcomitans* strain FDC 2112 (from ref. 57). Peaks: 1 = $C_{12:0}$; 2 = $C_{14:0}$; 3 = 3-OH-$C_{14:0}$; 4 = $C_{16:1}$; 5 = $C_{16:0}$.

qualitative rather than quantitative differences in the fatty acid content should be used as taxonomic criteria. Fatty acids can be present in bacteria in a free or bound form. Bound fatty acids cannot be extracted with organic solvents and require hydrolysis for removal from the cellular matrix [5, 6]. After hydrolysis of whole bacterial cells, as is being performed routinely in bacterial chemotaxonomy, free fatty acids can no longer be separated from bound fatty acids. Free fatty acids, if present, should be analysed separately because they may differ from bound acids (Fig. 1) and thus contribute to taxonomic differentiation. Hitherto, little interest has been paid to free fatty acids as diagnostic tools in bacteriology. In the present studies, free bacterial fatty acids were examined directly in a gas chromatograph, probably for the first time with a capillary column [7, 8].

1.3. Sugars

The sugar composition of the bacterial cell may help to distinguish between species [9]. Carbohydrate analyses have been made with whole microbial cells, cell walls, capsular polysaccharides and lipopolysaccharide (LPS). According to our findings, the composition of sugars in bacterial cells seems to be less influenced by external factors than are fatty acids. Several methods can be used to analyse bacterial sugars. Analysis of alditol acetates is frequently preferred. This procedure has the drawbacks of involving several steps and

requires aqueous depolymerization of oligosaccharides, which gives more decomposition products than does depolymerization in hydrochloric acid–anhydrous methanol [10]. Methanolysis does not cause significant destruction of sugars. Most glycosidic linkages are quantitatively broken and the reduced monosaccharides are stabilized as methyl glycosides [11, 12]. Trifluoroacetates are more volatile than both alditol acetates and trimethyl ethers, and chromatographic separation can therefore be carried out at lower temperatures [13, 14].

1.4. Proteins and enzymes

Closely related bacteria have similar or identical cellular proteins. Detailed protein profiles from bacterial cells can best be obtained by high-resolution, two-dimensional protein electrophoresis, by which up to 1200 different proteins have been resolved [15]. The protein pattern produced in this way is an expression of the genetic background of the examined strains, and thus can be used as an indication of relatedness.

Functional and structural patterns of certain bacterial enzymes, e.g. citrate synthases and succinate thiokinases, have proved to be useful in bacterial classification (for a review, see ref. 16). Not many investigations have been made so far in this field, but it is likely that the regulatory and molecular properties of enzymes will become of increasing value in chemotaxonomy. Even less attention has been devoted to enzymes of non-bacterial origin in taxonomy, despite the fact that such enzymes are often used by various hosts to counteract bacterial propagation. The present review will focus upon the potentialities of bacterial hydrogenases [17] and the non-bacterial enzyme lysozyme [18] in taxonomic work.

1.5. Pasteurellaceae

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are coccobacillary, capnophilic, Gram-negative rods normally present in dental plaque, although in small amounts. Before and during the development of gingivitis and periodontitis, the concentration of *A. actinomycetemcomitans* increases, particularly in localized juvenile periodontitis. This is a distinct clinical entity of developing periodontitis, which affects the supporting tissues of incisors and first molars in young people, and has been seen within families [19]. *A. actinomycetemcomitans* and *H. aphrophilus* isolated from localized juvenile periodontitis were originally included in group III and IV among five groups of bacterial species that were difficult to classify and identify [20].

There are several findings suggesting that *A. actinomycetemcomitans* is the major agent in localized juvenile periodontitis [21- 26]. The role that *H. aphrophilus* plays in the development of diseases in the supporting tissues of teeth is not clear. A common view up to now has been that *Haemophilus* species have a low periodontopathogenic potential. Both *A. actinomycetemcomitans* and *H. aphrophilus* may cause a number of diseases outside the oral cavity, some of them serious, e.g. endocarditis [27] and brain abscess [28]. Fastidious Gram-negative, facultatively anaerobic rods constituted 57% of the Gram-negative agents of bacterial endocarditis, among which *H. aphrophilus*,

other haemophili and *A. actinomycetemcomitans* were the most common organisms [29].

It may be difficult to differentiate between *A. actinomycetemcomitans* and *H. aphrophilus* in the microbial laboratory. Up to 1984, *A. actinomycetemcomitans* was listed in Bergey's manual as a species *incertae sedis* [30]. The taxonomic uncertainty existed especially in the relationship to *H. aphrophilus.* Some authors believed that *A. actinomycetemcomitans* and *H. aphrophilus* were the very same bacteria, and it had been suggested that *A. actinomycetemcomitans* should be included in the genus *Haemophilus* [31]. *A. actinomycetemcomitans* and *H. aphrophilus* are organisms with identical morphology and ultrastructure [32]. Relatively few biochemical tests were available for differentiation between them. The taxonomic studies that followed were unable to demonstrate differences in the total content of cellular fatty acids in these bacteria (e.g. ref. 33). With the great similarity in morphology, biochemical reactions and lipid content between *A. actinomycetemcomitans* and *H. aphrophilus*, these bacteria might be confused in clinical samples.

H. paraphrophilus is found as a member of the normal flora of the oral cavity and pharynx [34]. It may cause subacute endocarditis, paronychia and brain abscess, and has been isolated from osteomyelitis of the jaw, inflamed appendix, urine and vagina [35].

H. influenzae type b is a leading cause of bacterial meningitis in children, but it may also cause other diseases such as cellulitis, pneumonia, epiglottidis, septic arthritis and bacteriaemia (for a review, see ref. 36).

Pasteurellae are parasitic on the mucous membranes of the upper respiratory and digestive tracts of mammals (rarely man) and birds [37]. In humans, *P. multocida* has been reported as an opportunistic or secondary invader in a wide variety of sporadic infections: meningitis, encephalitis, otitis, septicemia, sinusitis, peritonitis, bronchiectasis and arthritis [38–40], and is more often involved in human infections than previously thought. *P. haemolytica* has been associated with only a few infections of man [37], and *P. ureae* with human bronchiectasis, bronchitis, pneumonia, meningitis, septicemia, sinusitis and ozaena [38].

Actinobacillus, Haemophilus and *Pasteurella* are included in the family *Pasteurellaceae*, and are now attracting renewed interest in microbiology [41]. The *Actinobacillus–Haemophilus–Pasteurella* group is taxonomically a vexed group. It may be problematic not only to distinguish between *A. actinomycetemcomitans* and *H. aphrophilus*, but also between *H. aphrophilus* and *H. paraphrophilus* [42, 43]. *H. influenzae* may be confused with *P. multocida* [44]. Furthermore, it has been claimed that the current classification of recognized actinobacilli and pasteurellas does not allow differentiation of the two genera, and their genetic relationship has shown that several species assigned to the genus *Pasteurella* are more closely related to *Actinobacillus* [45].

The aim of the present studies has been to develop chemotaxonomic methods for more rapid, accurate and specific differentiation between species of the *Actinobacillus–Haemophilus–Pasteurella* group, with emphasis on *A. actinomycetemcomitans* and *H. aphrophilus*. The present review will focus upon our own investigations in this field. A more comprehensive review on bacterial chemotaxonomy in general will be given elsewhere [46].

2. EXPERIMENTAL

2.1. Methanolysis and trifluoroacetic acid anhydride derivatization

Methanolysis and trifluoroacetic acid anhydride derivatization are not new procedures in microbiology. In the present experiments, we tried to improve existing routines [47—52]. Advances in derivatization procedures would be significant contributions to a more widespread use of gas chromatography (GC) in the clinical routine laboratory. Current procedures involve derivatization of methanolysates in a rather strong concentration of trifluoroacetic acid anhydride in acetonitrile (1:1), which is then diluted to 10% by addition of acetonitrile before GC analysis [11]. This 1:1 mixture of trifluoroacetic acid anhydride in acetonitrile may be difficult to analyse. In the present studies [50–52], it was found that a 1:3 mixture is optimal for derivatization before GC analyses. The fact that dilution could be avoided enabled a large number of samples to be analysed within a short time. Methanolysis and derivatization were carried out in the same vial without transfer of material. Preservation of material is important when small samples are analysed. In comparison, the alditol acetate method requires extraction. This is difficult to perform with small quantities of material and tends to prolong the experiment.

2.2. Sample size

Another striking feature of the present studies was the gradual decrease in sample size achieved, from the gram level used to make LPS, through the milli- to microgram levels used for analysis of whole cells, to the nanogram level of a single colony [47, 48, 52]. The single colony technique will enable large-scale analyses to be carried out in the routine laboratory within a short period of time and with a considerable reduction in media and technical assistance.

2.3. Lipopolysaccharide

Phenol—water extraction of whole cells for preparation of LPS is a resource-consuming procedure [47]. After preparation, LPS has to be purified by high-speed centrifugation, and its yield may be rather modest. In our experiments, phenol—water extraction provided chemically stable preparations, where the sugar composition gave precise differentiation between *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus* [47, 51] (Fig. 2).

2.4. Whole defatted cells

These preparations are intermediates between whole cells and isolated cell membranes. Whole defatted cells provided simpler gas chromatograms than did whole cells [48–50]. Methanolysed and trifluoroacetic acid anhydride derivatized whole defatted cells provided sugar patterns in *A. actinomycetemcomitans* and *H. aphrophilus*, enabling precise GC differentiation (Fig. 3). Defatting can be recommended as a modification of cells, which may facilitate identification and classification of relatively unknown bacteria.

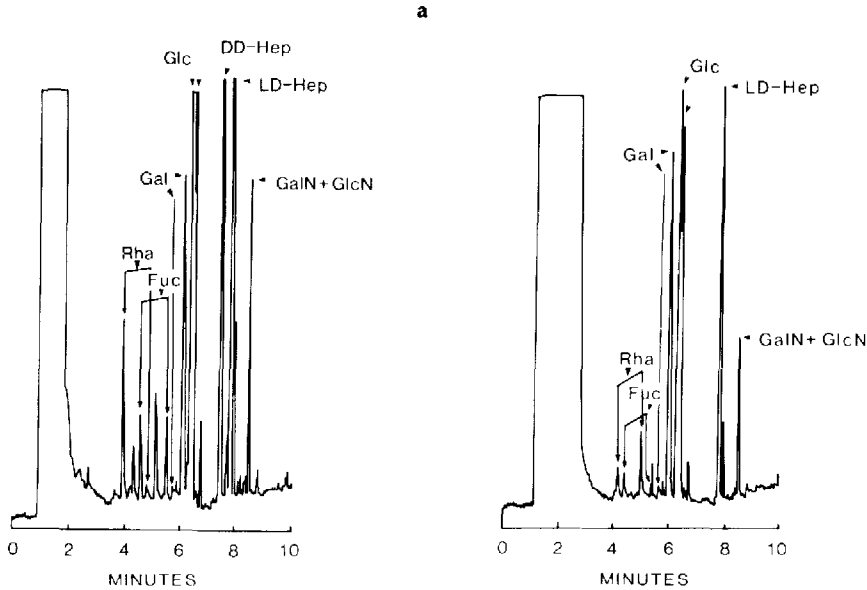

Fig. 2. (a) Typical gas chromatogram of the sugar composition in LPS from *A. actinomycetemcomitans*, as represented by strain ATCC 33384. (b) Typical gas chromatogram of the sugar composition in LPS from *H. aphrophilus*, as represented by strain ATCC 33389. Peaks: Rha = rhamnose; Fuc = fucose; Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose; GalN = galactosamine; GlcN = glucosamine. For chromatographic settings, see ref. 47. (From ref. 47.)

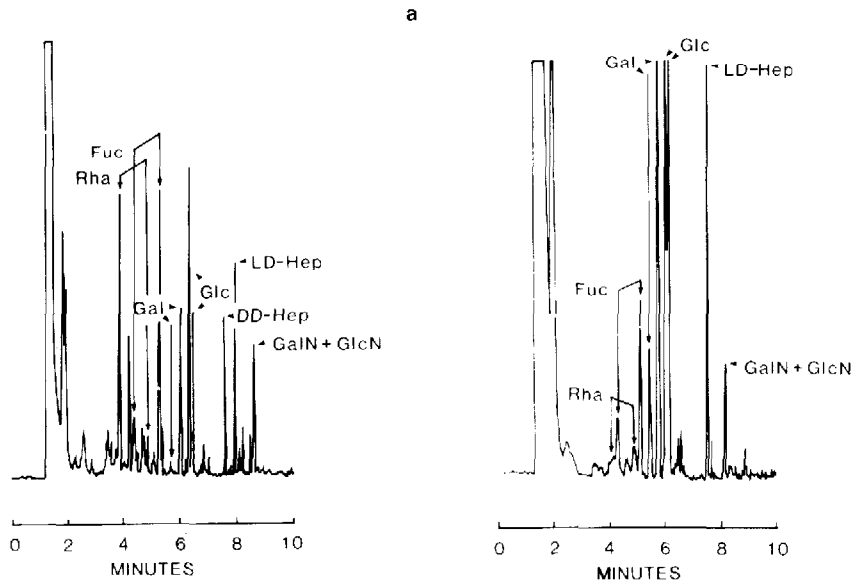

Fig. 3. (a) Typical gas chromatogram of the sugar composition of whole defatted cells of *A. actinomycetemcomitans*, as represented by strain ATCC 33384. (b) Typical gas chromatogram of the sugar composition of whole defatted cells of *H. aphrophilus*, as represented by strain ATCC 33389. Peaks: Rha = rhamnose; Fuc = fucose; Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose; GalN = galactosamine; GlcN = glucosamine. For chromatographic settings, see ref. 48. (From ref. 48.)

2.5. Whole cells

GC of methanolysed and trifluoroacetic acid anhydride derivatized whole cells provided chromatograms with a multitude of peaks [49, 50]. Their identification may be difficult, particularly when the constituents of the bacterial cells are unknown. However, for differentiation between closely related bacteria of the *Actinobacillus—Haemophilus—Pasteurella* group, whole-cell methanolysates would be well fitted in the clinical routine laboratory (Fig. 4). While the sugar patterns of these bacteria provided clear separation, their cellular fatty acids were of limited value [50].

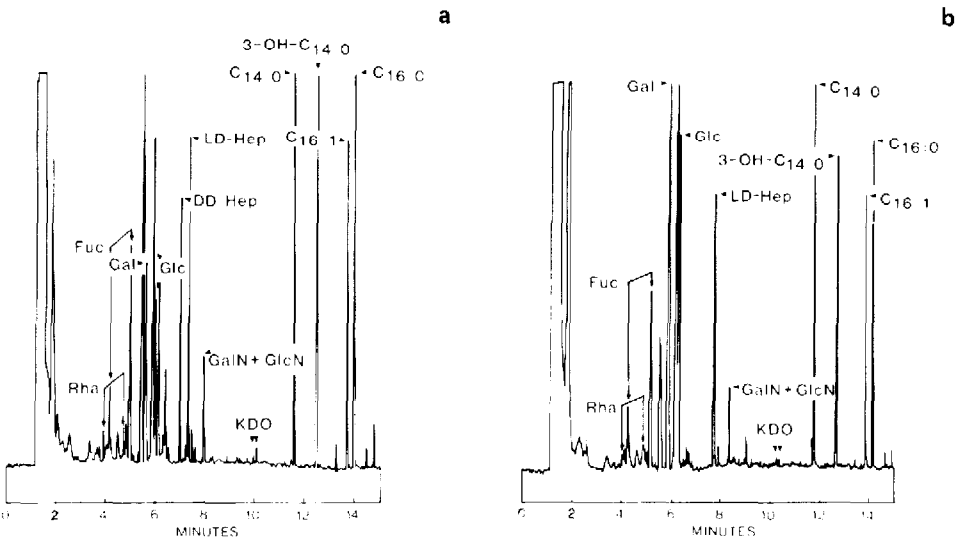

Fig. 4. (a) Gas chromatogram of sugars and acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates from *A. actinomycetemcomitans* strain ATCC 33384. (b) Gas chromatogram of sugars and acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates of *H. aphrophilus* strain ATCC 33389. Peaks: 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 methanolysed products; $C_{14:0}$ = myristic acid; 3-OH-$C_{14:0}$ = β-hydroxymyristic acid; $C_{16:1}$ = palmitoleic acid; $C_{16:0}$ = palmitic acid. For chromatographic settings, see ref. 49. (From ref. 49.)

2.6. Single bacterial colonies

Previously, a single bacterial colony has been considered of little value in chemotaxonomy because it contains too little biomass for analysis. Nevertheless, certain biochemical tests are being performed routinely with single colonies. In our investigations it was possible to analyse separately single colonies from *H. aphrophilus* with GC [52] (Fig. 5). The sugars and fatty acids detected in liquid cultures of *H. aphrophilus* were also detected in solid-grown cells [50, 52]. The amount of fatty acids in solid-grown cells was lower than that of sugars. Solid-grown cultures contained a number of unidentified substances, probably extracellular exopolymeric material. There was a larger

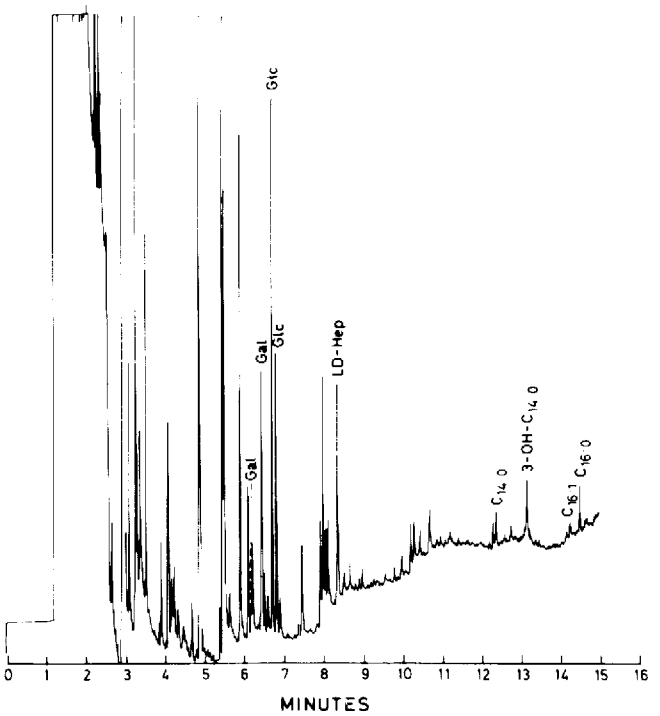

Fig. 5. Gas chromatogram of a single methanolysed and trifluoroacetic acid anhydride derivatized colony from *H. aphrophilus* strain ATCC 33389. Peaks: Gal = galactose; Glc = glucose; LD-Hep = L-glycero-D-mannoheptose; $C_{14:0}$ = myristic acid; 3-OH-$C_{14:0}$ = β-hydroxy-myristic acid; $C_{16:1}$ = palmitoleic acid; $C_{16:0}$ = palmitic acid. For chromatographic settings, see ref. 52. (From ref. 52.)

variation in the sugar and fatty acid content of solid cultures than of liquid cultures from *H. aphrophilus*.

2.7. Gas chromatography

The major advantages of GC are that it rapidly separates complex mixtures regardless of origin, at extreme sensitivity (down to picogram levels) and at relatively small costs, producing large amounts of data within a short time [53]. Already at the onset of gas–liquid chromatography, efforts were made to analyse free fatty acids directly. The main problem to overcome was the requirement of liquid phases to operate at high temperatures, molecular association of acids in the vapour state and adsorption on the column (for a review, see ref. 54). The last few years have seen a considerable improvement in GC columns, enabling stabilization of the stationary phase under relatively high temperatures. Improvements in capillary columns with the development of temperature-resistant stationary phases have led to satisfactory separation and symmetry of peaks from a mixture of fatty acids. In the present investigations, both saturated and unsaturated fatty acids were well resolved on capillary columns without peak tailing and significant adsorption [7, 8].

2.8. Mass spectrometry

Mass spectrometry (MS) is useful for confirmation of identities during structural analysis of microbial cells. Fragmentation of fatty acids gives a molecular ion, M^+, at an abundance of approximately 12% of the base peak, which may assist in their identification [8]. The fragment with *m/e* 60, which is acetic acid, is also characteristic of fatty acids and is a result of the McLafferty rearrangement [55]. The abundance (% of base peak) of this fragment in the spectrum of $C_{14:0}$ acid was 62.8%, of $C_{16:0}$ acid 62.1%, of iso-$C_{15:0}$ acid 50.2% and of $C_{16:1}$ acid 20.3% [8]. The differences in the percentage distribution of these fragments may be used as diagnostic criteria of bacterial fatty acids.

MS is also the most powerful identification technique for microbial sugars. It rarely provides the molecular ion, M^+, of monosaccharides, as it does with all polyols, because of molecular disintegration under electron bombardment. Yet the fragmentation pattern is highly characteristic of the sugar and also reveals whether a peak is due to a furanoside or pyranoside [56]. In the present studies, fragmentation schemes for heptoses from *A. actinomycetemcomitans* and *H. aphrophilus* were set up [47]. Fragmentations of trifluoroacetylated methyl glycosides were also used to identify other sugars.

3. RESULTS AND DISCUSSION

3.1. Differentiation by means of cellular acids

Usually, bacterial fatty acids are liberated by hydrolysis and derivatized before GC analysis. Unfortunately, both hydrolysis and derivatization may cause artifacts. In our experiments [7, 8], free fatty acids were extracted directly from the cells of *A. actinomycetemcomitans* and *H. aphrophilus*. By using a column consisting of a non-polar stationary phase, it was possible to analyse free fatty acids directly in the gas chromatograph without derivatization [7, 8]. While the investigated strains of *H. aphrophilus* were homogeneous in their fatty acid composition, *A. actinomycetemcomitans* strains could be divided into three groups [8]. The established technique may be useful in future taxonomic work with bacteria, provided that free fatty acids are present, owing to its simplicity, reproducibility and sensitivity.

It was not possible to differentiate taxonomically between *A. actinomycetemcomitans* and *H. aphrophilus* on the basis of bound cellular fatty acids [57]. The content of bound fatty acids, however, differed markedly from that of free fatty acids in both organisms (Fig. 1).

3.2. Differentiation by means of cellular sugars

The sugar content of LPS from *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus* differed markedly [47, 51]. All species contained rhamnose, fucose, galactose, glucose, galactosamine, glucosamine and L-glycero-D-mannoheptose. The content of galactose was approximately twice as high in LPS from *H. aphrophilus* as in 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. Contrary to LPS from *A. actinomycetemcomitans*, LPS from *H. paraphrophilus* contained only trace amounts of rhamnose and fucose. D-Glycero-D-mannoheptose was detected exclusively in LPS from *A. actinomycetemcomitans* (11.8—16.7%) and *H. paraphrophilus* (8.0%). This aldoheptose could therefore be used as a taxonomic marker.

Differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* was also made from the presence of sugars in whole defatted cells [48]. Defatting was made to produce more surveyable gas chromatograms (excluding the fatty acid profiles) and to reduce the time required for analysis. Defatting is also more easy to perform than preparing LPS and was achieved by extraction of free and bound fatty acids before the sugar analyses started. Whole defatted cells of both *A. actinomycetemcomitans* and *H. aphrophilus* contained rhamnose, fucose, galactose, glucose, galactosamine, glucosamine and L-glycero-D-mannoheptose. Contrary to *H. aphrophilus*, *A. actinomycetemcomitans* also contained D-glycero-D-mannoheptose. Both L- and D-glycero-D-mannoheptose were probably located in the LPS of these bacteria. *A. actinomycetemcomitans* seemed to have LPS as the only source of glucose. *H. aphrophilus* probably had glucose also in its microcapsule or as glycogen. The present study on sugars in whole defatted cells did not provide any clear basis for establishment of chemical groups corresponding to serogroups suggested for *A. actinomycetemcomitans.*

A. actinomycetemcomitans and *H. aphrophilus* were also distinguished by whole-cell methanolysates [49]. A characteristic of these preparations is that their sugar and fatty acid profiles are recorded on the same gas chromatogram. The present method was fast, sensitive, required little material and may be well fitted for differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* in the clinical routine laboratory. The study with whole-cell methanolysates confirmed our previous findings concerning distribution and differences in the content of sugars and fatty acids in *A. actinomycetemcomitans* and *H. aphrophilus.*

The system with whole-cell methanolysates was simplified later. At the same time, a series of clinically important species of the *Actinobacillus—Haemophilus—Pasteurella* group were included in the study [50]. These species, which constitute the family *Pasteurellaceae*, are isolated not only more frequently than in the past [58], but they are also recovered from sources previously considered unusual. Differentiation between species within *Pasteurellaceae* often meets with problems, and several bacteria have been isolated in this family which do not fit existing classification schemes. Our observations have facilitated differentiation between species of this family. It proved that *H. aphrophilus* could be differentiated from all the other species, i.e. *A. actinomycetemcomitans*, *H. paraphrophilus*, *H. influenzae* type b, *P. haemolytica*, *P. multocida*, and *P. ureae*, because it lacked D-glycero-D-mannoheptose. The pattern of cellular sugars in *P. ureae* and *P. haemolytica* resembled that seen in *A. actinomycetemcomitans*. It has been suggested that *P. ureae* and *P. haemolytica* should be transferred to *Actinobacillus* [37]. Whereas *P. multocida* showed only trace amounts of D-glycero-D-manno-

heptose, *P. ureae* and particularly *P. haemolytica* contained significant amounts of this taxonomic marker. *P. multocida* also differed from the other species of *Pasteurellaceae* through a particularly high content of L-glycero-D-manno-heptose. *H. influenzae* type b differed from *P. haemolytica* and *P. ureae* through a lower content of D-glycero-D-mannoheptose than in these organisms, and from *P. multocida* through a higher content of this aldoheptose. In both *A. actinomycetemcomitans* and *H. paraphrophilus*, the primary source of D-glycero-D-mannoheptose was LPS [47, 51].

3.3. Chemotaxonomy of a single bacterial colony

Recently, chemotaxonomy was performed on the basis of single bacterial colonies [52]. Despite the small biomass (nanogram) of a single colony, well separated peaks providing a multitude of information on the chemical composition of *H. aphrophilus* cells were obtained on the gas chromatograms. As in liquid-grown cultures of *H. aphrophilus* [49], sugars such as galactose, glucose and L-glycero-D-mannoheptose were detected, as well as fatty acids such as myristic, 3-hydroxymyristic, palmitic and palmitoleic acid. In addition, a multitude of unidentified peaks were resolved, which probably were methanolysed and derivatized extracellular exopolymeric material. Each colony tended to have a relatively distinct chemical composition.

Chemotaxonomy on solid-grown cells may offer several advantages to liquid-grown cells. The present technique avoids the disadvantages of multiple transfers of cultures, such as mutation and loss of culture. It enables rapid microbial diagnosis and specific treatment, selection of colonies with specific chemical properties, and diagnosis of organisms that cannot be grown in liquid culture. Solid cultures also maintain physiological associations between cells. Besides, they are completely free from particulate components of their nutritional medium, relatively free from small molecular nutrients and their own metabolic products, and yield preparations less liable to artifacts than liquid cultures. Furthermore, mixed cultures can be analysed by means of single colonies.

3.4. Differentiation by means of cellular proteins

A. actinomycetemcomitans and *H. aphrophilus* have also been differentiated by means of high-resolution, two-dimensional electrophoresis of cellular proteins [59]. This investigation revealed clear differences between *A. actinomycetemcomitans* and *H. aphrophilus* in their protein patterns, although there were also great similarities. Whereas *H. aphrophilus* in all GC analyses proved to be homogeneous, the present technique made it possible to delineate specific strains not only in *A. actinomycetemcomitans* but also in *H. aphrophilus*. This raised the question of making subdivisions in these species. Differentiation between microorganisms with this sensitive technique seems to have a high potential in chemotaxonomy, particularly in closely related organisms hard to distinguish with conventional methods.

3.5. Differentiation by means of cellular hydrogenase

A method based on the ability of cellular hydrogenases to reduce the redox

indicator methylene blue was set up to assist differentiation between closely related species of the *Actinobacillus—Haemophilus–Pasteurella* group [17]. After a standard growth period, the organisms were suspended in broth supplemented with methylene blue, and incubated at 37° C under air protection in a water bath. Changes in colour were assessed by comparing the suspension at regular intervals with a pre-made five-step colour scale based on methylene blue—broth and broth. With this system, it was possible to separate *P. haemolytica* and *P. ureae* from *A. actinomycetemcomitans*, *P. multocida*, *H. influenzae* type b, *H. aphrophilus* and *H. paraphrophilus*. *P. multocida* could not be distinguished from *H. influenzae* type b. Most strains of *A. actinomycetemcomitans—H. aphrophilus*, *H. aphrophilus—H. paraphrophilus* and *A. actinomycetemcomitans—H. influenzae* type b could be differentiated with methylene blue.

3.6. Differentiation by means of lysozyme and EDTA

The ability of lysozyme and EDTA to induce bacteriolysis was examined over a 50-min period in major species of the *Actinobacillus—Haemophilus—Pasteurella* group [18]. *A. actinomycetemcomitans* was more sensitive to bacteriolysis in this model system than was *H. aphrophilus*. *A. actinomycetemcomitans* could be divided into two groups of strains according to their lysis patterns, whereas *H. aphrophilus* was homogeneous. In group I, EDTA displayed a considerable lytic effect, which was not increased by supplementation with lysozyme. In group II, the lytic effect of EDTA was much lower. On the other hand, lysozyme had a considerable lytic effect in this group. Maximal lysis of *A. actinomycetemcomitans* in the presence of EDTA occurred at pH 8.0, and with EDTA—lysozyme, at pH 7.6. *H. aphrophilus* exhibited maximal lysis in the presence of EDTA at pH 9.0, and with EDTA—lysozyme, at pH 9.2. When the other species within the family *Pasteurellaceae* (*H. influenzae* type b, *H. paraphrophilus*, *P. multocida*, *P. haemolytica*, *P. ureae*) were tested in the same bacteriolysis model, it proved that the group I strains of *A. actinomycetemcomitans* were the most sensitive to EDTA of all the organisms examined. *H. paraphrophilus* was least sensitive to EDTA in the *Actinobacillus—Haemophilus—Pasteurella* group, but not so resistant as *Micrococcus luteus*, which was used as a control. On the other hand, *M. luteus* was the most sensitive to lysozyme of all the organisms examined, followed by *P. ureae* and the group II strains of *A. actinomycetemcomitans*. The group I strains of *A. actinomycetemcomitans*, *H. paraphrophilus* and *P. haemolytica* were least sensitive to lysozyme. The lysis pattern of *P. ureae* was identical to that of the group II strains of *A. actinomycetemcomitans*. Also, this finding supported the idea of transferring *P. ureae* to the genus *Actinobacillus*. Variations in the composition of cellular peptidoglycan, on which lysozyme acts specifically, are presently of taxonomic significance among Gram-positive bacteria. The results of the present study indicated that it may be worthwhile also to investigate the peptidoglycan composition of Gram-negative bacteria under classification and identification. It appeared that the present bacteriolysis system may assist in the differentiation between closely related species within *Pasteurellaceae*.

3.7. *Biomedical applications*

The present studies have made the clinical microbiologist more able to perform accurate and specific classification of major species within the *Actinobacillus—Haemophilus—Pasteurella* group. First of all, this may be achieved by using D-glycero-D-mannoheptose as a taxonomic marker. A more objective parameter for bacterial distinction can hardly be wished for. Precise bacterial diagnosis will enable better clarification of the individual roles of species within the *Actinobacillus—Haemophilus—Pasteurella* group in distinct clinical entities either in or outside the oral cavity. Secondly, the clinical microbiologist has been furnished with a set of methods, instrumental analytical, as well as bioenzymatic, to perform chemotaxonomy in the vexed family *Pasteurellaceae*. Some of these methods, e.g. the bioenzymatic methods, should be used only as supplements to conventional tests to assist classification and identification. GC analyses of single colonies and high-resolution, two-dimensional electrophoresis may become customary techniques of the clinical microbial laboratory in the future.

4. CONCLUSIONS

The present studies seemed to warrant the following conclusions.

(1) Soxhlet extraction is recommended for direct extraction of taxonomically important long-chain saturated, unsaturated, branched and unbranched free fatty acids from whole bacterial cells. These acids can be analysed directly by GC without derivatization.

(2) Bound fatty acids did not differ in *A. actinomycetemcomitans* and *H. aphrophilus*.

(3) Free and bound fatty acids differed markedly within *A. actinomycetemcomitans* and *H. aphrophilus*.

(4) GC of trifluoroacetylated whole-cell methanolysates may serve as a rapid method for differentiation between species within the *Actinobacillus—Haemophilus—Pasteurella* group.

(5) The sugar composition of cells from the *Actinobacillus—Haemophilus—Pasteurella* group had a greater potential for chemotaxonomic differentiation than had the fatty acid composition.

(6) D-Glycero-D-mannoheptose, which was absent in *H. aphrophilus*, may be used to differentiate this organism from other species of the *Actinobacillus—Haemophilus—Pasteurella* group, such as *A. actinomycetemcomitans*, *H. paraphrophilus*, *H. influenzae* type b, *P. haemolytica*, *P. multocida* and *P. ureae*, where it was present.

(7) LPS was the major localization of D-glycero- and L-glycero-D-mannoheptose in *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*.

(8) GC analysis of a single colony is a new method in bacterial chemotaxonomy with a promising future, which even enables analysis of organisms in a mixed culture.

(9) Cellular hydrogenase may be used as a supplementary criterion in the taxonomic differentiation between closely related species of the *Actinobacillus—Haemophilus—Pasteurella* group.

(10) Bacteriolysis induced by lysozyme and EDTA may serve as additional criteria in the differentiation between species of the *Actinobacillus—Haemophilus—Pasteurella* group.

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6. SUMMARY

Instrumental analytical and bioenzymatic methods were used to differentiate between species of the *Actinobacillus—Haemophilus—Pasteurella* group. Long-chain fatty acids were analysed directly with gas chromatography (GC) without derivatization. GC of trifluoroacetylated whole-cell methanolysates was a rapid method for differentiation. Cellular sugars were more suitable for differentiation than fatty acids. D-Glycero-D-mannoheptose, the major localization of which was lipopolysaccharide, distinguished *H. aphrophilus* from *A. actinomycetemcomitans*, *H. paraphrophilus*, *H. influenzae* type b, *P. haemolytica*, *P. multocida*, and *P. ureae*. GC of single colonies, which is a new chemotaxonomic method, was preferable to GC of liquid-grown cells. Lysozyme- and EDTA-induced bacteriolysis and reduction of methylene blue by cellular hydrogenase served as additional criteria for differentiation.

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