Comparison of the fatty acid profiles of *Borrelia*, *Serpulina* and *Leptospira* species

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Fatty acid methyl ester (FAME) derivatives were examined as a means of characterizing *Borrelia burgdorferi* isolates and distinguishing them from other spirochaetes. Analysis was performed using a gas liquid chromatography column in conjunction with Microbial Identification System (MIS) software. Reproducible FAME profiles were produced which distinguished *Borrelia* species, *Serpulina hyodysenteriae* and *Leptospira icterohaemorrhagiae*. Furthermore, the FAME profiles of four recognized *Borrelia* species (including two American isolates of *Borrelia burgdorferi*, B31 and JD1) were distinct from one another and from the BSK II medium in which they were grown. The results confirm previous reports that FAME profiles of bacteria represent a diagnostic phenotypic property and suggest that they may have applications in the chemotaxonomic classification of *Borrelia* species.

Introduction

Many workers have attempted the physical and genetic characterization of the spirochaetes. Their cellular ultrastructure is unique amongst the eubacteria (Canale-Parola, 1991) and they have several distinguishing features. The spirochaetal outer sheath has some features analogous to the outer membrane of Gram-negative bacteria and spirochaetes also possess internal periplasmic flagella which are organs of motility (Paster & Canale-Parola, 1980). The number of flagella varies according to the spirochaete species (Paster *et al.*, 1991). Spirochaetes are resistant to the antibiotic rifampin (Leschine & Canale-Parola, 1986) and possess ornithine in their cell wall peptidoglycan (with the exception of the *Leptospira*) (Joseph *et al.*, 1973).

Classification of spirochaetes has relied largely upon the use of genetic methods. rRNA oligonucleotide cataloguing has classified the spirochaetes into five major groups: *Treponema*, *Spirochaeta*, borrelias, leptospirae and *Treponema hyodysenteriae* (Paster *et al.*, 1984). Subsequently *Treponema hyodysenteriae* and *Treponema innocens* were reclassified in a new genus, *Serpulina* (Stanton *et al.*, 1991). Recent work, using analysis of

nearly complete 16S rRNA sequences has divided the spirochaetes into six groups: treponemes, *Spirochaeta*, borrelias, a spirochaete strain isolated from a shrew, *Serpulina* and *Leptospira* species (Paster *et al.*, 1991). *Borrelia burgdorferi*, the Lyme disease spirochaete, has been analysed at the phylogenetic level using DNA–DNA hybridization (Postic *et al.*, 1990), 16S rRNA sequencing (Marconi & Garon, 1992*a, b*) and multilocus enzyme electrophoresis (Boerlin *et al.*, 1992). Such methods are complex and time-consuming and, by comparison, analysis of FAME profiles is a simpler technique when used in conjunction with the MIDI microbial identification system. The *Leptospira* have been analysed using fatty acid profiles as a key for their classification (Cacciopuoti *et al.*, 1991; Moribayashi *et al.*, 1991). In this study we have used fatty acid profiles to distinguish selected members of the spirochaete family and to investigate the possibility of using the relatedness of profiles as a tool for the classification of borrelias.

Methods

Culture of bacterial strains. *Leptospira interrogans* (serogroup *icterohaemorrhagiae*) was cultured in EMJH medium as originally described by Ellinghausen & McCulloch (1965), 90% (v/v) EMJH base, 10% (v/v) EMJH supplement, 0.15% (w/v) agar, 6% (v/v) rabbit serum. The 0.15% agar and 6% rabbit serum were added immediately prior to use. The *L. icterohaemorrhagiae* were grown in 5 ml cultures at 30 °C for 7 d.

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Abbreviations: FAME, fatty acid methyl ester; MIS; microbial identification software.

Serpulina hyodysenteriae (S75/1 and B78 isolates) was cultured as described by Kent *et al.* (1988). The medium used consisted of Trypticase Soy Broth (TSB) supplemented with 0.2% (w/v) yeast extract, 0.25% (w/v) glucose, 0.05% (w/v) L-cysteine hydrochloride, 0.0001% (w/v) resazurin and 800 µg Spectam ml⁻¹ (400 µg soluble spectinomycin ml⁻¹). The medium was autoclaved, purged with deoxygenated nitrogen, stored at 4 °C and used within 7 d of preparation. Before use, the medium was supplemented with 0.2% (w/v) sodium bicarbonate and 10% (v/v) rabbit serum, under a stream of deoxygenated nitrogen with 5% (v/v) carbon dioxide. The *S. hyodysenteriae* were incubated in 2 ml cultures for 3–4 d at 37 °C then transferred to 50 ml cultures for a further 3–4 d.

Borrelia species were grown in a modified Barbour–Stoenner–Kelly medium as described by Barbour (1984). The medium contained bovine serum albumin fraction V (Boehringer) and CMRL 1066 without sodium bicarbonate and glutamine (Gibco). The media were sterilized using 0.22 µm filters. Immediately prior to use, 10% (v/v) rabbit serum and 20 ml 7% (w/v) gelatin per 100 ml were added. The media were dispensed into 10 × 100 mm tubes, where 6 ml occupied approximately 75% of the volume available. The *Borrelia* were incubated at 34 °C until a cell density of 10⁸ organisms per ml was reached. The same batches of medium enrichment (CMRL 1066) were used in all experiments.

Spirochaetes were harvested by centrifugation (1000 g, 15 min) when grown to a highly motile condition as determined by dark field microscopy. Fifty milligrams (wet wt) of spirochaetes were required for each fatty acid profile. The spirochaetes were washed three times in phosphate-buffered saline (pH 7.4) and finally resuspended in 0.2 ml phosphate-buffered saline.

Fatty acid methyl ester extraction. Long chain fatty acids extracted from bacteria require derivatization before GLC. FAME derivatives were prepared using the following procedure (Kloepper *et al.*, 1992): spirochaetes (50 mg, collected as described above) were saponified with 1 ml 3.75 M-NaOH in 50% aqueous methanol by heating at 100 °C for 30 min. Free fatty acids in the preparation were methylated with 2.0 ml 6 M-HCl in methanol, heated at 80 °C for 10 min, followed by rapid cooling to room temperature. FAMES were extracted from the aqueous phase with a 1:1 mixture of hexane and methyl t-butyl ether and the acidified lower phase discarded. The extract was washed and neutralized with 3.0 ml 0.3 M-NaOH, separation being aided by the addition of a few drops of saturated sodium chloride.

Gas liquid chromatography. FAMES were analysed with a Hewlett Packard series II gas chromatography model 5890A equipped with a 25 m × 0.22 mm × 0.33 µm phenyl methyl silicone capillary column. Gas chromatographic runs and the integration parameters, retention time, elution order and percentage of the total area of the FAME peaks were recorded via a Hewlett Packard integrator. Samples were processed with the HP 360 benchtop workstation using the Microbial Identification System software (MIS) and MIDI (Newark, Delaware) which calibrates the gas chromatograph with a commercial mixture of straight-chain saturated and hydroxy FAMES at the beginning of each analysis run and after every tenth sample [see Stead *et al.* (1992) for a description of typical conditions]. A library for the spirochaetes was constructed based on three replicates per isolate.

Reproducibility of FAME analysis. The reproducibility of FAME profiles for all the samples analysed was determined by analysing each isolate three times under standard conditions. For each peak in the chromatogram the coefficient of variation (standard deviation/mean) × 100 was calculated (Mukwaya & Welch, 1989). Peak area values for each fatty acid were calculated as percentages of the total peak area to eliminate the effect of inoculum size variation. MIS includes a statistical facility for the generation of dendrograms which groups bacteria (samples) according to the qualitative and quantitative

relatedness of FAMES. Similarity and relatedness are expressed as Euclidian distance values, as defined by Austin & Priest (1986). The fatty acid profile for *Pseudomonas aureofaciens* was obtained from the MIDI library as described by Thompson *et al.* (1992).

Results

Representative FAME profiles for *L. icterohaemorrhagiae*, *S. hyodysenteriae*, *B. burgdorferi*, *B. hermsii*, *B. turicatae* and *B. parkeri* are shown in Fig. 1. The FAME profile of *L. icterohaemorrhagiae* is similar to that published by Cacciopuoti *et al.* (1991).

In *L. icterohaemorrhagiae*, 26 peaks were recognized as typical of this isolate, of which 4 peaks had areas equal to or greater than 3% of the total percentage FAME area, and these peaks were identified using the MIDI system. In *S. hyodysenteriae*, a total of 21 peaks were recognized as typical of FAME profiles on the basis of their presence in replicates of the two isolates used in this study. Peak areas equal to or greater than 3% of the total percentage FAME area in at least one isolate were used in further analysis. Eight peaks had values above the cutoff point in the isolates tested and these peaks were identified using MIDI. A total of eight peaks were recognized as typical of *Borrelia burgdorferi* on the basis of their presence in repeated samples of the two isolates used in this study.

FAME peaks which had areas equal to or greater than 3% of the total FAME percentage area between *S. hyodysenteriae*, *L. icterohaemorrhagiae* and *Borrelia* species are compared in Table 1. Fatty acids present in *L. icterohaemorrhagiae*, *S. hyodysenteriae* (S75/1 isolate) and *B. burgdorferi* (B31 isolate) are shown in Table 2. Coefficients of variation were calculated between replicates and were found to be less than or equal to 7.52 for fatty acids present at greater than or equal to 3% of the total % FAME area.

In view of the reports that *Borrelia* species scavenge fatty acids (Johnson *et al.*, 1984), FAME profiles of fresh and 'spent' media (media in which *B. burgdorferi* had grown and then been removed) were analysed (Fig. 2) together with a representative *B. burgdorferi* profile (B31 reference strain, Fig. 1C). Different peaks (such as methyl tetradecanoate and parts of summed feature 6) occur in the *B. burgdorferi* profile than in the fresh and 'spent' media profiles, suggesting that *B. burgdorferi* can in fact modify fatty acids. Washings from the *Borrelia* were also analysed to check that fatty acids in the media were not adhering to the *Borrelia*.

The MIS software enabled a FAME relatedness tree to be constructed (Fig. 3). Clustering was accomplished by the unweighted pair group method for arithmetic averages (Sneath & Sokal, 1973). This showed a clear distinction between the species investigated in this study. Inclusion of *P. aureofaciens* in the analysis as an

Fig. 1. Representative FAME profiles of: (A) *Leptospira icterohaemorrhagiae*; (B) *Serpulina hyodysenteriae*; (C) *Borrelia burgdorferi*; (D) *Borrelia hermsii*; (E) *Borrelia turicatae*; (F) *Borrelia parkeri*. Peaks labelled a–h correspond to the fatty acids in Table 1.

Table 1. Comparison of the fatty acids present in *Leptospira icterohaemorrhagiae*, *Serpulina hyodysenteriae* and *Borrelia* species (greater than or equal to 3% of the total percentage FAME area)

The letter assigned to each fatty acid correlates with the peaks shown in Fig. 1.

Name	Shorthand Designation	<i>Leptospira icterohaemorrhagiae</i>	<i>Serpulina hyodysenteriae</i>	<i>Borrelia</i> species
a Tetradecanoate	C14:0		×	×
b 13-Methyltetradecanoate	C15:0 iso		×	
c 3-Hydroxy-12-methyltridecanoate	3-OH i-14:0		×	
d Hexadecanoate	C16:0	×	×	×
e <i>cis</i> -Hexadec-9-enoate	C16:1(9c)	×		
f <i>cis</i> -Octadec-9-enoate	C18:1(9c)	×	×	×
g Octadecanoate	C18:0		×	
h Methylenehexadecanoate	C17:0 cyclo	×		

'outgroup' confirmed that the different spirochaete species formed a common cluster. The Euclidian distance can be used to estimate the taxonomic distances between the species used in the study. Thus the results indicate

that *B. turicatae* and *B. parkeri* are more closely related to each other than they are to *B. hermsii* and that all three species show a closer evolutionary relationship than they do to *B. burgdorferi*.

Table 2. Fatty acids present in the bacterial isolates used in this study

Figures are percentages of total FAME area ($\pm$ SD). A summed feature refers to a group of fatty acids not resolved by the procedure used in this study.

Name	Shorthand designation	<i>Leptospira</i> <i>ictero-</i> <i>haemorrhagiae</i>	<i>Serpulina</i> <i>hyodysenteriae</i>	<i>Borrelia</i> <i>burgdorferi</i>
3-Hydroxydecanoate	3OH C10:0	1.70 $\pm$ 0.81		
Dodecanoate	C12:0	2.71 $\pm$ 0.76		
2-Hydroxydodecanoate	2OH C12:0	1.01 $\pm$ 0.92		
3-Hydroxydodecanoate	3OH C12:0	1.67 $\pm$ 0.61		
Tridecanoate	C13:1		1.61 $\pm$ 0.43	
Unknown C13	C13		2.09 $\pm$ 0.81	
Tetradecanoate	C14:0	2.61 $\pm$ 0.65	5.60 $\pm$ 0.92	3.00 $\pm$ 0.22
3-Hydroxy-12-methyltridecanoate	3OH i-14:0		8.68 $\pm$ 2.13	
2-Hydroxytetradecanoate	2OH C14:0		0.78 $\pm$ 0.31	
Pentadecanoate	C15:0			1.09 $\pm$ 0.07
13-Methyltetradecanoate	C15:0 iso		19.29 $\pm$ 4.25	
12-Methyltetradecanoate	C15:0 anteiso		1.87 $\pm$ 0.35	
Hexadecanoate	C16:0	25.75 $\pm$ 2.21	35.75 $\pm$ 1.02	58.74 $\pm$ 2.28
Hexadecenoate	C16:1	2.19 $\pm$ 0.63		
<i>cis</i> -Hexadec-9-enoate	C16:1 (9c)	10.54 $\pm$ 0.53	0.77 $\pm$ 0.23	1.38 $\pm$ 0.24
3-Hydroxyhexadecanoate	3OH C16:0		2.17 $\pm$ 0.62	
Heptadecanoate	C17:0		0.50 $\pm$ 0.04	
15-Methylhexadecanoate	C17:0 iso		0.72 $\pm$ 0.20	
14-Methylhexadecanoate	C17:0 anteiso		0.74 $\pm$ 0.09	0.37 $\pm$ 0.12
Methylenehexadecanoate	C17:0 cyclo	8.30 $\pm$ 1.64		
Octadecanoate	C18:0	1.93 $\pm$ 0.94	3.98 $\pm$ 1.91	2.79 $\pm$ 0.69
<i>cis</i> -Octadec-9-enoate	C18:1(9c)	32.09 $\pm$ 1.03	4.54 $\pm$ 1.55	23.77 $\pm$ 0.42
Arachidonate	C20:4(5c,8c,11c,14c)		0.44 $\pm$ 0.24	
Summed feature 2			2.70 $\pm$ 1.35	
Summed feature 5			1.89 $\pm$ 0.06	
Summed feature 6		2.60 $\pm$ 0.75	6.44 $\pm$ 2.08	6.07 $\pm$ 0.43
Summed feature 7		5.24 $\pm$ 0.53		
Summed feature 9		0.83 $\pm$ 0.12		

Discussion

A fatty acid profile is a stable phenotypic expression of a bacterial genotype when the bacteria under study are grown in controlled culture conditions (Welch, 1991). Fatty acid metabolism is constitutive and directed by the chromosome and is not known to be under plasmid control. The presence of certain fatty acids in bacteria has been shown to correlate with taxonomic conventions (Drucker, 1976; Lechevalier, 1977). Hence the patterns of fatty acids, once identified, have resulted in the production of 'fingerprints' of bacteria. This has led to the use of FAME profiles as a key for chemotaxonomic classification (Cacciopuoti *et al.*, 1991). Although fatty acid analysis has been used to classify *Leptospira* species (Cacciopuoti *et al.*, 1991) it has not been applied to other spirochaetes. One possible reason for this may be, in the case of *Borrelia* species, that spirochaetes have typically been considered to 'scavenge' fatty acids rather than synthesize or modify fatty acids (Johnson *et al.*, 1984). Although the FAME profiles of the *Borrelia* species used in this study do contain fatty acids present in the medium, they also contain novel fatty acids absent from the medium, suggesting that *Borrelia* species have the

capacity to modify fatty acids, e.g. *B. hermsii*, *B. parkeri* and *B. turicatae* contain > 0.4% arachidonate [C20:4(5c,8c,11c,14c)] which has a higher number of carbon atoms than any of the fatty acids present in BSK II medium. This is indicative of metabolism and synthesis although this fatty acid was not identified other than by retention time.

Borrelia species and *S. hyodysenteriae* contain many of the cellular fatty acids known to be present in *T. pallidum* and *Leptospira* species (Kondo & Ueta, 1972; Matthews *et al.*, 1979). In *Leptospira*, up to 50% of the cellular fatty acids are methyl hexadecanoate (C16:0), the remainder being unsaturated, whilst *T. pallidum* contains mainly methyl hexadecanoate, methyl octadecenoate (C18:1) and methyl octadecanoate (C18:0) (Welch, 1991). The major fatty acid components of the *Borrelia* species used in this study are methyl tetradecanoate (C14:0), methyl hexadecanoate and methyl *cis*-octadec-9-enoate [C18:1(9c)]. In addition, minor variations in the fatty acid composition of the different *Borrelia* species studied were recorded. These 'fingerprints' of the *Borrelia* species may have an application for determining their genetic and taxonomic relationships.

The dendrogram in Fig. 3, based on FAME profiles,

Fig. 2. FAME profiles generated on extracted cells and media analysed by GLC of (a) BSK II medium (fresh) (b) 'spent' medium (supernatant collected by centrifugation of *B. burgdorferi* B31 cultures).

shows the relatedness of the spirochaetes used in this study. Furthermore, from the data based on *Borrelia* species, FAME profiles can be used to distinguish *Borrelia* at the inter-species level (between *B. parkeri*, *B. hermsii*, *B. turicatae* and *B. burgdorferi*). Preliminary work with isolates of *S. hyodysenteriae* and *B. burgdorferi* suggests that FAME profile relatedness may be extended to distinguish isolates at the intra-species level (or intra-species variation).

Interestingly, the two isolates of *B. burgdorferi* used in this study (B31 and JD1) cluster separately from the other *Borrelia* species, *B. hermsii*, *B. turicatae* and *B. parkeri*. A major difference between *B. burgdorferi* and the other *Borrelia*s examined is shown in their tick vectors. Ticks of the ixodid species are vectors of *B. burgdorferi*, primarily *Ixodes ricinus* and *I. hexagonus* in Europe and *I. dammini* and *I. pacificus* in north America (Lane *et al.*, 1991), whereas *B. hermsii*, *B. turicatae* and *B. parkeri* are transmitted by the argasid tick species, *Ornithodoros hermsi*, *O. turicata* and *O. parkeri* (Kelly, 1984). However, the taxonomic relationships of *B. hermsii*, *B. turicatae* and *B. parkeri*, based on FAME analysis, does not correspond with their known similarities in carbohydrate metabolism. *B. turicatae* ferments only glucose, raffinose and dextrin whereas *B. hermsii* and *B. parkeri* ferment glucose, maltose, trehalose, starch, dextrin and glycogen, but not raffinose (Kelly, 1984).

All typical Gram-negative bacteria contain endotoxins which are lipopolysaccharide (LPS)-containing compounds usually found in the outer envelope and which have been implicated in the pathogenesis of several diseases (Rietschel *et al.*, 1982). Hydroxy fatty acids,

Fig. 3. Dendrogram of FAME profile demonstrating relatedness (Euclidean distance) of the species and isolates used in this study.

particularly 3-hydroxymyristic acid, (3OH C14:0) are associated with LPS (Galanos *et al.*, 1977). LPS has been found in *L. interrogans* serogroup *copenhageni* (Vinh *et al.*, 1986) and also in *S. (Treponema) hyodysenteriae* (Nuessen *et al.*, 1982; Bailey *et al.*, 1985). Furthermore LPS from *L. interrogans* serogroup *copenhageni* has been shown to contain several hydroxy fatty acids (3OH C10:0, 3OH C12:0, OH C15:0, 2OH C16:0, OH C17:0) although no 3-hydroxymyristic acid was shown to be present (Vinh *et al.*, 1986). Table 2 shows that hydroxylated fatty acids are present in *S. hyodysenteriae* and *L. interrogans* serogroup *icterohaemorrhagiae*, which is consistent with the presence of LPS in such organisms. By contrast, *B. burgdorferi* contains no hydroxylated fatty acids, which is consistent with the proposed absence of LPS from *B. burgdorferi* suggested by Takayama *et al.* (1987). Thus fatty acid analysis may provide a tool for analysis of the chemotaxonomy of the spirochaetes and for the study of fatty acids present in molecules conferring pathogenicity.

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