Analysis of intra-specific variation in the fatty acid profiles of *Borrelia burgdorferi*

M. ANNE LIVESLEY,¹ IAN P. THOMPSON,¹ LISE GERN² and PATRICIA A. NUTTALL^{1*}

¹NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR, UK

²Université de Neuchâtel, Institut de Zoologie, Chantemerle 22, Case Postale 2, 2007 Neuchâtel, Switzerland

(Received 11 March 1993; revised 13 May 1993; accepted 4 June 1993)

Analysis of the fatty acid methyl esters (FAMES) of bacteria is a commonly used chemotaxonomic technique. Application of this methodology to spirochaetes associated with Lyme borreliosis revealed distinct clusters corresponding to three genetically distinguished groups: *Borrelia burgdorferi sensu stricto*, *B. garinii*, and the VS461 group. However, *B. garinii* formed a common group with *B. hermsii*, a relapsing fever spirochaete, and VS461 grouped with *B. turicatae* and *B. parkeri*, two other relapsing fever spirochaetes. The diversity in fatty acid profiles of Lyme disease spirochaetes has implications for the protean clinical manifestations of the disease.

Introduction

Many methods have been used to compare different isolates of *Borrelia burgdorferi*, the aetiological agent of Lyme borreliosis. Apparently unique isolate-specific cleavage patterns of restriction-enzyme-digested whole *B. burgdorferi* DNA have been obtained (Le Febvre *et al.*, 1989). Plasmid profiles have been found to differ between isolates but also to change upon prolonged cultivation (Schwan *et al.*, 1988). The use of the polymerase chain reaction (PCR) has shown differences in the reactivity of different isolates of *B. burgdorferi* with chromosomally derived primers. Southern blot analysis of restriction patterns of the genes coding for rRNA have also revealed differences between isolates (Postic *et al.*, 1990). Differences in the levels of reactivity of *B. burgdorferi* isolates with monoclonal antibodies have also been shown (Wilske *et al.*, 1988).

More recently phylogenetic analysis based on 16S rRNA sequences of *B. anserina*, *B. coriaceae*, *B. hermsii* and *B. burgdorferi* (North American and European isolates) has been performed (Marconi & Garon, 1992*a, b*). Further attempts to classify *B. burgdorferi* isolates have involved the use of multilocus enzyme electrophoresis (Boerlin *et al.*, 1992), polymorphism of outer surface membranes (Peter & Bretz, 1992) and PCR

(Rosa *et al.*, 1991; Welsh *et al.*, 1992). Three DNA groups (genospecies) have been delineated based on rRNA gene restriction patterns, protein electrophoresis patterns and patterns of reactivity with murine monoclonal antibodies – *B. burgdorferi sensu stricto*, *B. garinii* sp. nov. and Group VS461 (Baranton *et al.*, 1992).

The classification of *B. burgdorferi* has been extensively investigated in view of the clinical manifestation of *B. burgdorferi* infection in humans. The symptoms of Lyme borreliosis differ in Europe when compared to North America (Steere, 1989). In Europe, acrodermatitis chronica atrophicans (ACA; a chronic skin disorder) and neuroborreliosis are common (Ackermann *et al.*, 1988; Asbrink & Hovmark, 1988). By contrast, in North America Lyme arthritis is common whereas this is comparatively rare in Europe (Steere, 1989). The classification of *Borrelia* species associated with relapsing fevers has not been properly investigated and it has been suggested by some workers that *B. hermsii*, *B. turicatae* and *B. parkeri* are not valid species on the basis of DNA–DNA hybridization studies (Hyde & Johnson, 1984).

Most of the methods which have been used to classify *B. burgdorferi* have relied upon the product of a single chromosomal locus, or a specific gene sequence, although multilocus enzyme electrophoresis has been utilized (Boerlin *et al.*, 1992). One phenotypic method of classification which has recently been applied to *Borrelia* species is based on the analysis of fatty acid profiles (Livesley *et al.*, 1993). The advantage of this method is that the fatty acid profile of bacteria is a stable indication

* Author for correspondence. Tel. (0865) 512361; fax (0865) 59962.

Abbreviations: ACA, acrodermatitis chronica atrophicans; MIS, microbial identification system; FAME, fatty acid methyl ester.

Table 1. Sources and suppliers of the *Borrelia* isolates used in this study

<i>Borrelia</i> isolate	Source	Country	Supplier*	Group†
<i>B. burgdorferi</i> ACA-1	ACA	Sweden	S. Cutler ¹	1
<i>B. burgdorferi</i> 328	<i>Ixodes dammini</i>	USA	S. Cutler	1
<i>B. burgdorferi</i> HB19	Human blood	USA	S. Cutler	1
<i>B. burgdorferi</i> HB4	Human blood	USA	S. Cutler	1
<i>B. burgdorferi</i> B31	<i>I. dammini</i>	USA	ATCC 35210	1
type strain				
<i>B. burgdorferi</i> JD1	<i>I. dammini</i>	USA	B. Johnson ²	1
<i>B. turicatae</i>	<i>Ornithodoros turicata</i>	USA	T. Schwan ³	2
<i>B. parkeri</i>	<i>O. parkeri</i>	USA	T. Schwan	2
<i>B. burgdorferi</i> Ir210	<i>I. ricinus</i>	CIS	E. I. Korenberg ⁴	3
<i>B. burgdorferi</i> 21305	<i>I. dammini</i>	USA	S. Cutler	3
<i>B. burgdorferi</i> M32	<i>I. ricinus</i>	Netherlands	S. Carter ⁵	3
VS461	<i>I. ricinus</i>	Switzerland	D. Postic ⁶	3
<i>B. burgdorferi</i> M27	<i>I. ricinus</i>	Netherlands	S. Carter	3
<i>B. hermsii</i> CON-1	Human	USA	T. Schwan	4
<i>B. hermsii</i> HS1	<i>O. hermsii</i>	USA	T. Schwan	4
<i>B. burgdorferi</i> NE550	<i>I. ricinus</i>	Switzerland	L. Gern ⁷	5
<i>B. burgdorferi</i> NE2	<i>I. ricinus</i>	Switzerland	L. Gern	5
<i>B. burgdorferi</i> NE4R4	<i>I. ricinus</i>	Switzerland	L. Gern	5
<i>B. burgdorferi</i> NE58P8	<i>I. ricinus</i>	Switzerland	L. Gern	5
<i>B. burgdorferi</i> NE58	<i>I. ricinus</i>	Switzerland	L. Gern	5
<i>B. garinii</i> 20047	<i>I. ricinus</i>	France	D. Postic	5

* 1, Charing Cross Hospital, London, UK; 2, Centers for Disease Control, Fort Collins, USA; 3, Rocky Mountain Laboratories, Hamilton, Montana, USA; 4, Gamaleya Institute for Epidemiology & Microbiology, Moscow, CIS; 5, University of Liverpool, UK; 6, Institut Pasteur, Paris, France; 7, Université de Neuchâtel, Switzerland.

† The group number corresponds with that shown in Fig. 1.

of the phenotype when the bacteria under study are grown in controlled culture conditions (Welch, 1991). Fatty acid metabolism is not under plasmid control and the presence of certain fatty acids has been shown to correlate with taxonomic conventions (Drucker, 1976; Lechevalier, 1977). The analysis of fatty acid profiles is one of the techniques of chemotaxonomy (Cacciopuoti *et al.*, 1991) and has been used successfully with *Xanthomonas maltophilia*, *Klebsiella terrigena*, *Erwinia herbicola* and *Microbacterium lacticum* (Thompson *et al.*, 1993), numerous Gram-negative bacteria (Stoakes *et al.*, 1991), *Leptospira* spp. (Cacciopuoti *et al.*, 1991) and as a means of distinguishing members of the spirochaete family (Livesley *et al.*, 1993). In this paper we use fatty acid profiles to demonstrate inter- and intra-species variation within the genus *Borrelia*.

Methods

Culture of bacterial strains. *Leptospira interrogans* (serogroup icterohaemorrhagiae) and *Serpulina hyodysenteriae* (S75/1 and B78 isolates) were cultured as previously described (Livesley *et al.*, 1993).

Borrelia species (Table 1) including the *B. burgdorferi* reference strain B31 (ATCC 35210) were grown in a modified Barbour–Stoenner–Kelly medium as described previously (Livesley *et al.*, 1993). Microaerophilic conditions were established by dispensing the media into 10 × 100 mm

tubes, where 6 ml occupied approximately 75% of the volume available. The *Borrelia* were incubated at 34 °C for the period needed to generate a cell density of 10⁸ organisms ml⁻¹. 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 (Livesley *et al.*, 1993).

Fatty acid methyl ester (FAME) extraction. Long-chain fatty acids extracted from bacteria require derivatization before gas–liquid chromatography. FAME derivatives were prepared using the procedure described by Kloepper *et al.* (1992).

Gas–liquid chromatography. FAMES were analysed with a Hewlett Packard series II gas chromatograph model 5890A equipped with a 25 m × 0.22 mm × 0.33 µm phenyl methyl silicone capillary column and integrator 3392A as described by Livesley *et al.* (1993).

Reproducibility of FAME analysis. For the reference isolates the reproducibility of the FAME profiles was analysed by using repeated injections from extracts derived from three separate cultures. All remaining isolates were analysed by repeat injections of three separate extracts derived from one culture of the isolate, under standard conditions. For each peak in the chromatogram the coefficient of variation (standard deviation/mean) × 100 was calculated (Mukwaya & Welch, 1989). Clustering was accomplished by the unweighted pair group for arithmetic averages (Sneath & Sokal, 1973). Similarity and relatedness were 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 and Discussion

Previous work has demonstrated that FAME analysis can be used to distinguish spirochaetes at the sub-generic level (Livesley *et al.*, 1993). The use of this technique to demonstrate intra-species variation amongst *Borrelia* is shown in Fig. 1.

Analyses of the profiles of 17 isolates together with borreliae associated with tick-borne relapsing fevers (*B. hermsii*, *B. parkeri* and *B. turicatae*) revealed a number of groups (Fig. 1, Table 1). The FAME compositions of representatives of each group are summarized in Table 2. *Borrelia parkeri* and *B. turicatae* grouped together (group 2), but the two *B. hermsii* isolates (group 4) did not form an obvious cluster. Further analyses need to be undertaken with isolates of *B. hermsii* and with *B. turicatae* and *B. parkeri* when more isolates of these latter two species become available. For the Lyme disease spirochaetes, clusters shown in the dendrogram (Fig. 1) support the delineation of *B. burgdorferi sensu stricto* (group 1), *B. garinii* (group 5), and Group VS461

(group 3), proposed by Baranton *et al.* (1992). The FAME profiles of reference members of these groups (*B. burgdorferi sensu stricto* B31, *B. garinii* 20047 and VS461) are shown in Fig. 2. FAME profiles of *B. hermsii*, *B. parkeri* and *B. turicatae* are shown in Livesley *et al.* (1993).

Geographical clustering of Lyme disease spirochaetes was observed. All the isolates from the Neuchâtel and Staatswald areas of Switzerland clustered together with *B. garinii* 20047, an isolate from France (group 5). North American isolates clustered together (group 1), as did another group of European isolates: M32, M27, VS461 and Ir210 (group 3). The exceptions were the American isolate 21305 in group 3, and the Swedish isolate ACA-1 in group 1. Few of the isolates used in the FAME analysis have been examined by other techniques. The grouping of the Swiss isolate NE2 with *B. garinii* 20047 was also observed by multilocus enzyme electrophoresis (Boerlin *et al.*, 1992) but, in the same study, the Swedish isolate ACA-1 clustered with VS461 rather than with *B. burgdorferi sensu stricto* (group 1; Fig. 1).

The clustering of American and European isolates of *B. burgdorferi* has been demonstrated by other methods such as 16S rRNA sequencing (Marconi & Garon, 1992*a, b*). These workers distinguished between *B. burgdorferi* on one branch of a phylogenetic tree and *B. hermsii*, *B. coriaceae* and *B. anserina* on a separate branch. The results of the cluster analysis shown in Fig. 1 represent the first reported attempt to group Lyme disease spirochaetes on the basis of their fatty acid content. Clustering of isolates based on other phenotypic characteristics, such as multilocus enzyme electrophoresis (Boerlin *et al.*, 1992) and antigenic variability (Wilske *et al.*, 1988), has been demonstrated. However, the former study (Boerlin *et al.*, 1992) did not include *B. hermsii*, *B. parkeri* and *B. turicatae*, and the latter study (Wilske *et al.*, 1988) included these species but utilized monoclonal antibodies which react with a single epitope, hence the results are not directly comparable with those shown in Fig. 1. None of the methods used to date have demonstrated a complete correlation between clustering pattern and either the source (e.g. species of tick, clinical sample) or the geographical location of the isolate.

Surprisingly, borreliae associated with Lyme disease were not distinguished as a group from the relapsing fever spirochaetes examined. Group 5 (containing *B. garinii*) showed a closer relationship with *B. hermsii* than with the other two groups of Lyme disease spirochaetes, and group 3 (containing VS461) was closest to *B. turicatae* and *B. parkeri*. Apart from their differences in disease associations, Lyme disease spirochaetes are transmitted by ixodid ticks (*Ixodes* spp.) and relapsing fever spirochaetes are transmitted by argasid ticks (*Ornithodoros* spp.) (Kelly, 1984). Considerable varia-

Fig. 1. Dendrogram of the FAME profile relatedness of reference isolates of *Borrelia* used in this study. Isolates of *Leptospira interrogans* (serogroup *icterohaemorrhagiae*) and *Serpulina hyodysenteriae* were included as representative examples of other spirochaetes. *Pseudomonas aureofaciens* was included in the dendrogram as an outgroup.

Table 2. Fatty acids (as percentage of the total FAME area $\pm$ SD) present in representative isolates of each of the groups delineated in Fig. 1

A summed feature refers to a group of fatty acids not resolved by the extraction procedure used in this study. Coefficients of variation were ≤ 8.01 for peaks which occupied $\geq 3.00\%$ of the total FAME percentage area.

Fatty acid	Shorthand designation	<i>B. burgdorferi</i> <i>sensu stricto</i> (1)	<i>B. turicatae</i> <i>B. parkeri</i> (2)	VS461 (3)	<i>B. hermsii</i> (4)	<i>B. garinii</i> (5)
Dodecanoate	12:0			0.30 $\pm$ 0.06		0.39 $\pm$ 0.06
Tetradecanoate	14:0	1.34 $\pm$ 0.22	1.10 $\pm$ 0.07	2.34 $\pm$ 0.22	1.20 $\pm$ 0.08	1.78 $\pm$ 0.03
12-Methyltetradecanoate	15:0 anteiso					0.12 $\pm$ 0.02
Pentadecanoate	15:0	1.09 $\pm$ 0.07	0.75 $\pm$ 0.03	1.16 $\pm$ 0.15	0.74 $\pm$ 0.06	0.80 $\pm$ 0.02
Hexadecanoate	16:1	0.23 $\pm$ 0.01		0.38 $\pm$ 0.06	0.22 $\pm$ 0.12	0.35 $\pm$ 0.03
14-Methylpentadecanoate	16:0 iso				0.13 $\pm$ 0.02	0.16 $\pm$ 0.07
<i>cis</i> -Hexadec-9-enoate	16:1 (9c)	1.39 $\pm$ 0.23	0.61 $\pm$ 0.03	2.23 $\pm$ 0.15	1.14 $\pm$ 0.09	1.93 $\pm$ 0.09
Hexadecanoate	16:0	57.62 $\pm$ 2.18	48.93 $\pm$ 1.29	50.88 $\pm$ 2.09	43.29 $\pm$ 1.63	39.83 $\pm$ 0.12
14-Methylhexadecanoate	17:0 anteiso	0.37 $\pm$ 0.12	0.21 $\pm$ 0.02	0.45 $\pm$ 0.14	0.17 $\pm$ 0.02	
Heptadecenoate	17:1		0.22 $\pm$ 0.03		0.34 $\pm$ 0.04	0.36 $\pm$ 0.02
Heptadecanoate	17:0	1.08 $\pm$ 0.08	1.25 $\pm$ 0.05	1.33 $\pm$ 0.12	1.18 $\pm$ 0.07	1.14 $\pm$ 0.03
<i>cis</i> -Octadec-9-enoate	18:1 (9c)	23.28 $\pm$ 0.62	31.11 $\pm$ 1.27	22.56 $\pm$ 1.08	26.48 $\pm$ 1.03	23.02 $\pm$ 0.08
Octadecanoate	18:0	2.79 $\pm$ 0.68	6.39 $\pm$ 0.32	6.24 $\pm$ 0.50	0.02 $\pm$ 0.01	9.70 $\pm$ 0.68
Arachidonate	20:4 5,8,11,14c		0.41 $\pm$ 0.02	0.18 $\pm$ 0.06	0.59 $\pm$ 0.03	0.28 $\pm$ 0.03
Summed feature 6		5.30 $\pm$ 0.32	9.25 $\pm$ 0.63	11.67 $\pm$ 0.35	15.31 $\pm$ 0.75	19.52 $\pm$ 0.57

Fig. 2. Representative FAME profiles of reference isolates of: (1) *B. burgdorferi sensu stricto* B31, (2) *B. garinii* 20047, (3) VS461. Three fatty acid peaks are labelled for purposes of comparison: (a) tetradecanoate, (b) hexadecanoate, (c) *cis*-octadec-9-enoate.

bility in the peptidase, glycosidase and esterase activities of nine Lyme disease spirochaetes, and similarities with *B. hermsii*, have been reported (Stanek *et al.*, 1992).

Some metabolic enzymes are likely to be involved in fatty acid synthesis and metabolism and the diversity in specific enzyme activities may help explain the clusters derived from analyses of fatty acid profiles. Currently, there are insufficient published data to determine whether the FAME clusters result from a convergence in phenotype of the different borrelial types, or if they reflect genetic relationships.

Besides their application in chemotaxonomy, fatty acids are of interest because of their role in the pathogenesis of several bacterial diseases (Lechevalier, 1977). The differentiation of *Borrelia* using fatty acids, given their involvement in membrane structure, may possibly aid an explanation of the differing manifestations of Lyme borreliosis, for example arthritis being a more common symptom in the United States (Steere, 1989) and ACA and neuroborreliosis being more common in Europe (Ackermann *et al.*, 1988; Asbrink & Hovmark, 1988). In *Leptospira* the presence of serovar-specific glycolipids has been demonstrated (Ono *et al.*, 1987) whilst in *B. burgdorferi* Wheeler *et al.* (1993) showed antibody reactivity to fractions of chloroform/methanol extracts, using sera from Lyme disease patients, and speculated on the possible role of this lipid-containing antigen in autoreactive-mediated pathogenesis. Obviously, fatty acids provide a source of diversity among Lyme disease spirochaetes that needs to be explored further in relation to the protean clinical manifestations of the disease.

We are grateful to the Health and Safety Executive (HSE) for funding M.A.L., the Department of the Environment for funding I.P.T. and the Swiss National Science Foundation (SNSF) for funding

L.G. The collaboration was supported by the SNSF/British Council Joint Research Programme. We also thank all the suppliers of the *Borrelia* isolates as shown in Table 1.

References

- ACKERMANN, R., REHSE-KUPPER, B., GOLLMER, E. & SCHMIDT, R. (1988). Chronic neurologic manifestations of Erythema Migrans borreliosis. *Annals of the New York Academy of Sciences* **539**, 16–23.
- ASBRINK, E. & HOVMARK, A. (1988). Early and late cutaneous manifestations in *Ixodes* borne borreliosis (Erythema migrans borreliosis, Lyme borreliosis). *Annals of the New York Academy of Sciences* **539**, 4–15.
- AUSTIN, B. & PRIEST, F. (1986). *Modern Bacterial Taxonomy*. Wokingham, UK: Van Nostrand Reinhold.
- BARANTON, G., POSTIC, D., SAINT GIRONS, I., BOERLIN, P., PIFFARETTI, J. C., ASSOUS, M. & GRIMONT, P. A. D. (1992). Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov. and group VS461 associated with Lyme Borreliosis. *International Journal of Systematic Bacteriology* **42**, 378–383.
- BOERLIN, P., PETER, O., BRETZ, A. G., POSTIC, D., BARANTON, G. & PIFFARETTI, J. C. (1992). Population genetic analysis of *Borrelia burgdorferi* by multilocus enzyme electrophoresis. *Infection and Immunity* **60**, 1677–1683.
- CACCIOPUOTI, B., CICERONI, L. & ATTARD BARBINI, D. (1991). Fatty acid profiles – a chemotaxonomic key for classification of strains of the *Leptospiraceae*. *International Journal of Systematic Bacteriology* **41**, 295–300.
- DRUCKER, D. B. (1976). Gas liquid chromatographic chemotaxonomy. *Methods in Microbiology* **9**, 51–125.
- HYDE, F. W. & JOHNSON, R. C. (1984). Genetic relationship of Lyme disease spirochaetes to *Borrelia*, *Treponema* and *Leptospira* spp. *Journal of Clinical Microbiology* **20**, 151–154.
- KELLY, R. T. (1984). Spirochetes. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 57–61. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- KLOEPPER, J. W., RODRIGO, R., MCINROY, J. A. & YOUNG, R. W. (1992). Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root knot (*Meloidogyne incognita*) nematodes: identification by fatty acid analysis and frequency of biological control activity. *Plant and Soil* **139**, 75–84.
- LECHEVALIER, M. P. (1977). Lipids in bacterial taxonomy. A taxonomist's view. *Critical Reviews in Microbiology* **5**, 109–210.
- LE FEBVRE, R. B., PERNG, G. C. & JOHNSON, R. C. (1989). Characterization of *Borrelia burgdorferi* isolates by restriction endonuclease analysis and DNA hybridization. *Journal of Clinical Microbiology* **27**, 636–639.
- LIVESLEY, M. A., THOMPSON, I. P., BAILEY, M. J. & NUTTALL, P. A. (1993). Comparison of the fatty acid profiles of *Borrelia*, *Serpulina* and *Leptospira* species. *Journal of General Microbiology* **139**, 889–895.
- MARCONI, R. T. & GARON, C. F. (1992a). Identification of a third genomic group of *Borrelia burgdorferi* through signature nucleotide analysis and 16S rRNA sequence determination. *Journal of General Microbiology* **138**, 533–536.
- MARCONI, R. T. & GARON, C. F. (1992b). Phylogenetic analysis of the genus *Borrelia*: a comparison of North American and European isolates of *Borrelia burgdorferi*. *Journal of Bacteriology* **174**, 241–244.
- MUKWAYA, G. M. & WELCH, D. F. (1989). Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. *Journal of Clinical Microbiology* **27**, 2646–2649.
- ONO, E., TAKASE, H., NAIKI, M. & YANAGAWA, R. (1987). Purification, characterization and serological properties of a glycolipid antigen reactive with a serovar specific monoclonal antibody against *Leptospira interrogans* serovar canicola. *Journal of General Microbiology* **133**, 1329–1336.
- PETER, O. & BRETZ, A. G. (1992). Polymorphism of outer surface proteins of *Borrelia burgdorferi* as a tool for classification. *Zentralblatt für Bakteriologie* **277**, 28–33.
- POSTIC, D., EDLINGER, C., RICHAUD, C., GRIMONT, F., DUFRESNE, Y., PEROLATE, P., BARANTON, G. & GRIMONT, P. A. D. (1990). Two genomic species in *Borrelia burgdorferi*. *Research in Microbiology* **141**, 465–475.
- ROSA, P. A., HOGAN, D. & SCHWAN, T. G. (1991). Polymerase chain reaction analyses identify two distinct classes of *Borrelia burgdorferi*. *Journal of Clinical Microbiology* **29**, 524–532.
- SCHWAN, T. G., BURGDORFER, W. & GARON, C. F. (1988). Changes in infectivity and plasmid profile of the Lyme spirochaete *Borrelia burgdorferi* as a result of *in vitro* cultivation. *Infection and Immunity* **56**, 1831–1836.
- SNEATH, P. H. A. & SOKAL, R. R. (1973). *Numerical Taxonomy: the Principles and Practice of Numerical Classification*, pp. 230–234. San Francisco: W. H. Freeman.
- STANEK, G., MANAFI, M. & KOCH, C. (1992) cited by PENN, C. W., BASSFORD, P. J., YELTON, D. B., DUNN, J., NELSON, D. R., FUKUNAGA, M. & STANEK, G. Genetic approaches to cell biology and metabolism of spirochetes. *Research in Microbiology* **143**, 605–613.
- STEELE, A. C. (1989). Lyme disease. *New England Journal of Medicine* **321**, 586–596.
- STOAKES, L., KELLY, T., SCHIEVEN, B., HARLEY, D., RAMOS, M., LANNIGAN, R., GROVES, D. & HUSSAIN, Z. (1991). Gas liquid chromatographic analysis of cellular fatty acids for identification of Gram-negative anaerobic bacilli. *Journal of Clinical Microbiology* **29**, 2636–2638.
- THOMPSON, I. P., BAILEY, M. J., ELLIS, R. J. & PURDY, K. J. (1993). Subgrouping of bacterial populations by cellular fatty acid composition. *FEMS Microbiology Ecology* **102**, 75–84.
- WELCH, D. (1991). Applications of cellular fatty acid analysis. *Clinical Microbiological Reviews* **4**, 422–438.
- WELSH, J., PRETZMAN, C., POSTIC, D., SAINT GIRONS, I., BARANTON, G. & McLELLAND, M. (1992). Genetic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. *International Journal of Systematic Bacteriology* **42**, 370–377.
- WHEELER, C. M., MONCO, J. C. G., BENACH, J. L., GOLIGHTLY, M. G., HABICHT, G. S. & STEERE, A. C. (1993). Non-protein antigens of *Borrelia burgdorferi*. *Journal of Infectious Diseases* **167**, 665–674.
- WILKE, B., PREAC-MURSIC, V., SCHIERZ, G., KUHBECK, R., BARBOUR, A. G. & KRAMER, M. (1988). Antigenic variability of *Borrelia burgdorferi*. *Annals of the New York Academy of Sciences* **539**, 126–143.