Phylogenetic diversity of intra-amoebal legionellae as revealed by 16S rRNA gene sequence comparison

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Following a recent phylogenetic analysis of all 39 species of the genus Legionella and related organisms, the name Legionella lytica has been proposed for bacteria previously considered either as Sarcobium lyticum or as ‘Legionella-like amoebal pathogens’ (LLAPs). To investigate the phylogenetic integrity of this newly proposed species, we determined the 16S rRNA gene sequences of 10 LLAPs isolated from various environmental sources. All 10 isolates clustered within a monophyletic group containing all other members of the genus Legionella. Eight of the 10 isolates formed a monophyletic subgroup within the genus which also included the two previously characterized L. lytica strains. Four of these 10 isolates shared a specific and very close relationship with the L. lytica type strain (> 99% sequence similarity). However, although clearly legionellae, the remaining two LLAP strains bore no specific evolutionary relationship to either L. lytica or any other Legionella species (< 96% sequence similarity). Both isolates lay on their own relatively deep-rooted branches within the radiation of the Legionella cluster. LLAPs do not, therefore, represent a unique species or even a single line of descent within the genus, and investigation of more isolates may reveal them to be as evolutionarily diverse as the other presently recognized Legionella species.

Keywords: Legionella lytica, LLAPs, amoebae, 16S rRNA gene, phylogeny

INTRODUCTION

In 1954 Drozanski isolated from Polish soil an acanthamoeba infected with motile bacilli. The fastidious nature of one of the strains so obtained (designated L2) prevented its culture on artificial media, which in turn prevented it from being studied using conventional microbiological techniques, and thus excluded it from classification. However, subsequent propagation, in cultured Acanthamoeba castellanii, allowed its basic characterization in terms of microscopic morphology, cell wall peptidoglycan structure, cellular oxygen consumption, and DNA base composition. As the organism did not appear to bear the significant traits of either other amoebal pathogens or other bacterial genera, after many years of study it was assigned to a new genus, Sarcobium, as the species Sarcobium lyticum (Drozanski, 1991).

Rowbotham (1980) proposed that amoebae may act as natural hosts for the then recently characterized organism Legionella pneumophila. Subsequent work demonstrated in vitro interaction between Legionella species and amoebae and ciliates (Rowbotham, 1983; Fields et al., 1984; Barbaree et al., 1986), and led Rowbotham to coin the term ‘Legionella-like amoebal pathogens’ (LLAPs) for bacteria that caused legionella-like infections in amoebae, but could not be grown on agar media. The natural interaction between amoebae and legionellae was exploited in the development of methods for the isolation of the bacteria from clinical specimens and environmental samples (Rowbotham, 1983; Fallon & Rowbotham, 1990). These methods have sometimes been successful when conventional cultures have failed. In 1986 an LLAP

Abbreviation: LLAP, ‘Legionella-like amoebal pathogen’.

The EMBL accession numbers for the 16S rRNA gene sequences reported in this study are: LLAP1, X97355; LLAP2, X97365; LLAP4, X97357; LLAP6, X97359; LLAP7, X97365; LLAP8, X97361; LLAP9, X97360; LLAP10, X97363; LLAP11, X97362; LLAP12, X97366; L. lytica strain L2, X97364; L. lytica strain L2, X97358.
L. \textit{pneumoniae} was added, as a food-source for the amoebae, to each well between 2 and 24 h later. The plates were incubated at either 30 °C (LLAPs 1, 4, 5, 7 and 11) or 35 °C (L2, LLAPs 2, 3, 6, 8, 9 and 10) for a further 2–3 d. Following this incubation, 10–20 ml washed trophozoites (again at 10^8 ml^-1) in tissue culture flasks were inoculated with infected amoebae taken from the microtitre wells containing the most dilute sample to cause infection. After a further 2−3 d incubation, 1 ml aliquots of a heavily infected co-culture suspension were transferred to cryotubes and frozen to −70 °C. Aliquots (0.1 ml) were also inoculated onto buffered charcoal yeast extract agar (BCYE) and unheated horse blood agar then incubated at 30 and 35 °C for 7 d to check for the presence of axenically-culturable legionellae and other micro-organisms.

From 1987, \textit{L. \textit{pneumoniae}} species (Fry \textit{et al.}, 1991a) were amplified from these extracts using a PCR incorporating universal eubacterial primers, as previously described (Birtles \textit{et al.}, 1991). Amplification products were purified using QIAquick purification kits (Qiagen) according to the manufacturer's instructions, for use as templates in cycle sequencing reactions. Sequencing reactions used the reagents of the Amplicycle kit (Eurogentec), according to the manufacturer's instructions. The primers employed were universal eubacterial 16S rRNA sequencing primers as previously described (Fry \textit{et al.}, 1991a). The sequencing products were resolved on 0.35 mm READIMIX acrylamide gels (Pharmacia), then detected and translated into sequence data using an ALF automated sequencer (Pharmacia) and associated software.

**16S rRNA sequence analysis.** The primary sequences of the test strains were generated by aligning then combining the sequences generated by each primer using DNAsis (Hitachi Software Engineering America). Primary sequences were then aligned with each other, and with 16S rRNA sequences of the recognized \textit{Legionella} species and other \textit{Proteobacteria} using the CLUSTAL multisequence alignment program (Higgins \textit{et al.}, 1992) supported within the BISANCE workstatck (Dessen \textit{et al.}, 1990). Phylogenetic analysis of this alignment was achieved using programs within the PHYLIP 3.5c2 Phylogenetic Inference Package (J. Felsenstein, University of Washington).
The assumptions of Jukes & Cantor (1969), and a phylogenetic tree was inferred from this matrix using the criteria of Fitch & Margoliash (1967) (FITCH). The stability of the branching order proposed in this tree was determined by bootstrap analysis using the programs SEQUWith CONSENSE to yield a strict majority-rule consensus tree based on 100 samples. Parsimony analysis of the alignment was carried out using DNASPars, and the stability of the tree derived from this analysis was assessed by bootstrap analysis as described above.

RESULTS AND DISCUSSION

Primary sequence data of between 1427 and 1507 bp were determined for each of the 10 test strains and for the L. \textit{lytica} strains L2 and LLAP3. The percentage similarities between these sequences of each of the test strains are presented in Table 2. Included in this table are the \textit{Legionella} species with which each test strain shares the highest sequence similarity. The sequences of LLAP9 and L. \textit{lytica} L2 were found to be identical, as were those of LLAP1 and LLAP12. A recent taxonomic proposal has suggested that bacterial strains of the same species should share more than 97\% 16S rRNA sequence similarity, and thus strains sharing less than this level of similarity represent different, albeit possibly very closely related, species (Stackebrandt & Goebel, 1994). As none of the test strains shared more than 96.2\% similarity with any of the axenically-cultured \textit{Legionella} species, application of this criterion indicates them not to be unculturable forms of a recognized taxon. Furthermore, as strains LLAP1 and LLAP8 share less than 97\% similarity with any of the other strains included in this study, they can be considered representatives of different and previously unrecognized \textit{Legionella} species. LLAP10 also exhibits only moderate sequence similarity with other L. \textit{lytica} strains (< 97.2\%).

The sequence of LLAP4 is most similar to that of LLAPs 11 and 12 (98.7\%). The remaining strains, LLAPs 2, 6 and 7, all share more than 99.0\% similarity with both the L. \textit{lytica} type strain and LLAP3; this similarity is of the level observed between subspecies and serogroups of other \textit{Legionella} species (Fry et al., 1991b; Hooker et al., 1996).

The alignment used as a basis for phylogenetic analysis comprised the test sequences, the 16S rRNA gene sequences of the \textit{Legionella} species, and that of \textit{Coxiella burnetii} (included as an outgroup on which phylogenetic trees would be rooted). The 16S rRNA gene sequences available for some of the \textit{Legionella} species were not as complete as those obtained in this study, usually through a lack of data on the 3' extremity of the gene. The length of the alignment was thus limited to 1345 sites. An evolutionary distance matrix was calculated from pairwise comparison of sequences in this alignment and a phylogenetic tree (Fig. 1) was inferred from this matrix. The inclusion of data from the 10 LLAP strains has had no significant effect on the overall topology of this tree, which is in concordance with those proposed previously (Fry et al., 1991b; Hookey et al., 1996). Seven of the 10 LLAPs form a well-defined, strongly supported (98/100 significant) monophyletic group within the genus, which also includes both previously characterized L. \textit{lytica} strains (L2 and LLAP3). Four of the uncharacterized isolates (LLAPs 2, 6, 7 and 9) exhibit a particularly intimate phylogenetic relationship with L2 and LLAP3, and the monophyletic group formed by the six strains is one of the few within the genus which had full statistical support in bootstrap analyses. The three other LLAPs

Table 1. Sources of LLAP strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host*</th>
<th>Source*</th>
<th>Location of source</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLAP1</td>
<td>Acanthamoeba sp.</td>
<td>Deposit from bottom of tank of water well</td>
<td>Pontefract, W. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP2</td>
<td>Acanthamoeba sp.</td>
<td>Deposit from bottom of a garage steam-cleaning pit</td>
<td>Sheffield, S. Yorkshire, UK</td>
</tr>
<tr>
<td>L. \textit{lytica} LLAP3</td>
<td>Acanthamoeba sp.</td>
<td>Amoebal enrichment of sputum from a patient with persistent pneumonia</td>
<td>Scarborough, N. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP4</td>
<td>Acanthamoeba sp.</td>
<td>Material from the nozzle of a hospital whirlpool bath</td>
<td>Bradford, W. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP6</td>
<td>Acanthamoeba sp.</td>
<td>Water and sludge from an industrial liquifier tower</td>
<td>Hull, Humberside, UK</td>
</tr>
<tr>
<td>LLAP7</td>
<td>Acanthamoeba sp.</td>
<td>Biofilm from tidemark area of a hotel whirlpool spa</td>
<td>Leicester, Leicestershire, UK</td>
</tr>
<tr>
<td>LLAP8</td>
<td>H. vermiciformis</td>
<td>Biofilm of hospital shower</td>
<td>Leeds, W. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP9</td>
<td>Acanthamoeba sp.</td>
<td>Factory cooling system</td>
<td>Wakefield, W. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP10</td>
<td>Acanthamoeba sp.</td>
<td>Condensate water of a cruise liner air-conditioning system; \textit{L. pneumophila} serogroup 3, \textit{V. aberdonica}, \textit{H. vermiciformis}, \textit{A. astronys} and a fourth unidentified amoeba were isolated from the same sample</td>
<td>Eastern Mediterranean</td>
</tr>
<tr>
<td>LLAP11</td>
<td>Acanthamoeba sp.</td>
<td>Cooling tower water; \textit{L. maceachernii}, \textit{M. cultura} and \textit{Acanthamoeba} species isolated from the same sample</td>
<td>Leeds, W. Yorkshire, UK</td>
</tr>
<tr>
<td>LLAP12</td>
<td>Acanthamoeba sp.</td>
<td>Cooling tower water; \textit{L. maceachernii}, a high-temperature \textit{Acanthamoeba} sp., \textit{V. platypodia} and a non-motile coccus were isolated from the same sample</td>
<td>Leeds, W. Yorkshire, UK</td>
</tr>
<tr>
<td>L. \textit{lytica} L2 (type strain)</td>
<td>Acanthamoeba sp.</td>
<td>Soil</td>
<td>Lublin, Poland</td>
</tr>
</tbody>
</table>

*Key to genus names: \textit{A.}, Acanthamoeba; \textit{H.}, Hartmannella; \textit{L.}, Legionella; \textit{M.}, Mayorella; \textit{V.}, Vannella; \textit{Vk.}, Valkampfia.
### Table 2. 16S rRNA gene sequence similarities (%) for LLAPs, strains of *L. lytica*, and other *Legionella* species

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLAP1</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP2</td>
<td>94.7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lytica</em> LLAP3</td>
<td>94.6</td>
<td>99.1</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP4</td>
<td>94.7</td>
<td>97.2</td>
<td>97.6</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP6</td>
<td>94.7</td>
<td>99.9</td>
<td>99.1</td>
<td>97.0</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>LLAP7</td>
<td>94.7</td>
<td>99.4</td>
<td>99.9</td>
<td>97.5</td>
<td>99.3</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP8</td>
<td>94.7</td>
<td>95.5</td>
<td>95.3</td>
<td>95.6</td>
<td>95.5</td>
<td>95.4</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP9</td>
<td>94.5</td>
<td>99.3</td>
<td>99.8</td>
<td>97.6</td>
<td>99.2</td>
<td>99.9</td>
<td>95.2</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP10</td>
<td>95.2</td>
<td>97.1</td>
<td>96.6</td>
<td>96.2</td>
<td>97.1</td>
<td>96.7</td>
<td>95.5</td>
<td>96.7</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLAP11</td>
<td>95.0</td>
<td>97.3</td>
<td>97.6</td>
<td>98.7</td>
<td>97.3</td>
<td>97.6</td>
<td>96.0</td>
<td>97.6</td>
<td>96.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>LLAP12</td>
<td>95.0</td>
<td>97.3</td>
<td>97.6</td>
<td>98.7</td>
<td>97.3</td>
<td>97.6</td>
<td>96.0</td>
<td>97.6</td>
<td>96.5</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td><em>L. lytica</em> L2</td>
<td>94.5</td>
<td>99.3</td>
<td>99.8</td>
<td>97.6</td>
<td>99.2</td>
<td>99.9</td>
<td>95.2</td>
<td>100</td>
<td>96.7</td>
<td>97.6</td>
<td>97.6</td>
</tr>
<tr>
<td><em>L. feeleii</em></td>
<td>95.5</td>
<td>94.3</td>
<td>93.5</td>
<td>93.0</td>
<td>94.0</td>
<td>93.8</td>
<td>94.3</td>
<td>93.7</td>
<td>94.3</td>
<td>93.6</td>
<td>93.6</td>
</tr>
<tr>
<td><em>L. tucsonensis</em></td>
<td>94.4</td>
<td>96.5</td>
<td>96.0</td>
<td>95.0</td>
<td>95.0</td>
<td>96.0</td>
<td>95.5</td>
<td>96.0</td>
<td>96.0</td>
<td>96.6</td>
<td>96.6</td>
</tr>
<tr>
<td><em>L. wadsworthii</em></td>
<td>93.9</td>
<td>95.6</td>
<td>95.0</td>
<td>95.6</td>
<td>95.6</td>
<td>95.0</td>
<td>95.4</td>
<td>95.7</td>
<td>95.7</td>
<td>95.7</td>
<td>95.7</td>
</tr>
</tbody>
</table>

* *L. feeleii* shares highest similarity with ‘Glasgow’ 86/35784 (98.2%), *L. tucsonensis* shares highest similarity with *L. parisiensis* (97.2%), and *L. wadsworthii* shares highest similarity with *L. cebrii* (97.2%).

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**Fig. 1.** Phylogenetic tree reflecting evolutionary relationships between LLAPs, members of the genus *Legionella* and *Coxiella burnetii*. The tree was inferred from a distance matrix using the criteria of Fitch & Margoliash (1967) as described in the text. The numbers shown next to the nodes indicate bootstrap values of 100 replicates. Only those supported by more than 50% of the samples are indicated.
within the clade (LLAPs 4, 11 and 12) form their own
distinct and well-supported monophyletic group, sepa-
rated from the L. lytica-containing group by an evolu-
tionary distance at least as long as those observed
between other closely related species within the genus.
Although LLAP10 lies on the same deep-rooted branch as
the L. lytica group of isolates discussed above, it forms a
distinct lineage, lying on a branch which diverged from
that carrying the L. lytica group relatively soon after their
shared deep-rooted branch diverged from those carrying
the other Legionella species. Both LLAP1 and LLAP8 lie
on their own deep-rooted branches within the tree, and
neither demonstrates any specific evolutionary relation-
ship to either other LLAPs or to other Legionella species.
Parsimony analysis of the aligned sequences confirmed
the evolutionary relationships between the LLAP and L.
lytica strains proposed by distance matrix analysis. The
lineage carrying all isolates except LLAPs 1 and 8 was
retained in this analysis, as was the clustering of the L.
lytica strains with LLAPs 2, 6, 7 and 9. Again LLAPs 1 and
8 demonstrated no specific evolutionary links with either
L. lytica or the LLAP strains (data not shown).

The legionellae are one of the most recently discovered
groups of pathogenic bacteria, having been first
recognized following a large outbreak of what is now
known as legionnaires' disease, in 1976. The failure to
identify either the disease or its agent previously probably
resulted from the fastidious nature of legionellae, which
do not grow on media conventionally used for the
isolation of bacterial pathogens (McDade et al., 1977).
Indeed, agars able to support the growth of Legionella
pneumophila, the legionnaires' disease agent, were only
developed, with considerable effort, following initial
characterization of the bacterium in infected guinea pigs.
As legionellae remain, above all, organisms of medical
interest, laboratory media have generally evolved to
enhance the isolation of Legionella pneumophila, which is by far the
most clinically important species. However, it is now
increasingly likely that legionellae are protozoan pathogens, coexisting in vivo with protozoan hosts, and
only occasionally infecting man, and that their physiology
is adapted to the specific environmental niche they fill. It
is therefore not surprising that a medium suitable for the
growth of one species is not always appropriate for
others. Indeed, although none of the L. lytica strains or
the LLAPs grow on conventional BCYE, Giles et al.
(1995) have very recently reported preliminary data indicating that supplementation of this medium with
amino acids enabled the cultivation of L. lytica strain L2.
With the inclusion of L. lytica in the genus Legionella
comes the realization that not all legionellae pathogenic to
man grow on BCYE.

The assumption that the evolutionary divergence of
'BCYE-culturable' Legionella strains is presently broader
than that of 'BCYE-unculturable' strains (and that
'unculturability' has evolved from 'culturability') may
itself be misleading, and again highlights the extremely
biased view of bacterial biodiversity which has arisen
from our historical reliance on culture as an essential
prerequisite for microbial characterization. The advent of
PCR methodologies and establishment of easily-accessed
public-domain databases now provides the means to more
accurately assess microbial populations without requiring
their axenic cultivation. More substantial surveys of
environmental Legionella/LLAP populations are now
feasible and may demonstrate a genus in which species
that grow on presently used media are exceptions rather
than the norm. The L. lytica L2 strain has been shown to
react with the widely used Perkin-Elmer Legionella genus-
specific PCR test (Giles et al., 1995); thus specimens
positive with this test but negative by conventional
culture may be useful sources for new LLAPs.

The recognition of diversity among LLAPs also has
medical implications; although to date the ability of
LLAPs to cause human respiratory infection has only
been confirmed once, serological evidence suggests that
they may play a significant role in the aetiology of atypical
pneumonias (Benson et al., 1995; Rowbotham, 1993). As
diagnosis of these infections is most commonly made
using serological tests incorporating specific antigens to
detect specific antibodies (Harrison & Taylor, 1988), the
isolation and characterization of the agents to be tested for
is a prerequisite. Unless all unculturable Legionella species
share common immunoreactive antigens (most culturable
species do not), different species need to be defined in
order that all can be included in future serological surveys
or diagnostic screening schemes. That all species be
included in such studies is important: of the presently
recognized Legionella species, several were only implicated
in human disease following their initial isolation from
environmental sources.

Although the name L. lytica is appropriate for the two
strains to which it is presently applied, the phylogenetic
diversity of the organisms tested in this study clearly
indicates that LLAPs cannot be considered as belonging
to a single species and thus the taxonomic definition of
L. lytica needs to be revised. Indeed a description of
'Legionella-like' for these organisms is itself now a
misnomer and should perhaps be abandoned. The novel
strains included in this study are presently undergoing
phenotypic and genotypic characterization prior to pro-
posals for their formal incorporation into Legionella
classification.

REFERENCES

Barbaree, J. M., Fields, B. S., Feeley, J. C., Gorman, G. W. &
Martin, W. T. (1986). Isolation of protozoa from water associated
with a legionellosis outbreak and demonstration of intracellular
multiplication of Legionella pneumophila. Appl Environ Microbiol 51,
422-424.

Benson, R. F., Drozanski, W. J., Rowbotham, T. J., Bialkowska, L.,
Losos, D., Butler, J. C., Lipman, H. B., Plouffe, J. F. & Fields, B. S.
amoebal pathogens in pneumonia patients. 95th ASM General
Meeting, Washington DC, USA, 21–25 May 1995, Abstract C-200,
p. 35.

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based on phylogenetic and phenotypic characteristics. FEMS


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