Legionella drancourtii sp. nov., a strictly intracellular amoebal pathogen

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A *Legionella*-like amoebal pathogen (LLAP), formerly named LLAP12^T, was characterized on the basis of microscopic appearance, staining characteristics, growth in *Acanthamoeba polyphaga* at different temperatures, DNA G+C content, serological cross-reactivity and 16S rRNA and macrophage infectivity potentiator (*mip*) gene sequence analysis. LLAP12^T was found to be a motile, Gram-negative bacterium that grew within cytoplasmic vacuoles in infected amoebae. The infecting bacteria induced lysis of their amoebal hosts and time taken to do so was dependent on incubation temperature. Recovery of LLAP12^T from amoebae onto axenic media could not be achieved. Phylogenetic analysis of LLAP12^T, based on 16S rRNA and *mip* gene sequence analysis, indicated that it lay within the radiation of the *Legionellaceae* and that it clustered specifically with *Legionella lytica* and *Legionella rowbothamii*. The divergence observed between LLAP12^T and these two species was of a degree equal to, or greater than, that observed between other members of the family. In support of this delineation, LLAP12^T was found not to cross-react serologically with any other *Legionella* species. The *mip* and 16S rRNA gene sequence-based analyses also indicated that LLAP12^T was related very closely to two other previously identified LLAP isolates, LLAP4 and LLAP11. Taken together, these results support the proposal of LLAP12^T as the type strain of *Legionella drancourtii* sp. nov.

Intracellular parasitism of protozoa by bacteria is an increasingly recognized phenomenon. Adaptation to this niche has occurred independently among different groups of bacteria: extant intra-protozoal parasites are phylogenetically diverse. Five lineages have been identified among the obligate endosymbionts (Horn *et al.*, 2001) and this diversity is matched by that of facultative intra-amoebal parasites, which include numerous established human pathogens (Barker & Brown, 1994; Horn *et al.*, 2001). Following the demonstration that legionellae exploit amoebae as natural hosts (Rowbotham, 1980, 1983), amoebal co-culture methods have been introduced for the recovery of legionellae from environmental samples

and have led to the isolation of several organisms that could not be cultured on axenic media (Rowbotham, 1983; Barbaree *et al.*, 1986). As these organisms could not be subjected to conventional methods of bacterial identification, their designation remained *Legionella*-like amoebal pathogens (LLAPs). The introduction of 16S rRNA gene PCR-based methods finally permitted partial characterization of LLAPs and, on the basis of 16S rRNA gene sequence comparison, many were found to share specific evolutionary homology with members of the *Legionellaceae* (Fry *et al.*, 1991; Birtles *et al.*, 1996). Although several LLAPs have now been grown axenically (Adeleke *et al.*, 2001), cultivation of others has failed. In this study, an LLAP isolate that had previously been characterized only by 16S rRNA gene sequence analysis (Birtles *et al.*, 1996) was subjected to polyphasic assessment, thereby permitting its accurate classification.

LLAP12^T was isolated by T. J. Rowbotham (Public Health Laboratory, Leeds, UK) in co-culture with an *Acanthamoeba*

Abbreviations: LLAP, *Legionella*-like amoebal pathogen; *mip*, macrophage infectivity potentiator.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *L. drancourtii* LLAP12^T is X97366 and that for the *mip* gene sequence is AF407571.

species from an environmental water source in West Yorkshire, UK (Fallon & Rowbotham, 1990). In our laboratory, LLAP12^T was co-cultured routinely with *Acanthamoeba polyphaga* strain Linc AP-1 in peptone/yeast extract/glucose medium under previously described conditions at 32 °C (Rowbotham, 1983). All attempts to isolate LLAP12^T by using buffered charcoal/yeast extract agar failed, despite the use of media from different manufacturers (bioMérieux, Diagnostic Pasteur) and prolonged incubation at 30 or 35 °C, with or without the presence of 5 % CO₂.

Phenotypic characterization

The microscopic appearance of LLAP12^T was verified by direct observation of infected amoebae using an inverted microscope, and of fixed and stained bacteria by using conventional microscopy. Both approaches revealed large numbers of motile bacteria within amoebal cytoplasmic vacuoles. These organisms stained weakly with Gram stain (negative), but well with Giménez stain. Ultrastructural analysis using transmission electron microscopy (La Scola *et al.*, 2001) indicated that LLAP12^T was a polymorphic, Gram-negative organism and confirmed that clusters of bacteria lay within amoebal cytoplasmic vacuoles (Fig. 1).

Purification of LLAP12^T (for inoculation into animals) and serology were performed as described previously (La Scola *et al.*, 2001), as was the production of polyvalent mouse antibodies (La Scola *et al.*, 2001). This antiserum

Fig. 1. Transmission electronic micrograph of LLAP12^T within vacuoles of *A. polyphaga*. Bar, 1 µm.

was adsorbed to the amoebae by mixing the sera with washed amoebae, then shaking the mixture overnight at room temperature. After centrifugation at 10 000 g for 10 min, the supernatant was removed and frozen at -80 °C. The immunofluorescent test (Harrison & Taylor, 1988) was used to assess cross-reactivity between antisera that were raised against LLAP12^T and 69 different *Legionella* antigens, and cross-reactivity between LLAP12^T and rabbit antisera that were raised against 80 *Legionella* antigens. Despite a high (>1/3200) homologous antibody titre to LLAP12^T, the mouse antiserum failed to react significantly (>1/100) with any other *Legionella* antigen preparation. Furthermore, all antisera that were raised against other *Legionella* taxa failed to react significantly (>1/100) with LLAP12^T antigen.

Our observations of LLAP12^T morphology and its intra-amoebal position are in keeping with those reported for other legionellae. Although serological reactivity among members of the *Legionellaceae* varies, this approach has long served as a routine method for species identification. It is reasonable to suspect that an isolate that demonstrates no reactivity with a panel of antisera that represents the serological spectrum of the current genus is likely to be a member of a novel taxon.

Sequence analysis

Methods used for amplification and determination of 16S rRNA and macrophage infectivity potentiator (*mip*) gene sequences and the procedure for determination of DNA G+C content have been detailed elsewhere (Birtles *et al.*, 1996; Ratcliff *et al.*, 1998; La Scola *et al.*, 2001). A partial *mip* gene sequence for *Legionella taurinensis* Turin I^T was also determined. A 585 bp fragment of the *mip* gene of LLAP12^T was sequenced and used for further analysis. Sequence comparison revealed that the LLAP12^T sequence was most similar (86–88 %) to those of *Legionella lytica* and *Legionella rowbothamii*. Phylogenetic reconstruction, derived from an alignment of the LLAP12^T *mip* gene sequence fragment with those of *Legionella* species with validly published names by using previously described methods (Birtles *et al.*, 1996; Ratcliff *et al.*, 1998), revealed that LLAP12^T clustered specifically with, but had diverged from, *L. lytica* and *L. rowbothamii* (Fig. 2). This branching order was supported strongly by bootstrap analysis. Interestingly, the LLAP12^T *mip* gene sequence was found to be indistinguishable from that of LLAP4, an organism that was isolated in the same manner as LLAP12^T and was characterized partially by 16S rRNA gene sequence analysis (Birtles *et al.*, 1996). Although this earlier study found that among the *Legionellaceae*, LLAP4 was related specifically to LLAP12^T, there was, nonetheless, 1.3 % 16S rRNA gene sequence dissimilarity between them (19 mismatches in an alignment of 1482 bp). This was the first time that interstrain 16S rRNA gene sequence dissimilarity had been found to be greater than that observed between *mip* gene sequences. Furthermore, the extent of 16S rRNA gene sequence dissimilarity between LLAP4 and LLAP12^T was greater than that seen between some different *Legionella*

Fig. 2. Dendrogram representing phylogenetic relationships between *L. drancourtii* LLAP12^T and other closely related members of the genus *Legionella*. Dendrograms were generated from *mip* gene fragments (right-hand tree) and the 16S rRNA gene (left-hand tree) by using the neighbour-joining method. Support of each branch, as determined from 1000 bootstrap resamples, is indicated by the value at the node when ≥ 750 .

species (the 16S rRNA genes of *L. rowbothamii* and *L. lytica* differ by <1 %). Concern about these observations led us to repeat 16S rRNA gene sequencing for LLAP4 and LLAP12^T. Although LLAP4 was no longer extant, DNA preparations remained. The 16S rRNA gene sequences we obtained from LLAP12^T and LLAP4 were different from those submitted to GenBank (accession numbers X97366 and X97357, respectively). The new LLAP12^T sequence differed at only two positions from that submitted to GenBank (in a 1348 bp alignment), whereas the new LLAP4 sequence differed at 12 positions (in a 1350 bp alignment). Comparison of the new LLAP4 and LLAP12^T 16S rRNA gene sequences indicated that they differed at only four sites (<0.3 % of a 1348 bp alignment). Our experiences serve as a warning that sequences held in databases, particularly those deposited in the 1980s and early 1990s when sequencing methodologies were somewhat cruder than those that are currently available, cannot always be relied on. The LLAP4 and LLAP12^T 16S rRNA gene sequences held by GenBank have now been revised.

Comparison of 16S rDNA sequences reveals that LLAP12^T shares 97 % similarity with *L. lytica* L2^T and *L. rowbothamii* LLAP-6^T and 98 % similarity with *L. lytica* LLAP7. As it has been demonstrated previously that organisms sharing > 97 % similarity may belong to the same species (Ash *et al.*, 1991; Fox *et al.*, 1992; Stackebrandt & Goebel, 1994), this analysis should not be relied on in the justification of species status for LLAP12^T. However, the 16S rRNA gene sequences of other *Legionella* species are more similar to one another than that of LLAP12^T is to that of *L. lytica*. Furthermore, phylogenetic inference derived from 16S rRNA gene sequence data demonstrates clearly that LLAP12^T has diverged significantly from its close relatives. A previous study using these data (Birtles *et al.*, 1996) included LLAP4 and LLAP11, organisms that are no longer viable and thus could not be included in the current study. LLAP11 was found to possess a 16S rRNA gene sequence that was indistinguishable from that of LLAP12^T, whereas LLAP4 was shown in this study to possess a sequence that shares > 99 % similarity with that of LLAP12^T. Taken together,

these data provide further evidence that LLAP12^T is not unique, but rather represents a distinct clade of legionellae.

Phylogenetic analyses derived from 16S rRNA and *mip* gene analysis are generally congruent (Ratcliff *et al.*, 1998), although with variation within the genus *Legionella* of up to 30 %, *mip* gene sequences potentially offer a more reliable and sensitive basis for comparative analysis. Nonetheless, the combined taxonomic value of 16S rRNA and *mip* gene sequence analyses is greater than the sum of their individual worth, as congruence in bi- or multilocus analyses provides a good indication that evolutionary relationships inferred from individual genes or gene fragments do indeed represent true phylogenies for the organism. Thus, these data and those derived from the serological assessment provide unequivocal evidence that LLAP12^T is a representative of a novel *Legionella* species. The data also support the inclusion of LLAP4 and LLAP11 in this species, a hypothesis that was first put forward solely on the basis of 16S rRNA gene sequence data (Adeleke *et al.*, 1996).

Effect of temperature on the physiology of intra-amoebal LLAP12^T

To assess the effect of temperature on the nature of the association between LLAP12^T and its amoebal host, six flasks that each contained 10 ml of a suspension of washed amoebae ($5 \times 10^5 \text{ ml}^{-1}$) in Page's amoeba saline (PAS) solution were seeded with 1 ml supernatant from a 7-day-old infected flask to yield a bacteria:cell ratio of 10:1. Infected flasks were incubated at 25, 28, 30, 32, 35 and 37 °C. Every day for 8 days, flasks were shaken, then 100 µl culture was removed, mixed with 300 µl trypan blue (0.13 %) (Sigma-Aldrich) in PAS solution, then transferred to a Nageotte counting chamber. Microscopic

examination of amoebae permitted the enumeration of intact cells. Six uninfected amoebal cultures that were prepared concurrently with those described above and incubated and handled in the same way were used as controls.

A. polyphaga grew at all temperatures tested, but higher numbers of cells survived for longer periods at lower incubation temperatures (Fig. 3). Conversely, when infected with LLAP12^T, amoebae incubated at these lower temperatures (<35 °C) lysed more rapidly (Fig. 3). After 48 h, LLAP12^T infection led to lysis of 53 % of amoebae at 25–32 °C, as opposed to 0 % at 35–37 °C ($P < 0.05$). This observation was confirmed by phase-contrast microscopic observation of infected amoebal cultures in culture flasks. After 48 h, co-cultures that were incubated at 25 and 28 °C already included high numbers of rounded, detached cells that contained a single, large vacuole filled with motile bacilli. This cytopathic effect was not observed in cultures that were incubated at 35–37 °C, even after prolonged incubation, demonstrating that LLAP12^T maintained stable infection of amoebae at higher temperatures. This ability may contribute to the propensity of man-made water supplies, which are commonly maintained at higher temperatures than that of natural aquatic environments, to serve as sources of *Legionella* infection. Previous work has demonstrated that the virulence of legionellae increases when they are associated with amoebae (Cirillo *et al.*, 1999); thus, exposure to conditions that are capable of prolonging this association must serve to enhance this pathogenic potential.

Concluding remarks

Legionellae have been traditionally classified by using polyphasic means, with DNA–DNA hybridization as the

Fig. 3. Effect of temperature on the growth and survival of uninfected (open symbols) and LLAP12^T-infected (filled symbols) *A. polyphaga*.

cornerstone of species delineation. Although a variety of different methods can be utilized in the characterization of intra-amoebal organisms (as demonstrated in this study), the application of techniques that require large amounts of DNA, such as DNA–DNA hybridization, is not feasible. However, it seems foolhardy to exclude organisms such as LLAP12^T from classification, or to even deny them full taxonomic status, merely because of a lack of DNA–DNA hybridization data, if a proposal is well-supported by data derived from other assessments. Clearly, for some intracellular organisms that cannot be sustained in *in vitro* cultures of eukaryotic cells, concerns could be raised about the uniqueness of isolates, but in the case of LLAP12^T and other LLAPs that have been subjected to limiting dilution and multiple passage, such concerns recede. Furthermore, cultures of LLAP12^T have been accepted for deposition in culture collections (World Health Organization Collaborative Center for Rickettsial Reference and Research, Marseilles, France, and the American Type Culture Collection, Manassas, VA, USA), thereby providing reference strains for future studies. We propose the name *Legionella drancourtii* sp. nov. for this organism, in honour of Michel Drancourt, a prolific French microbiologist who has contributed significantly to our understanding of the diversity and nature of intracellular bacteria of medical importance.

Description of *Legionella drancourtii* sp. nov.

Legionella drancourtii (dran.cour'ti.i. N.L. gen. n. *drancourtii* in honour of Michel Drancourt for his work on intracellular micro-organisms, especially *Rickettsiae*).

Gram-negative. Does not grow on axenic media. Exists naturally as a strictly intracellular parasite of free-living amoebae. Cytopathic effect on *A. polyphaga* is restricted to co-cultures that are incubated at lower temperatures (25–32 °C). May be observed as motile rods within vacuoles of amoebae. DNA G + C content is 39.4 mol%.

The type strain is LLAP12^T = ATCC 50991^T.

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