'*Candidatus Odysella thessalonicensis*' gen. nov., sp. nov., an obligate intracellular parasite of *Acanthamoeba* species

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An intracellular bacterium, strain L13, was observed infecting an environmental isolate of an *Acanthamoeba* species. The bacterium could not be recovered on axenic medium but was recovered and cultivated *in vitro* using cultures of *Acanthamoeba polyphaga*. The 16S rRNA gene sequence of L13 was found to be new, sharing less than 84% similarity with other sequences in the GenBank/EMBL database. L13 was found to be a member of the α -Proteobacteria, sharing an evolutionary line of descent with a group of uniquely obligate intracellular organisms comprised of *Caedibacter* and *Holospira* species and the NHP bacterium. Viable bacteria appeared to be highly motile within amoebae. Ultrastructural analysis of the bacterium demonstrated that it is rod-shaped and possesses a typical Gram-negative cell wall, but has no other outstanding features except small vesicle-like structures often associated with the outer surface of each bacterium. The host range of L13 was found to be limited to the genus *Acanthamoeba*. In *A. polyphaga*, L13 infection was slow to manifest when cultures were incubated below 30 °C, but at higher temperatures bacteria multiplied prolifically and induced host cell lysis. The protein profile of the bacterium purified from the amoebae was assessed by SDS-PAGE and its G+C content was estimated to be 41 mol%. Although these results support the proposal of L13 as a new species, its obligate intracellular nature prevented isolation of a definitive type strain. L13 is therefore proposed as '*Candidatus Odysella thessalonicensis*' gen. nov., sp. nov.

Keywords: '*Candidatus Odysella thessalonicensis*', obligate intracellular bacterium, α -Proteobacteria, *Acanthamoeba*

INTRODUCTION

Among the diverse bacteria that make up the staple diet of free-living protozoa, some have evolved the ability to survive grazing and exploit their predators as hosts. Over the past 25 years there have been numerous observations of such bacteria, although for most, their obligate intracellular nature has prevented their axenic culture and has thus excluded them from charac-

terization and taxonomic placement (Fritzsche *et al.*, 1993; Hall & Voelz, 1985; Michel *et al.*, 1995b; Preer & Preer, 1984; Proca-Ciobanu *et al.*, 1975). Such organisms have generally been distinguished from one another solely on the basis of either morphological features or differences in host specificity. The advent of molecular identification methods, involving PCR-based amplification then sequence analysis of 16S rRNA genes, has permitted meaningful taxonomic assessments to be applied to these organisms for the first time. Recent studies have demonstrated the evolutionary diversity of intraprotzoal bacteria, with meaningful phylogenetic placements being proposed for amoeba-infecting *Legionella* species and *Chlamydia*-like organisms (Amann *et al.*, 1997; Birtles

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The GenBank/EMBL accession number for the 16S rRNA sequence of '*Candidatus Odysella thessalonicensis*' strain L13 is AF069496.

et al., 1996), and the ciliate-infecting species *Holospira obtusa* and *Caedibacter caryophilus* (Amann *et al.*, 1991; Springer *et al.*, 1993).

Such taxonomic studies have been accompanied by *in vitro* demonstrations of the ability of a number of recognized human pathogens to survive in or exploit protozoan hosts. Pathogens including *Legionella pneumophila*, *Listeria monocytogenes*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Burkholderia pickettii* and *Edwardsiella tarda* have been shown to be able to multiply within free-living amoebae (King & Shotts, 1988; Ly & Müller, 1990; Michel *et al.*, 1995a; Michel & Hauröder, 1997; Rowbotham, 1980; Thom *et al.*, 1992), whereas other species have been shown to be able to survive protozoal uptake and to be viably maintained within them (Jardin, 1975; King *et al.*, 1988; Tyndall *et al.*, 1991). Taken together, these reports signify an increased awareness of the role protozoa are likely to play in the environmental maintenance of a spectrum of micro-organisms, and thus the importance of their study.

In this paper we characterize a novel acanthamoebal parasite and, on the basis of this characterization, propose it as a member of a new genus under the name '*Candidatus Odysella thessalonicensis*' gen. nov., sp. nov.

METHODS

Primary isolation of L13. Microscopic examination of a water sample collected from the drip-tray of the air conditioning unit of a hospital in Thessalonika, Greece, revealed an *Acanthamoeba* sp. infected with rod-shaped bacteria. The recovery and cultivation of these bacteria in *Acanthamoeba polyphaga* Linc Ap-1 (Fallon & Rowbotham, 1990) was achieved as previously described for legionellae (Birtles *et al.*, 1996).

Axenic culture of L13 was attempted by lysing and then inoculating infected amoebae onto a range of media, including buffered charcoal yeast extract agar, trypticase soy agar, heated horse blood agar, Columbia blood agar, chocolate agar, chocolate polyvitex agar, McConkey's agar and nutrient agar. Inoculated media were incubated at ambient temperature and at 37 °C under aerobic and anaerobic conditions for up to 2 weeks.

Morphological characteristics. The microscopic appearance of L13 within infected amoebae was determined by Gimenez staining. Ultrastructural analysis of L13 was achieved using transmission electron microscopy. A 4-d-old culture of *A. polyphaga*, observed to be heavily infected with L13 by Gimenez staining, was harvested by centrifugation at 5000 g for 10 min, washed twice in Page's amoebal saline (PAS; Page, 1976), then pre-fixed in 2.5% (v/v) glutaraldehyde in PAS for 2 h at 4 °C. Amoebae were harvested again as above, washed twice in PAS, dehydrated in an ethanol series (70, 90 and 100%, v/v) and embedded in Epon 812. Thin transverse sections were cut using an LKB Ultratome III microtome, then attached to microscope grids and stained with a saturated solution of methanol/uranyl acetate and lead citrate in water. Grids were examined using JEOL JEM 1200EX apparatus.

Host range of L13. The host range of L13 was assessed in 23 strains of free-living amoebae. These strains, which belong to 19 species and nine genera, are listed in Table 1. All amoebae were isolated and identified by R. Michel, except *Naegleria gruberi* CCAP1518/1^e and *Naegleria lovaniensis* Aq/9/1/4D, which were kindly provided by Dr J. De Jonckheere, Wetenschappelijk Instituut voor Volksgezondheid-Louis Pasteur, Brussels, Belgium, *Balamuthia mandrillaris*, kindly provided by Dr K. Janitschke, Robert Koch Institut, Berlin, Germany, and the *Acanthamoeba* group III Rink strain, kindly provided by Dr H. Seitz, Institut für Medizinische Parasitologie, Bonn, Germany. Two protocols were used to determine host range, one for amoebae which could be maintained *in vitro* in axenic media and one for those which could only be maintained in the laboratory through grazing on bacterial lawns (see Table 1). For both methods, an L13-infected culture of *A. polyphaga* was harvested by centrifugation at 5000 g for 10 min. The pellet was resuspended in SCGYE medium (De Jonckheere, 1977), frozen for 3 h at -20 °C and thawed rapidly in a water bath at 37 °C. Liberated bacteria were separated from amoebal debris by passage through a 1.2 µm filter (Minisart NML; Sartorius) and the filtrate was used immediately to seed cultures of amoebal species, grown either in SCGYE medium or on lawns of enterobacteria cultivated on NN agar (NNA) plates (Page, 1976). Infection of each host species was monitored by microscopic examination using phase-contrast microscopy. If neither infected cells nor any marked reduction in amoebal numbers (which may have resulted from the action of the parasite) were observed after 21 d incubation at 30 °C, the host was considered resistant to L13 infection.

Effect of temperature on bacterial virulence. Cell culture flasks (Rowbotham *et al.* 1983) containing 20 ml PYG were seeded with 1.5×10^3 *A. polyphaga* then incubated at 24, 30, 32 or 37 °C. After 3 d incubation, the concentration of intact amoebae in each flask was determined. This was achieved by first shaking the flask to disrupt the monolayer, then removing 200 µl suspension. The suspension was diluted 1:3 by the addition of equal volumes of PAS and trypan blue (Sigma), then transferred to a counting chamber. The number of amoebae within each grid of the chamber was assessed by microscopic observation using $\times 20$ objective and $\times 10$ eyepiece lenses. At least three grids were counted for each sample. Estimates were repeated on day 4, after which cultures were split into two 10 ml aliquots in new 20 cm² flasks and one was seeded with L13. The inoculum used was 200 µl of a 500 amoeba µl⁻¹ suspension drawn from a 3-d-old culture of *A. polyphaga* infected with L13 (incubated at 30 °C). Microscopic observation of this culture following Gimenez staining indicated about 1 in 30 amoebae were infected with 20–50 bacteria within each amoeba, thus the infecting inoculum contained in the order of 6.6×10^4 – 1.5×10^5 intracellular bacteria. Following inoculation, the amoebal concentrations within infected and uninfected cultures at each of the four temperatures were monitored each day (as described above) for the next 12 d, then subsequently at 15, 18 and 21 d.

Amplification and base sequence determination of the 16S rRNA gene of L13. A crude DNA extract was prepared from a heavily infected amoebal culture as described previously (Birtles *et al.*, 1996). This extract was then used as template in a PCR incorporating broad-spectrum 16S rRNA gene primers (Lane, 1991). The amplification product was purified using reagents of the QIAquick purification kit (Qiagen), according to manufacturer's instructions, for use as template

Table 1. Results of L13 co-cultivation tests using different strains of free-living amoebae

Species	Strain	Intracytoplasmic multiplication of L13	Growth requirement
<i>Acanthamoeba polyphaga</i>	Linc A1	+++	SCGYE
<i>Acanthamoeba lenticulata</i> (group III)	89	+	SCGYE
<i>Acanthamoeba lenticulata</i> (group III)	45	++	SCGYE
<i>Acanthamoeba lenticulata</i> (group III)	118	++	SCGYE
<i>Acanthamoeba</i> spp. (group II)	HLA	++	SCGYE
<i>Acanthamoeba</i> spp. (group II)	Rink	+	SCGYE
<i>Acanthamoeba castellanii</i> (group II)	C3	+++	SCGYE
<i>Acanthamoeba quinalugdunensis</i> (group II)	312-1	++	SCGYE
<i>Acanthamoeba comandoni</i> (group I)	Am23	—	NNA
<i>Acanthamoeba comandoni</i> (group I)	WBT	—	NNA
<i>Naegleria gruberi</i>	CCAP1518/1e	—	SCGYE
<i>Naegleria gruberi</i>	aflag	—	SCGYE
<i>Naegleria lovaniensis</i>	Aq/9/1/4D	—	SCGYE
<i>Willaertia magna</i>	NI4CL1	—	SCGYE
<i>Willaertia magna</i>	JIIIPW	—	SCGYE
<i>Vahlkampfia ustiana</i>	A1PW	—	NNA
<i>Vahlkampfia ovis</i>	Rhodos	—	NNA
<i>Vahlkampfia avara</i>	TWA	—	NNA
<i>Hartmannella vermiformis</i>	Os/101	—	SCGYE
<i>Comandonia operculata</i>	WBT	—	NNA
<i>Vannella</i> spp.	Aun Vano	—	NNA
<i>Saccamoeba limax</i>	SL1CL1	—	NNA
<i>Balamuthia mandrillaris</i>	CDC VO39	—	SCGYE

in cycle sequencing reactions. Sequencing reactions used reagents of the Amplicycle kit (Pharmacia), incorporating fluorescein 5'-labelled primers. The primers employed were universal eubacterial 16S rRNA sequencing primers (Lane, 1991). The products of sequencing reactions were resolved on 0.35 mm Readimix acrylamide gels (Pharmacia), then detected and converted into sequence data using an ALF automated sequencer (Pharmacia) and associated software. The nucleotide sequence of the 16S rRNA gene was determined in both forward and reverse directions and in triplicate.

Phylogeny of L13. The primary sequence of the 16S rRNA gene of L13 was generated by aligning and then combining the sequences generated by each primer using DNASIS (Hitachi Software Engineering). The validity of this sequence was verified by assessing theoretical nucleotide base pairing in a eubacterial secondary structure model (Neefs *et al.*, 1993). The similarity values between the verified L13 sequence and those of other eubacterial 16S rRNA sequences were assessed using a BLAST search of the GenBank/EMBL databases (24 June 1998).

All phylogenetic studies of the L13 16S rRNA gene sequence were carried out on programs supported within the BISANCE workstation (Dessen *et al.*, 1990). The sequence was aligned with those of other chosen *Proteobacteria* using the multi-sequence alignment program CLUSTAL (Higgins *et al.*, 1992). Phylogenetic inferences were derived from this alignment once overhanging sequences at either end of the alignment, gaps and ambiguities had been removed. Three approaches were used, all of which used programs within the PHYLIP suite

(J. Felsenstein, University of Washington, Seattle, USA). First, a matrix of evolutionary distances was generated from the alignment using DNADIST under the assumptions of Jukes & Cantor (1969) and a phylogenetic tree was derived from this matrix using the criteria of Fitch & Margoliash (1967) (FITCH). Second, the alignment was subjected to parsimony analysis (DNAPARS) and third, maximum-likelihood analysis (DNAMLK). The stabilities of the topologies proposed by each approach were assessed by bootstrap analysis using the programs SEQBOOT and CONSENSE to yield a strict majority-rule consensus tree based on 200 samples.

Purification of L13 from *A. polyphaga* cultures. The entire procedure for the recovery of L13 from infected *A. polyphaga* was carried out at 4 °C. L13-infected *A. polyphaga* was harvested from ten cultures (40 ml) by centrifugation at 10400 *g* for 12 min at 4 °C. Pellets from each harvest were then resuspended and pooled into 10 ml PAS. This suspension was centrifuged at 300 *g* for 15 min to pellet intact amoebae, which were then resuspended in 5 ml PAS and disrupted by sonication at 4 °C for 2 min. The efficiency of this step was assessed by centrifugation at 300 *g* for 15 min: if a pellet reformed, sonication was repeated. When a pellet was no longer formed, all the supernatants were pooled, centrifuged at 10400 *g* for 12 min at 4 °C and the pellet was resuspended in 10 ml PAS. This suspension was then layered onto an equal volume of 25% (w/v) sucrose in PAS and centrifuged at 8000 *g* for 30 min. The pellet was washed twice with PAS, resuspended in 2 ml PAS, then carefully layered onto a 45, 36, 28% (w/v in PAS) renograffin gradient. The gradient was centrifuged at 25000 *g* for 60 min, then bacteria were recovered from the 45:36%

(intact bacteria) and 36:28% (broken bacteria) interfaces using a spinal puncture needle. These bacteria were then washed twice in PBS, resuspended in PBS and stored at -20°C until required. The presence and purity of recovered bacteria were assessed by Gimenez staining.

Analysis of cellular proteins. Purified bacteria, unpurified bacteria and uninfected amoebae (each at a concentration of 20–50 μg protein) were each mixed 1:1 with Laemmli buffer (0.625 M Tris/HCl, pH 8.0; 2%, w/v, SDS; 5%, v/v, mercaptoethanol; 10%, v/v, glycerol; 0.002%, w/v, bromophenol blue). This mixture was then treated in one of three ways, being either (i) held on ice, (ii) held at room temperature for 30 min or (iii) boiled for 5 min. These preparations were then subjected to SDS-PAGE as described by Laemmli (1970) using a 3.0% stacking gel with 0.5% SDS and a 15% resolving gel. Separated proteins were visualized by staining with Coomassie brilliant blue. Prestained SDS-PAGE standards (Bio-Rad) were included for estimation of protein band sizes.

Extraction of DNA and estimation of mean base composition. Purified L13 cells were resuspended in 1 ml TNE buffer (10 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl, pH 8.0) containing 0.5% SDS and 100 μg proteinase K ml^{-1} . The suspension was incubated at 37°C for 30 min then 5 μl 1 mg DNase-free RNase ml^{-1} was added and incubation was continued for a further 30 min. DNA was extracted from the lysed cell suspension using, sequentially, TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) saturated with phenol, phenol/chloroform/isoamylalcohol (25:24:1, by vol.) and finally chloroform. Purified DNA was precipitated from the final aqueous phase by addition of 10% (v/v) 3.3 M sodium acetate and 2 vols ice-cold absolute ethanol. After washing in ethanol, the DNA was dried, resuspended in TE buffer and quantified spectrophotometrically.

The base composition of extracted DNA was estimated spectrophotometrically by the thermal denaturation temperature method as described by Owen *et al.* (1978). The G+C content was calculated from the denaturation temperature and expressed relative to the control value of a previously calculated value of 50.9 mol% for *Escherichia coli* NCTC 9001. The estimation was carried out in triplicate.

Nucleotide sequence accession numbers. The following eubacterial 16S rRNA gene sequences were retrieved from the GenBank database for inclusion in this study: *Afpia felis*, M65248; *Agrobacterium tumefaciens*, M11223; *Azospirillum lipoferum*, Z29619; *Bartonella bacilliformis*, X60042; *Beijerinckia indica*, M59060; *C. caryophilus*, X71837; *Ehrlichia sennetsu*, M73225; *Erythrobacter longus*, M59062; *Escherichia coli*, J01695; *H. obtusa*, X58198; *Legionella brunensis*, Z32636; *Magnetospirillum magnetotacticum*, M58171; NHP bacterium, U65509; *Rhodobacter capsulatus*, D16428; *Rhodobacter sphaeroides*, D16425; *Rhodospirillum rubrum*, D30778; *Rickettsia prowazekii*, M21789; *Rickettsia rickettsii*, M21293; *Sphingomonas paucimobilis*, D13725; *Wolbachia pipientis*, X61768.

RESULTS

Morphological characteristics

Observation of infected amoebae using light or phase-contrast microscopy revealed the presence of large numbers of highly mobile rod-shaped bacteria within the cytoplasm. Gimenez staining of heat-fixed infected

Fig. 1. Transmission electron micrograph of a section *A. polyphaga* infected with L13, demonstrating the ultrastructural features of the bacterium. V, vesicle-like structures associated with bacterial outer membrane; T, electron-translucent halo surrounding bacteria; M, amoebal mitochondrion. Bar, 0.25 μm .

amoebae confirmed the intracytoplasmic presence of these bacteria. Electron microscopic observation revealed details of their ultrastructure (Fig. 1). Bacteria appeared to lie free within the cytoplasm, but often surrounded by an electron-translucent area (see Fig. 1). The bacterial cell wall appeared typically Gram-negative, although often vesicle-like structures were closely associated with it. Vesicles also appeared detached from the bacterial surface, lying within the surrounding translucent zone (Fig. 1). L13 was estimated to be typically 0.2–0.5 μm wide and 0.7–1.0 μm long, although some degree of polymorphism was observed. On release from lysed host amoebae, L13 appeared to take on a more elongated morphology (data not shown). Electron microscopy failed to identify any flagella on L13 despite observation of co-cultures of different ages and incubated at different temperatures. L13 appeared to lack flagella even when released following host-cell lysis (data not shown). No specific features could be observed within the bacteria.

Host range

Of the 23 strains of free-living amoebae in which cultivation of L13 was attempted, only those belonging to groups II and III of the genus *Acanthamoeba* were susceptible to infection (Table 1). Multiplication of L13 occurred best in *A. polyphaga* and *Acanthamoeba castellanii*. *Acanthamoeba comandoni*, a member of the group I *Acanthamoeba* species, was the only member of the genus tested which was not susceptible to infection.

Effect of temperature on bacterial virulence

A. polyphaga grew at temperatures between 22 and 37 °C, but rapidly encysted at 42 °C. As demonstrated in Fig. 2, lower temperatures favoured better growth of the amoeba, with cultures incubated at 22 °C being over twice as dense as those grown at 37 °C. Higher temperatures enhanced the virulence of L13. At 37 °C virtually all amoebae were lysed within 5 d of bacterial infection, whereas at 22 °C intact amoebae could be observed over 3 weeks post-infection. Even growth at 32 or 30 °C resulted in destruction of virtually all amoebae within 10 d of bacterial infection. At all of the three higher temperatures tested, the rate of decline of intact amoebae in infected cultures appeared to be very similar and very rapid (taking 3–4 d), and decreasing temperature served only to delay the onset of this decline. However, at 22 °C the rate at which amoebae were destroyed was markedly slower; after an initial sharp rise, the number of intact amoeba fell gradually throughout the 21 d of the experiment. Importantly, viable L13-infected amoebae could be recovered from the cultures incubated at 22 °C over 4 months after the experiment described above was completed (data not shown).

16S rRNA gene amplification and analysis

A primary 16S rRNA gene sequence of 1454 bp was determined for L13 and its validity was supported by secondary structure modelling (data not shown). A FASTA search of the GenBank database indicated a proteobacterial origin for the bacterium. The 16S rRNA gene of L13 was found to be most similar to those of members of the α -Proteobacteria, sharing between 80 and 84% similarity. However, within this subclass, L13 did not share a specifically high similarity with any particular species, nor with the members of any particular group.

The alignment used as a basis for phylogenetic analysis comprised the L13 sequence, the sequences of diverse representatives of the α -Proteobacteria and those of two γ -Proteobacteria (*Escherichia coli* and *Legionella brunensis*). The latter two were included as out-grouping species on which trees could be rooted. The 16S rRNA gene sequences available for some of the α -Proteobacteria were not as complete as that obtained in this study, usually through the lack of data at the 3' extremity of the gene. Some sequences also included a relatively large number of base ambiguities. The length of the alignment was thus limited to 1177 bp. An evolutionary distance matrix was calculated from pairwise comparison of sequences in this alignment and a phylogenetic tree (Fig. 3) was inferred from this matrix. The inclusion of L13 had no significant effect on the overall topology of this tree, which is in concordance with those previously proposed (e.g. Loy *et al.*, 1996). The evolutionary position proposed for L13 was within a cluster of obligate intracellular bacteria, which, although within the α -subclass of the Proteobacteria, lies apart from any of the four recognized subgroups (α 1–4) and the lineage carrying

Fig. 3. Phylogenetic tree comparing L13 with various members of the α - and γ -Proteobacteria. The numbers at each node are the results of bootstrapping; each value is derived from 200 samples as an indication of semi-statistical support for the proposed branching order. The first value is derived from distance matrix analysis and the second value is derived from parsimony analysis. Values of >140 (70% of samples) are usually considered as indicating good support for the proposed branching order at that node. An asterisk appearing after the two bootstrap values indicates that the cluster supported by that node was also proposed by maximum-likelihood analysis. The vertical bars to the right of the tree indicate representatives of various subclasses of the Proteobacteria ($\alpha 1$ – $\alpha 4$, γ) and representatives of the Rickettsiales (Ri). Scale bar, 0.04 K_{nuc} .

members of the *Rickettsiales* (Fig. 3). A specific evolutionary relatedness between L13 and the three other members of the identified cluster (*H. obtusa*, *C. caryophilus* and the NHP bacterium) was also supported by parsimony- and maximum-likelihood-based phylogenetic inferences, although in none of these three reconstructions was there overwhelming support from bootstrapped sampling (see Fig. 3). Likewise, although all three inferences suggested that the L13-containing cluster shared specific evolutionary descent with the *Rickettsiales* lineage, bootstrap support was again only moderately strong. However, within the cluster defined above, L13 branched apart from the lineage carrying the other three members, a divergence which was well supported (Fig. 3).

Analysis of cellular proteins

SDS-PAGE profiles obtained for L13 are presented in Fig. 4. The profiles obtained for purified bacteria were distinct from those of uninfected amoebae, demonstrating the success of the purification protocol used.

Unpurified bacteria yielded a profile which, although dominated by L13-specific bands, also included amoebal proteins. The profiles yielded by untreated bacteria included heavy smears at the top of the gel, indicating the presence of very large proteins that were unable to enter the gel. These profiles also did not have clearly distinguishable bands larger than 36 kDa with the exception of one of an estimated 39 kDa. However, below this size numerous strongly staining bands were present. There was little difference between the profiles derived from intact and broken bacteria, although it appeared that a protein of approximately 33 kDa was more abundantly liberated from broken cells (Fig. 4). Heat treatment resulted in denaturation of very high molecular mass proteins to yield profiles more complex than those derived from untreated organisms. A number of larger bands appeared in the profiles, including an intensely staining protein of about 50 kDa. Two further particularly strongly staining bands were present at approximately 25 and 18 kDa, although at least 12 other bands were also discernible. Bacteria incubated with Laemmli buffer at 37 °C for

Fig. 4. SDS-PAGE profiles derived from L13 and *A. polyphaga*. Samples in lanes 2–5 were boiled prior to loading, those in 6–9 were held at 37 °C for 30 min and those in lanes 10–13 were untreated. Lanes: 2, 6 and 10, purified intact L13; 3, 7 and 11, purified non-intact L13; 4, 8 and 12, *A. polyphaga* heavily infected with L13; 5, 9 and 13, uninfected *A. polyphaga*; 1 and 14, molecular mass markers.

30 min yielded profiles which, in general, consisted both of bands present in profiles derived from untreated bacteria and bands present in profiles derived from boiled bacteria. However, only very few bands were present in profiles derived from both untreated and heat-treated bacteria (see Fig. 4), with the co-migrating bands of approximately 33 and 27 kDa being perhaps the best candidates for heat-stable proteins.

Estimate of mean base composition

The G+C content of L13 was estimated to be 41 ± 1 mol %.

DISCUSSION

The ability to exploit protozoan hosts offers bacteria access to a protected, nutrient-rich niche suitable for long-term survival in the environment. That this opportunity has been seized by a wide spectrum of bacteria is now becoming more apparent. Protozoa-incorporating *in vitro* co-cultivation methods are known to be able to support an increasing number of recognized pathogens and, following the introduction of PCR-based characterization methods, an increasing number of newly recognized obligate intraprotzoal organisms are being reported. In this study we add to this knowledge by reporting the isolation and characterization of another previously unrecognized bacterium.

Although the current taxonomic criteria for the creation of a new species are well-defined (Murray *et al.*, 1990; Stackebrandt & Goebel, 1994), those required for the proposal of a new genus are more subjective

(Murray *et al.*, 1990). Thus, whereas the moderate level of 16S rRNA sequence similarity shared by L13 with previously recognized species can be used to justify its proposal as a new species, that this new species warrants placement in a new genus is another question. Perhaps the best way of resolving this issue is to assess whether L13 can be satisfactorily accommodated within the genera with which it shares most evolutionary homology, namely *Holospora* and *Caedibacter*. Although both these genera are comprised endosymbionts of protozoa, these have to date been associated solely with infection of ciliates. *Holospora* species specifically infect the micronucleus or macronucleus of their *Paramecium* hosts and possess a life cycle comprised two distinct morphological forms. In a refractile form, the bacteria form short (1–3 µm) fusiform rods, which undergo binary fission and give rise to long infective forms of up to 20 µm (Preer & Preer, 1984). The G+C content has not been measured for any *Holospora* species. *Caedibacter* are moderately sized (up to 1 µm diam. and 4 µm long) rod-shaped or coccobacilli which are characterized by possession of distinctive refractile inclusions, termed R bodies. From these descriptions it is clear that L13 is somewhat smaller than *Holospora* and *Caedibacter* species and shares none of the distinctive morphological features of either genus. Although somewhat pleomorphic, L13 present a typical Gram-negative rod-like morphology. No elongated forms comparable to *Holospora* infective forms were observed and no refractile bodies characteristic of *Caedibacter* were present within L13 bacteria. Furthermore, the host range of L13 was limited to *Acanthamoeba* species. Although the G+C content of L13 was found to be similar to that reported for *Caedibacter* species (Preer & Preer, 1984), this cannot

be used to justify that the organisms share a specific taxonomic relationship. A low G+C content is in common with many species both within the α -*Proteobacteria* and beyond. Thus, from these comparisons it is clear that the proposal of a new genus to accommodate L13 is undoubtedly justified.

Whether L13 is the same organism as any of those observed in previous studies is difficult to assess. Proca-Ciobanu *et al.* (1975) reported *Acanthamoeba* SN endosymbionts which were Gram-negative, intracytoplasmic and of a similar size to L13. An *Acanthamoeba* endosymbiont (HN-3) described by Hall & Voelz (1985) was very similar in appearance to the SN endosymbionts but was surrounded by an electron-translucent area, similar in appearance to that of L13. Hall & Voelz (1985) demonstrated that this translucent area resulted from the presence of bacterial capsular material. Although we have not investigated if the area surrounding L13 is of the same origin, we did note that in several electron micrographs L13 did not possess this translucent halo and that this absence was more common when L13 was cultured at higher temperatures (37 °C). More recently, Fritsche *et al.* (1993) reported two forms of endosymbionts in *Acanthamoeba*. One, a coccal form, appeared indistinguishable from 'Candidatus Parachlamydia acanthamoebae' (Amann *et al.*, 1997), whereas the second bore a marked microscopic and ultrastructural resemblance to L13. This organism was, however, reported to be non-motile. The same workers have subsequently reported preliminary 16S rRNA sequence data for these two organisms (Gautom & Fritsche, 1996); the first form was indeed confirmed as sharing high levels of sequence similarity with the sequences of *Chlamydia* and *Parachlamydia* species, whereas the second was found to share highest similarity levels with the *Rickettsiales*. Although distinct from the *Rickettsiales*, we have shown that L13 may well share an evolutionary lineage with α -proteobacterial members of this polyphyletic family and that these species are among those with which L13 shares the highest 16S rRNA similarity levels.

The results we report add to other recent findings in broadening the evolutionary spectrum of bacteria now recognized as being capable of exploiting *Acanthamoeba* as hosts. The importance of these protozoa to the natural cycles of legionellae is now well-established (Barker & Brown, 1994) and 16S rRNA methods have been used to demonstrate a diversity of *Legionella* species which can be recovered using *in vitro* acanthamoebal co-cultivation methods, but not axenic media (Birtles *et al.*, 1996). More recently, Amann *et al.* (1997) used phylogenetic methods to demonstrate the *Chlamydia*-like nature of another endosymbiont and named it 'Candidatus Parachlamydia acanthamoebae'. Thus, it is now apparent that bacteria from at least three distinct lineages have evolved mechanisms for the exploitation of *Acanthamoebae*. In addition to these well-characterized organisms, other amoebal endosymbionts have been observed, notably

an *Ehrlichia*-like bacteria (Michel *et al.*, 1995b) and even possible *Archaea* (Hoffmann *et al.*, 1998). In light of these findings, a phylogenetic assessment of such organisms would be interesting and we are pursuing these goals.

Evidence for a wider role of protozoa as natural hosts for pathogenic bacteria is also supported by numerous laboratory studies. *Legionella pneumophila*, *Listeria monocytogenes*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Burkholderia pickettii* and *Edwardsiella tarda* have all been shown to be able to multiply within laboratory protozoal cultures (King & Shotts, 1988; Ly & Müller, 1990; Michel *et al.*, 1995a; Michel & Hauröder, 1997; Rowbotham, 1980; Thom *et al.*, 1992), whereas other species, including *Mycobacterium leprae*, *Pseudomonas* and *Bacillus* species, some coliforms and *Chlamydia pneumoniae* (Jardin, 1975; Tyndall *et al.*, 1991; King *et al.*, 1988; Essig *et al.*, 1997) have been shown to be able to survive protozoal uptake and to be viably maintained within them. Additionally, evidence of the pathogenic potential of 'Candidatus Parachlamydia acanthamoebae' and intra-amoebal legionellae in atypical pneumonia has now been presented (Fry *et al.*, 1991; Birtles *et al.*, 1997). It also appears that such endosymbionts are common. Fritsche *et al.* (1993) observed intra-*Acanthamoeba* bacteria in 14 out of 57 (24%) clinical and environmental isolates examined. Taken together, these findings add further weight to previous indications (Amann *et al.*, 1997) that the role of protozoa, and possibly small free-living amoebae in particular, in the epidemiology of human diseases may presently be significantly underrated.

Investigation of the effect of temperature on the virulence of L13 led to some interesting observations. Higher incubation temperatures resulted in enhanced virulence, whereas at 22 °C, L13 appeared to form a far more stable relationship with its host. Whether the virulence of L13 itself is enhanced at temperatures over 30 °C, or whether its apparent virulence results from physiological changes within its host is not known. It is clear from our experiments that uninfected *A. polyphaga* did not grow as well at elevated temperatures and these suboptimal conditions may lead to disruption of the host-parasite equilibrium and the onset of bacterial virulence. The longevity of the L13-host relationship at lower temperatures has not been assessed, although we have observed it to be ongoing after 4 months of co-cultivation.

Although we have applied polyphasic methods to the characterization of L13, its obligate intracellular nature has prevented the unequivocal isolation of a unique strain for proposal as the type strain. Thus, according to Murray & Schleifer (1994), we propose provisional classification of L13 as 'Candidatus Odysseella thessalonicensis' gen. nov., sp. nov. The genus name is derived from the eloquent analogy drawn by Barker & Brown (1993) of protozoa as 'Trojan horses of the microbial world'. Odysseus is the

Latin name for the warrior who, according to Greek mythology, thought up the strategy of penetrating the walls of Troy by hiding himself and fellow Greek warriors inside the infamous wooden horse. The species name reflects the location from where the isolate was obtained, the Greek city of Thessalonika.

Description of '*Candidatus Odysella thessalonicensis*' gen. nov., sp. nov.

'*Candidatus Odysella thessalonicensis*' (Od.y.s.el'la. dim. fem. n. pertaining to Odysseus; thess.al.lon'i. cen'sis. M.L. masc. n. adj. pertaining to the Greek city Thessalonika from where the organism was isolated).

Obligate intracellular bacterium of *Acanthamoeba* species which cannot be cultivated on cell-free media, only by co-cultivation with a number of *Acanthamoeba* species at temperatures at and below 37 °C. At temperatures over 30 °C the organism multiplies rapidly destroying its host, whereas at lower temperatures, it is far less virulent. Host range appears limited to group II and III *Acanthamoeba* species. Organism is motile and microscopic appearance is typically Gram-negative and rod-shaped. Electron microscopy reveals that the organism lies intracytoplasmically and is usually surrounded by an electron-translucent layer in which vesicle-like structures derived from the cell envelope can be observed. The G+C content is 41 mol%. The phylogenetic position, inferred from comparison of the 16S rRNA gene sequence, is within the α -*Proteobacteria*.

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