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Estrella lausannensis, a new star in the *Chlamydiales* order

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Abstract

Originally, the *Chlamydiales* order was represented by a single family, the *Chlamydiaceae*, composed of several pathogens, such as *Chlamydia trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. abortus*. Recently, 6 new families of *Chlamydia*-related bacteria have been added to the *Chlamydiales* order. Most of these obligate intracellular bacteria are able to replicate in free-living amoebae. Amoebal co-culture may be used to selectively isolate amoeba-resisting bacteria. This method allowed in a previous work to discover strain CRIB 30, from an environmental water sample. Based on its 16S rRNA gene sequence similarity with *Criblamydia sequenensis*, strain CRIB 30 was considered as a new member of the *Criblamydiaceae* family. In the present work, phylogenetic analyses of the genes *gyrA*, *gyrB*, *rpoA*, *rpoB*, *secY*, *topA* and 23S rRNA as well as MALDI-TOF MS confirmed the taxonomic classification of strain CRIB 30. Morphological examination revealed peculiar star-shaped elementary bodies (EBs) similar to those of *Criblamydia sequanensis*. Therefore, this new strain was called "*Estrella lausannensis*". Finally, *E. lausannensis* showed a large amoebal host range and a very efficient replication rate in *Acanthamoeba* species. Furthermore, *E. lausannensis* is the first member of the *Chlamydiales* order to grow successfully in the genetically tractable *Dictyostelium discoideum*, which opens new perspectives in the study of chlamydial biology.

Keywords: *CRIBLAMYDIACEAE*; TAXONOMY; MALDI-TOF MASS SPECTROMETRY; *CHLAMYDIAE;* INTRACELLULAR BACTERIA.

1. Introduction

The order *Chlamydiales* was proposed in 1971 by Storz and Page [1]. *Chlamydiales* are obligate intracellular bacteria that exhibit a unique developmental cycle with two bacterial forms: the infectious elementary body (EB) and the replicative reticulate body (RB), which exhibit condensed and decondensed nucleoid respectively. The *Chlamydiales* order initially contained a single family, the *Chlamydiaceae* composed of a single genus *Chlamydia* [2, 3], that includes well established human and animal pathogens such as *Chlamydia trachomatis*, *C. pneumoniae*, *C. psittaci* or *C. abortus*. During the past few years, the discovery of new *Chlamydia*-related species divided in different families has expanded the *Chlamydiales* order [4, 5]. The *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae* families were proposed in 1999 [6]. More recently, three additional family-level lineages were identified, the *Rhabdochlamydiaceae* [7, 8], the *Piscichlamydiaceae* [9] and the *Criblamydiaceae* [10]. Some of these *Chlamydia*-related bacteria represent possible emerging pathogens. For instance, *Parachlamydia acanthamoebae* is considered as an agent of respiratory tract infections [4, 11] whereas *Waddlia chondrophila* might cause miscarriage in humans [12-14]. In addition, both *P. acanthamoebae* and *W. chondrophila* have been identified as possible agents of abortion in ruminants [15-19].

Most of these "*Chlamydia*-like" bacteria are able to replicate in many free-living amoebal species. The ability to resist digestion by amoebae which can be used as a replicative niche, suggests that these bacteria have developed possible virulence mechanisms also used to resist mammalian professional phagocytic cells, an essential component of the innate immune system [20]. Thus, amoebal co-culture may be applied to selectively isolate amoeba-resisting microorganisms representing potential pathogens (reviewed in [21]). Several new members of the *Chlamydiales* order were isolated using this technique [22], including strain CRIB 30 which was found in raw surface water, taken upstream of a water treatment plant fed by the river Llobregat

in Spain [22]. Best BLAST hit for the 16S ribosomal RNA (rRNA) gene showed 93% similarity with *Criblamydia sequanensis* (a member of the *Criblamydiaceae* family). Based on Everett's criteria [6], it suggested that strain CRIB 30 represents a new genus (<95%) within the *Criblamydiaceae* family (>90%).

In the present work, taxonomic classification of strain CRIB 30 was further specified using phenotypic and phylogenetic approaches. Phenotypic studies included a MALDI-TOF mass spectrometry (MS) analysis (protein profiling) of strain CRIB 30 and of 6 other *Chlamydia*-related bacteria. Phylogenetic studies were also performed, based on 8 different core genes. The growth of strain CRIB 30 and of its closest relative (*C. sequanensis*) in different amoebal strains (*Acanthamoeba castellanii*, *A. comandoni*, *Hartmannella vermiformis* and *Dictyostelium discoideum*) was also studied (using different cell culture conditions).

2. Materials and methods

2.1. Amoebal culture

Acanthamoeba castellanii ATCC 30010, *Acanthamoeba comandoni* strain WBT and *Hartmannella vermiformis* ATCC 50237 were cultured in peptone yeast extract glucose (PYG) medium [23] in 75-cm^2-surface cell culture flasks (Becton Dickinson, Allschwill, Switzerland) at 28°C [21]. *Dictyostelium discoideum* DH1-10 was grown in HL5 medium at room temperature [24].

2.2. Bacterial culture and purification

Parachlamydia acanthamoebae strain Hall's coccus, *Estrella lausannensis* strain CRIB 30, *Criblamydia sequanensis* strain CRIB 18 and *Waddlia chondrophila* strain WSU 86-1044 (ATCC VR1470) were co-cultured with *A. castellanii* ATCC 30010 at 32°C, in 75-cm^2-surface

cell culture flasks (Becton Dickinson, Allschwill, Switzerland), with 30 mL of PYG medium [23]. Co-cultures were harvested when complete amoebal lysis was observed. Bacteria were purified successively with sucrose and gastrographin gradients, as described previously [25].

2.3. Infection procedure

Amoebae were harvested from fresh cultures. The number of amoebae was adjusted with PYG or HL5 medium to have about 40 x 10^4 *A. castellanii* ATCC 30010/ml, 12 x 10^4 *A. comandoni* strain WBT/ml, 33 x 10^4 *H. vermiformis* ATCC 50237/ml or 33 x 10^4 *D. discoideum* DH1-10/ml. Amoebae were distributed into cell culture flasks or 24-wells microplates. When homogenous monolayers of amoebae were observed, they were infected with *E. lausannensis* or *C. sequanensis* with a multiplicity of infection (MOI) of 1 to 10. Microplates or cell culture flasks were centrifuged at room temperature during 15 min at 1790 x g (to promote physical contact between bacteria and amoebae). The infected amoebae were then incubated during 1 h at 28°C for *Acanthamoeba* species and *H. vermiformis* ATCC 50237 and at room temperature for *D. discoideum* DH1-10. The infected amoebal monolayers were washed carefully with 1 ml of PBS and fresh medium was added. Infected amoebae were then incubated without CO_2 at 32°C (or at room temperature for *D. discoideum*).

2.4. Morphological examination

Amoebae were cultured and infected with *E. lausannensis* strain CRIB 30 directly in 75-cm^2-surface cell culture flasks (Becton Dickinson, Allschwill, Switzerland) as described above.

For DiffQuick and Gram staining, 1 ml of co-culture was centrifuged 7500 x g during 5 min. The pellet was re-suspended in 15 µl of PBS, spread on glass slides and air-dried. For Gram staining, preparations were previously fixed with ice-cold methanol during 5 min and washed three times

with PBS. Cell preparations were then stained with the GRAM kit (Becton Dickinson, Allschwill, Switzerland) or DiffQuick kit (Polymed Medical, Mont-sur-Lausanne, Switzerland) according to the manufacturer's protocol. Images were taken using LEICA microscope and LEICA DFC420 camera (Leica Microsystems, Heerbrugg, Switzerland).

For immunofluorescence and confocal microscopy, amoebae were infected in 24-wells microplates as described before. Amoebae were harvested and centrifuged 4 min at 7500 x g at different times post infection. Supernatant was discarded and about 5 µL of the pellet was scattered on autoclaved coverslips, fixed and permeabilized with ice-cold methanol during 10 min and washed three times with PBS. The remaining pellet was re-suspended in 1 ml of PBS and frozen at -20°C for DNA extraction. Fixed amoebae were incubated in blocking solution (0.1% saponin (Sigma-Aldrich, Buchs, Switzerland), 0.2 % NaN3 (Acros, Geel, Belgium) and 10 % FCS (PAA Laboratories, Pasching, Austria)) at room temperature for at least 1 h. After a washing step (twice with PBS 0.1% saponin (Sigma-Aldrich, Buchs, Switzerland) and once with PBS), *E. lausannensis* strain CRIB 30 or *C. sequanensis* strain CRIB 18 bacteria were stained 1 h at room temperature with a mouse polyclonal primary antibody (Eurogentec, Seraing, Belgium), diluted 1:1000 in blocking solution. Cells were then washed and incubated with a goat anti-mouse AlexaFluor 488 secondary antibody (Eurogentec, Seraing, Belgium) diluted 1:1000 in blocking solution for 1 h at room temperature. Amoebae were counterstained with Concanavalin A (Invitrogen, Basel, Switzerland) and DNA with 300nM of 4',6-diamidino-2-phenylindole, DAPI (Invitrogen, Basel, Switzerland). Coverslips were mounted in moewiol. Fluorescence was analyzed with a Zeiss LSM710 (Carl Zeiss MicroImaging, Göttingen, Germany) confocal fluorescence microscope.

Ten milliliters co-culture of *A. castellanii* ATCC 30010 infected with *E. lausannensis* strain CRIB 30 was prepared for electron microscopy. Infected amoebae harvested 30h post infection

were centrifuged 10 min at 1500 x g and the pellet was fixed with 4 % glutaraldehyde (Fluka Biochemika, Buchs, Switzerland) overnight at 4°C as described previously [10]. Preparations were then observed under a MEM201C electron microscope (Philips, Zurich, Switzerland).

2.5. Phylogenetic analyses

Genomic DNA was extracted from 1 ml of purified *E. lausannensis* strain CRIB 30 or *C. sequanensis* strain CRIB 18 with the Wizard genomic DNA purification kit (Promega, Duebendorf, Switzerland). Sequencing was performed for both bacteria using the Solexa/Illumina technology on the GA-IIx system.

From raw genome sequences assembled using EDENA assembly software [26], 8 conserved genes were selected for phylogenetic analyses : the 16S rRNA, the 23S rRNA, the DNA gyrase subunits A and B (*gyrA* and *gyrB*), the DNA topoisomerase I (*topA),* the preprotein translocase subunit SecY (*secY*) and the DNA-directed RNA polymerase subunit alpha and beta (*rpoA* and *rpoB*). The homologous gene sequences of the bacterium *Parachlamydia acanthamoebae* strain Hall's coccus [27] were used to recover these 8 genes by BLAST (nr/nt) in the contigs of *E. lausannensis* strain CRIB 30 or *C. sequanensis* strain CRIB 18. Sequences from other bacteria (*C. trachomatis* D/UW-3/CX, *C. pneumoniae* CWL029, *C. muridarum* Nigg, *C. abortus* S26/3, *Protochlamydia amoebophila* UWE25, *Waddlia chondrophila* WSU 86-1044 and *Pirellula staleyi* strain DSM 6068) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) (see Accession number in supplementary Table S1). After a MUSCLE alignment [28] of individual or concatenated sequences, neighbor-joining [29], minimum-evolution [30] and maximum parsimony [31] phylogenetic trees were build with a p-distance model, based on both nucleotide and amino-acid sequences, using the MEGA software version 4.1 [32].

156

157 *2.6. MALDI-TOF Mass Spectrometry*

158 Proteins were extracted from 1 to 2 ml suspension of purified bacteria. Bacteria were washed
159 three times with PBS with centrifugations at 13, 000 x g for 3 min. Pellets were then re-
160 suspended with 300 µl of a mix (v/v) of 100% acetonitrile/70% formic acid and incubated 15
161 min at room temperature. Protein concentrations were evaluated by Bradford assay (Biorad,
162 Rheinach, Switzerland) according to the manufacturer's procedure. Bradford assay was measured
163 on a luminometer FLUOstarOmega (BMG Labtech, Offenburg, Germany) at 595 nm. Solutions
164 of extracted proteins were normalized to 200 µg/ml. Then, 1.5 µl of extracted proteins from each
165 bacterium were spotted in triplicate on a polished steel MALDI-TOF 96 target sample plate
166 (Bruker Daltonics). When air-dried, each spot was overlaid with 1.5 µl of HCCA MALDI-TOF
167 matrix (125 µg/ml of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5 %
168 trifluoroacetic acid (TFA)).

169

170 *2.7. MALDI-TOF MS data analysis*

171 The MALDI-TOF MS system used was the Microflex LT instrument (Bruker Daltonics, Leipzig,
172 Germany). Data were analyzed using the FlexControl 3.0 software (Bruker Daltonics). Using a
173 variability coefficient of 0.0002 m/z, peaks that were present in at least 2 independent generated
174 spectra were included in the cluster analysis. Then, for each bacterium, 150 peaks with the
175 highest intensity were selected. A total of 864 different peaks were collected (corresponding to
176 864 different m/z values) for the 7 analyzed *Chlamydiales* bacteria. A binary matrix was
177 constructed with values of 1 when peaks were present and 0 when absent. Using the binary
178 matrix, similarity and distances measures (see supplementary Table S2) were calculated with
179 Dice's coefficient using the PAST software (http://palaeo-

electronica.org/2001_1/past/issue1_01.htm) [33]. These measures were used to build a neighbor joining phyletic tree with bootstrap test (300 replicates).

2.8. Growth kinetics of C. sequanensis and E. lausannensis in 4 amoebal strains

Amoebae were infected in 24-well microplates as described before. Then, they were harvested at different times post infection and frozen at -20°C until genomic DNA extraction. DNA was extracted with 100 µL of the co-culture using the QIAmp DNA extraction kit (Qiagen, Hombrechtikon, Switzerland) and eluted in 100 µL of the provided elution buffer. Detection of *E. lausannensis* strain CRIB 30 DNA and *C. sequanensis* strain CRIB 18 DNA was done by quantitative real-time PCR.

Primers and probe for quantitative real-time PCR assays were designed using the Geneious software v.5 to specifically detect *E. lausannensis* strain CRIB 30 or *C. sequanensis* CRIB 18. The PCRs amplified DNA fragments of 161 bp and 105 bp of the 16S rRNA gene of *E. lausannensis* and *C. sequanensis*, respectively. Primers and probe (Eurogentec, Seraing, Belgium) used were EstF (5'- ACACGTGCTACAATGGCCGGT-3'), EstR (5'-CCGGGAACGTATTCACGGCGTT-3') and EstS (5'-FAM-CAGCCAACCCGTGAGGG-BHQ1-3') for *E. lausannensis* and CrF (5'FAM-CGGTAATACGGAGGGTGCAAG- BHQ1-3'), CrR2 (5'-CGTTCCGAGGTTGAGCCC-3') and CrS (5'-FAM-ACCCGACTTGTGTTTCCGCCTGCG-BHQ1-3') for *C. sequanensis*. Probes contained Locked Nucleic Acids underlined in the above sequences. Quantitative real-time PCR assays were performed in a total volume of 20 µl, with 10 µl of iTaq supermix (Bio-Rad, Rheinach, Switzerland), 100 nM of probe, 200 nM of forward primer, 200 nM of reverse primer, molecular biology grade water (Sigma-Aldrich, Buchs, Switzerland) and 5 µl of DNA sample. Cycling conditions were enzyme activation at 95°C during 3 min and 45 cycles with 15 sec at 95°C and 1

min at 60°C. Quantitative PCR assays were performed on the Step One (Applied Biosystems, Zug, Switzerland) PCR system. Water was used as a negative control. Positive control and quantification were assessed using a plasmid constructed as previously described [34]. Negative controls, standard curve and samples were analyzed in duplicate. Growth kinetic was also analyzed by immunofluorescence, as described above.

3. Results

3.1. Morphology of strain CRIB 30

The morphology of strain CRIB 30 was analyzed from a co-culture with *Acanthamoeba castellanii* 30h post infection using different staining and microscopic approaches. Gram staining showed that EBs are Gram positive whereas RBs appear Gram negative (Fig. 1A). Strain CRIB 30 could also easily be stained with Giemsa using the DiffQuick® procedure (Fig. 1B). Intra-amoebal inclusions of *Acanthamoeba castellanii* infected with strain CRIB 30 were observed by immunofluorescence confocal microscopy (Fig. 1C) and inclusions containing both EBs and RBs were observed by electron microscopy 30h post infection (Fig. 1D, E, F). Following glutaraldehyde fixation, the peculiar membrane of EBs (Fig. 1E) was characterized by irregular branching that resembled the membrane of star-shaped EBs of *Criblamydia sequanensis* [10]. Similar to other *Chlamydia*-related bacteria, EBs and RBs are characterized by a condensed and decondensed nucleoid and by a diameter between 0.5-1 µm and 0.8µ-1.8 µm, respectively. Unlike *C. sequanensis*, no electron-translucent lamellar structure was observed in the cytoplasm of strain CRIB 30's EBs [10].

3.2. Phylogenetic analysis of strain CRIB 30

Complete DNA sequences of 7 well conserved bacterial genes were selected to confirm the 16S rRNA taxonomic classification of strain CRIB 30. The 7 selected genes were *gyrA*, *gyrB* (DNA gyrase subunits A and B), *topA* (DNA topoisomerase I), *secY* (preprotein translocase subunit secY), *rpoA*, *rpoB* (DNA-directed RNA polymerase subunits alpha and beta) and the 23S rRNA. Several *Chlamydiales* bacteria were used for sequence comparison. *Pirellula staleyi* strain DSM 6068 (a *Planctomycetales*) was used as an outgroup. The Fig. 2A shows the percentage of similarity between *E. lausannensis*, 8 other members of the *Chlamydiales* order and the outgroup *P. staleyi*. Except for the outgroup *P. staleyi*, the percentage similarity of the 16S rRNA and 23S rRNA genes is above 85% whereas it is ≤75.2% for the 6 additional genes, demonstrating the higher taxonomial discriminating power conferred by these conserved genes. The highest percentage similarity for each *E. lausannensis* core genes was always obtained with *C. sequanensis* orthologous genes. Moreover, a higher gene/protein sequence similarity was observed between *E. lausannensis* and *Chlamydia*-related bacteria compared to *Chlamydiaceae*. Based on individual gene/protein sequences (Supplementary Fig. 1) or on concatenated DNA sequences (Fig. 2B) of the 6 coding genes, neighbor-joining (NJ) [29], minimum-evolution (ME) [30] and maximum parsimony (MP) [31] phylogenetic trees indicate a clustering of strain CRIB 30 with *C. sequanensis*, supported by high bootstraps values (≥72% for NJ method).

3.3. MALDI-TOF MS analysis of strain CRIB 30

In Fig. 3A, an illustration of the MALDI-TOF MS spectra of *E. lausannensis* and 6 *Chlamydia*-related bacteria is shown. As expected, the spectra indicate unique profiles for each *Chlamydia*-related bacterium, belonging to different families or species. The 150 peaks with the highest

intensity were selected for each chlamydial species and used to generate a binary matrix (presence or absence of peaks) composed of a total of 864 collected peaks for the 7 members of the *Chlamydiales* order. A schematic illustration of the 150 peaks for each bacterium is shown in Fig. 3B. A neighbor joining phyletic tree was built with similarity and distance Dice indices (see supplementary table S2) calculated using the binary matrix (Fig. 3C). *C. sequanensis* and strain CRIB 30 were in the same cluster with about 34% similarity. The clustering of the other *Chlamydia*-related bacteria was congruent with the results obtained following phylogenetic analysis (see Figure 2 and supplementary Figure S1).

3.4. Growth kinetic of strain CRIB 30 and C. sequanensis in different amoebal strains

Several amoebal strains, *Acanthamoeba castellanii*, *A. comandoni*, *Hartmannella vermiformis* and *Dictyostelium discoideum* were used to test the amoebal host range of strain CRIB 30. The permissivity and growth kinetic of strain CRIB 30 and *C. sequanensis* were assessed by quantitative real-time PCR (Fig. 4) and by immunofluorescence (Fig. 5).

Strain CRIB 30 grew in all tested amoebae, whereas *C. sequanensis* was only able to grow in *A. castellanii* (Fig. 4). The growth of strain CRIB 30 was of about 3 log in *Acanthamoeba* species (Fig. 4A and B) and reached titers 10-fold higher than *C. sequanensis* in *A. castellanii* (Fig. 4A). Unlike *C. sequanensis*, strain CRIB 30 was also able to replicate in *Hartmannella vermiformis* (Fig. 4C and 5) and in *Dictyostelium discoideum* (Fig. 4D), but to a lesser extent, since detection of bacterial DNA increased of 2 log and of 1 log in 48h in *H. vermiformis* and *D. discoideum*, respectively. EBs and RBs could be differentiated by DAPI staining, since EBs and RBs nucleoids are condensed and decondensed, respectively. About 50% of *E. lausannensis'* EBs differentiated into RBs at 2h post infection in *H. vermiformis*, followed by *A. comandoni* (at 5h), *A. castellanii* (at 8h) and *D. discoideum* (at 12h). Similarly, conversion of half of *C. sequanensis*

EBs into RBs in *A. castellanii* appeared within the 8 first hours of the infection. After differentiation of EBs into RBs, strain CRIB 30 replicated into inclusions and re-differentiated into EBs between 30 and 48h. Lysis of amoebae occurred between 48 to 96h depending on the amoebal strain (Fig. 5).

D. discoideum cannot be cultivated above 25°C, which may be a limiting factor for bacterial growth [24]. To be able to compare the growth kinetic of strain CRIB 30 in *A. castellanii* and *D. discoideum*, *A. castellanii* was also infected with strain CRIB 30 at 20°C (Fig. 4E). At this lower temperature, bacterial growth in *A. castellanii* was reduced of 1 log compared to the growth at 32°C, indicating that the growth kinetic of strain CRIB 30 in *A. castellanii* is temperature sensitive. However, the growth of CRIB 30 in *A. castellanii* at 20°C remained 10 fold higher than in *D. discoideum* (Fig. 4E).

4. Discussion

In this study, the taxonomic classification of strain CRIB 30 was performed based on phenotypic, phylogenetic analyses and proteomic characterization by MALDI-TOF mass spectrometry.

Using classical fixative methods, strain CRIB 30 exhibited peculiar star-shaped EBs observed by electron microscopy. This phenotypic characteristic similar to *Criblamydia sequanensis* EBs suggested its classification within the *Criblamydiaceae* family [10]. This original shape may be due to the fixative methods and additional investigations should be done to evaluate the impact of the fixative solutions on chlamydial EBs and RBs morphologies. However, this unique star-shaped phenotype indirectly indicates that strain CRIB 30 and *C. sequanensis* have membrane structures distinct from other members of the *Chlamydiales* order. Similar to other *Chlamydiales*, strain CRIB 30's EBs stained as Gram positive whereas RBs stained Gram negative demonstrating marked differences in the cell wall composition between the developmental stages.

No peptidoglycan has been detected in the cell wall of *Chlamydiales* bacteria [35]. However, the rigidity of EBs is assured by an alternative structure, which is the outer membrane complex [36]. This complex is constituted of a network of cystein-rich proteins cross-linked by disulfide bonds that are absent in osmotically fragile RBs [36, 37]. Thus, the specific cell wall composition of EBs retain crystal violet dye/Iodine complex of the Gram staining whereas RBs do not retain the dyes and are counterstained with safranine.

Using the 16S rRNA gene sequence, the first phylogenetic analysis of strain CRIB 30 has indicated 93% similarity with *C. sequanensis* [22]. According to Everett's criteria [6], strain CRIB 30 belongs to the *Criblamydiaceae* family (>90%), representing a new genus (<95%). The International Committee on Systematics of Prokaryotes recommends for the description of new species to use 16S and 23S rRNA genes, but also several other core genes which requires the availability of sequenced genome [2, 3]. For instance, this approach has already been validated for the description of *Bartonella* spp. [38]. Cut-offs for ribosomal RNA genes have already been described for *Chlamydiae* [4, 6] but need to be specified for other housekeeping genes. Genome sequencing has largely increased in the past few years and the importance of specific genes for taxonomy will be specified in the future. Concerning *E. lausannensis*, 7 other conserved core genes (*gyrA*, *gyrB*, *rpoA*, *rpoB*, *secY*, *topA* and 23S rRNA) were selected and the classification of strain CRIB 30 in the *Criblamydiaceae* family was confirmed using different building tree methods.

Instead of using classical proteomic methods such as SDS gels profiles [39, 40], MALDI-TOF MS was used and confirmed the clustering of *E. lausannensis* and *C. sequanensis* as already supported by phylogenetic approaches. For further taxonomic analyses, this method needs to be refined by optimizing the analyses algorithm, testing different matrix compositions, matrix to sample ratio and by including a larger number of *Chlamydiales* bacteria from different families

and species. However, these data suggest that MALDI-TOF MS may represent a rapid method that could improve chlamydial species and sub-species classification when used in association with 16S rRNA gene sequencing and multi-locus sequencing, as shown for other bacterial species [41-43].

Based on these phylogenetic and proteomic results, we suggest classifying strain CRIB 30 as the first member of a new genus in the *Criblamydiaceae* family and we propose to call it *Estrella lausannensis*. The genus' name "*Estrella*" (star in Spanish) is derived from the star-shaped EBs and the species' name "*lausannensis*" comes from the Latinized name of Lausanne, where the bacterium was isolated using amoebal co-culture.

The host range of *Estrella lausannensis* was evaluated using 4 different amoebal strains. The bacterium was able to grow in all amoebae tested compared to *C. sequanensis* that grew only in *A. castellanii*, suggesting that strain CRIB 30 is adapted to a larger amoebal host range. *E. lausannensis* grew also in *H. vermiformis* and *D. discoideum* but to a lesser extent compared to *Acanthamoeba* species. Similar to *Acanthamoeba* spp., *Hartmannella vermiformis* is often recovered from environmental samples [44-46] and could thus represent an additional reservoir and a vector for the dissemination of strain CRIB 30 in the environment.

The haploid social amoeba *Dictyostelium discoideum* is often used as a model to study bacterial virulence for several amoeba-resisting intracellular bacteria, such as *Mycobacterium* spp. and *Legionella* spp. [47, 48]. The ability of *E. lausannensis* to grow in this amoeba provides new perspectives in the study of chlamydial biology since no member of the *Chlamydiales* order has been successfully grown in this model host. Thus, the biology of *Chlamydiales* could be investigated using different mutants of *D. discoideum*.

Future isolations of new species will help in better evaluating chlamydial diversity, which is currently underestimated. As *Chlamydiales* can infect a very broad range of hosts [5], cell culture

methods must be as variable as possible. In fact, *Waddlia chondrophila* has yet never been isolated by amoebal co-culture, but only from mammalian cells [15, 49] and *Simkania negevensis* was highlighted as a contaminant of human and simian cell lines [50, 51]. Furthermore, DNAs of several new *Chlamydiales* members were recently detected in fish cells [9, 52]. Although amoebal co-culture may only isolate amoebal pathogens or amoebal symbionts, it offers the advantage to isolate obligate and facultative intracellular bacteria from heavily-contaminated samples such as river water. Moreover, recovered amoeba-resisting bacteria may also be resistant to other phagocytic cells, including mammalian macrophages, and may thus represent possible emerging pathogens.

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Fig. 1. **Morphology of *Estrella lausannensis* in *A. castellanii* (30h post infection), using different stainings and microscopy approaches**. (A) Gram staining; the arrowheads show Gram positive EBs and the arrow indicates Gram negative RBs. (B) Diff-Quick staining; the arrow indicates an inclusion filled with strain CRIB 30. (C) Co-culture visualized by immunofluorescence confocal microscopy. *E. lausannensis* bacteria are stained with a mouse polyclonal antibody (in green) and amoebae with Concanavalin A (in red). (D, E, F) Electron microscopy of *E. lausannensis*. (D) *E. lausannensis* replicates in several inclusions in the amoeba *A. castellanii*. An EB and a RB can be observed at higher magnification, respectively in (E) and (F). Magnification of 1'000 x (A to C), 4'500 x (D) and 70'000 x (E and F).

Fig. 2. **Phylogenetic analyses of *E. lausannensis***. (A) The graph represents the percentage of similarity of 8 genes between *E. lausannensis* and different bacteria. The predicted clustering of *E. lausannensis* with *C. sequanensis* using the 16S rRNA gene sequence was confirmed with the 7 additional genes (23S rRNA, *gyrA*, *gyrB*, *rpoA*, *rpoB*, *secY* and *topA*). (B) Phylogenetic trees built with neighbor joining [29], minimum evolution (ME) [30] and maximum parsimony (MP) [31] method, on concatenated DNA sequences of the genes *gyrA*, *gyrB*, *rpoA*, *rpoB*, *secY* and *topA*, using the p-distance model. The consensus tree with NJ method is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1'000 replicates) [53] are shown next to the branches (NJ/ME/MP methods). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Fig. 3. **Proteomic analysis of *E. lausannensis* using MALDI-TOF MS.** (A) MALDI-TOF MS spectra. The *y* axis (scale to y_{max} = 6'000) represents the absolute intensity of the peaks and the *x* axis (scaled to x_{max} = 14'000) indicates the mass-to-charge ratio (m/z). (B) Schematic representation of the presence (black bar) or absence (white bar) of peaks. The 150 peaks with the highest intensity were selected for each of the 7 investigated *Chlamydiales* bacteria giving a total of 864 different peaks. (C) Using the binary matrix, a neighbor joining phyletic tree of the 7 *Chlamydiales* members was built using the similarity and distance measures calculated with Dice's coefficient. Percentages of similarity are indicated in the *x* axis.

Fig. 4. **Growth of *Estrella lausannensis* and *Criblamydia sequanensis* in 4 amoebal species**. Bacterial growth was measured by real-time quantitative PCR. Co-culture was performed in *Acanthamoeba castellanii* (A, E), *A. comandoni* (B), *Hartmannella vermiformis* (C) and *Dictyostelium discoideum* (D, E). Infections were incubated at 32°C (A, B, C, E) or 20°C (D, E). Compared to *H. vermiformis* and *D. discoideum*, *E. lausannensis* showed growth rates 10 to 100-fold higher in *Acanthamoeba* species. The growth of *E. lausannensis* was compared in *A. castellanii* and *D. discoideum* at 20°C. *E. lausannensis* is able to grow 10-fold better in *A. castellanii* than in *D. discoideum*.

Fig. 5. **Growth kinetic of *Estrella lausannensis* assessed by confocal microscopy in the amoeba *Hartmannella vermiformis*.** Bacteria (in green) were stained with a mouse anti-*Estrella lausannensis* polyclonal antibody and amoebae (in red) with Concanavalin A. Inclusions filled with *E. lausannensis* are observed between 24 and 30h post infection. At 48h, amoebae are lysed by the bacteria. Confocal microscopy, 1'000 x magnification.

Figure 1

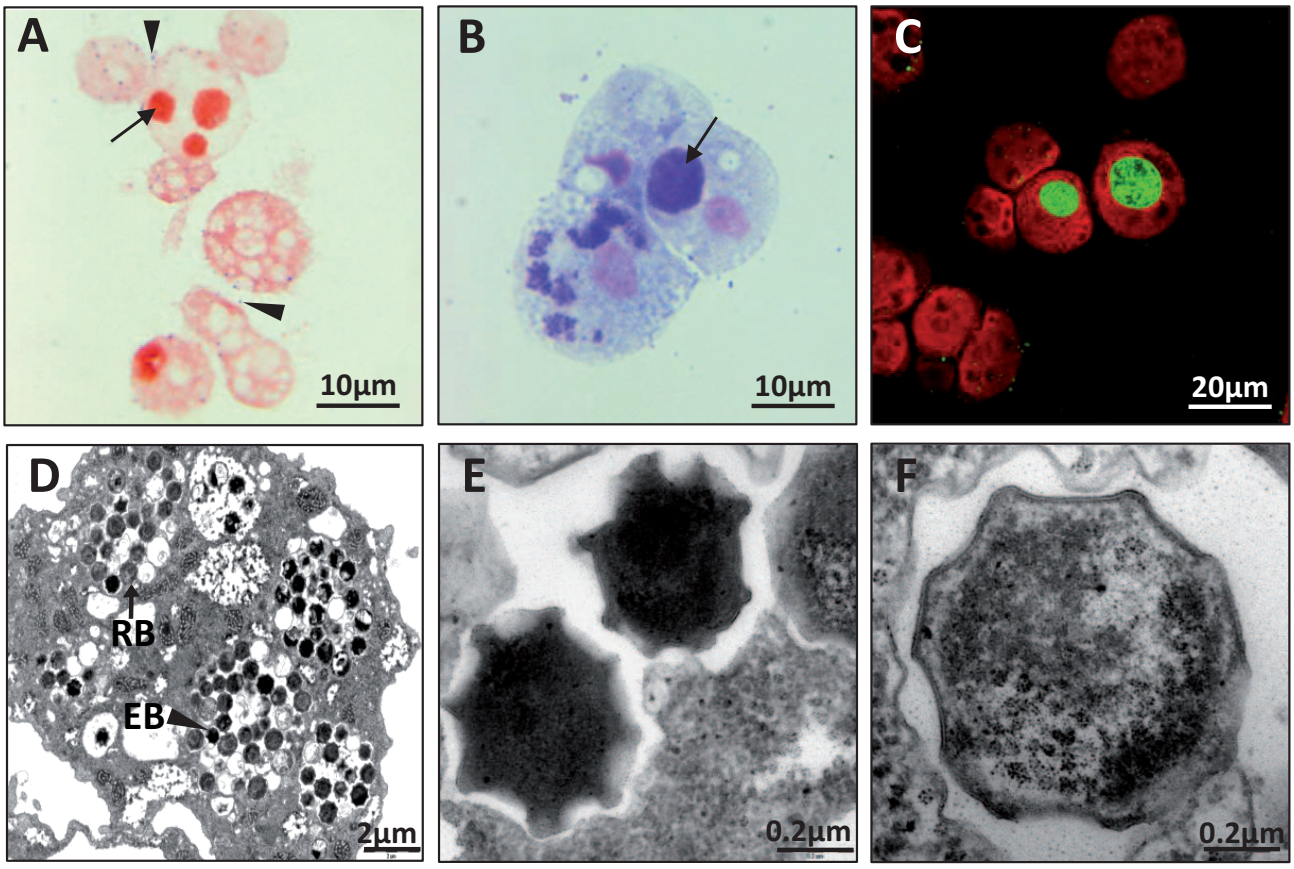


Figure 2

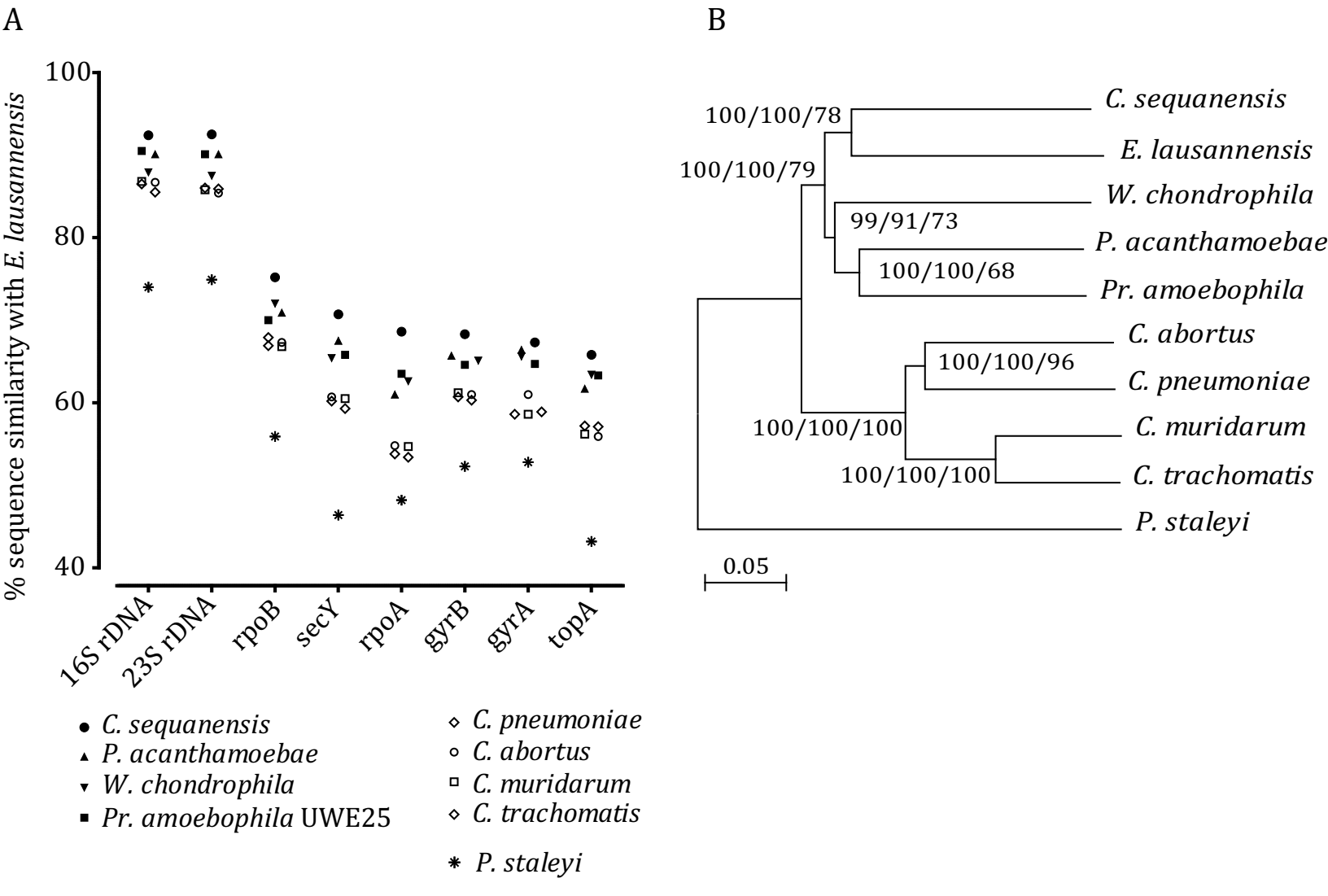
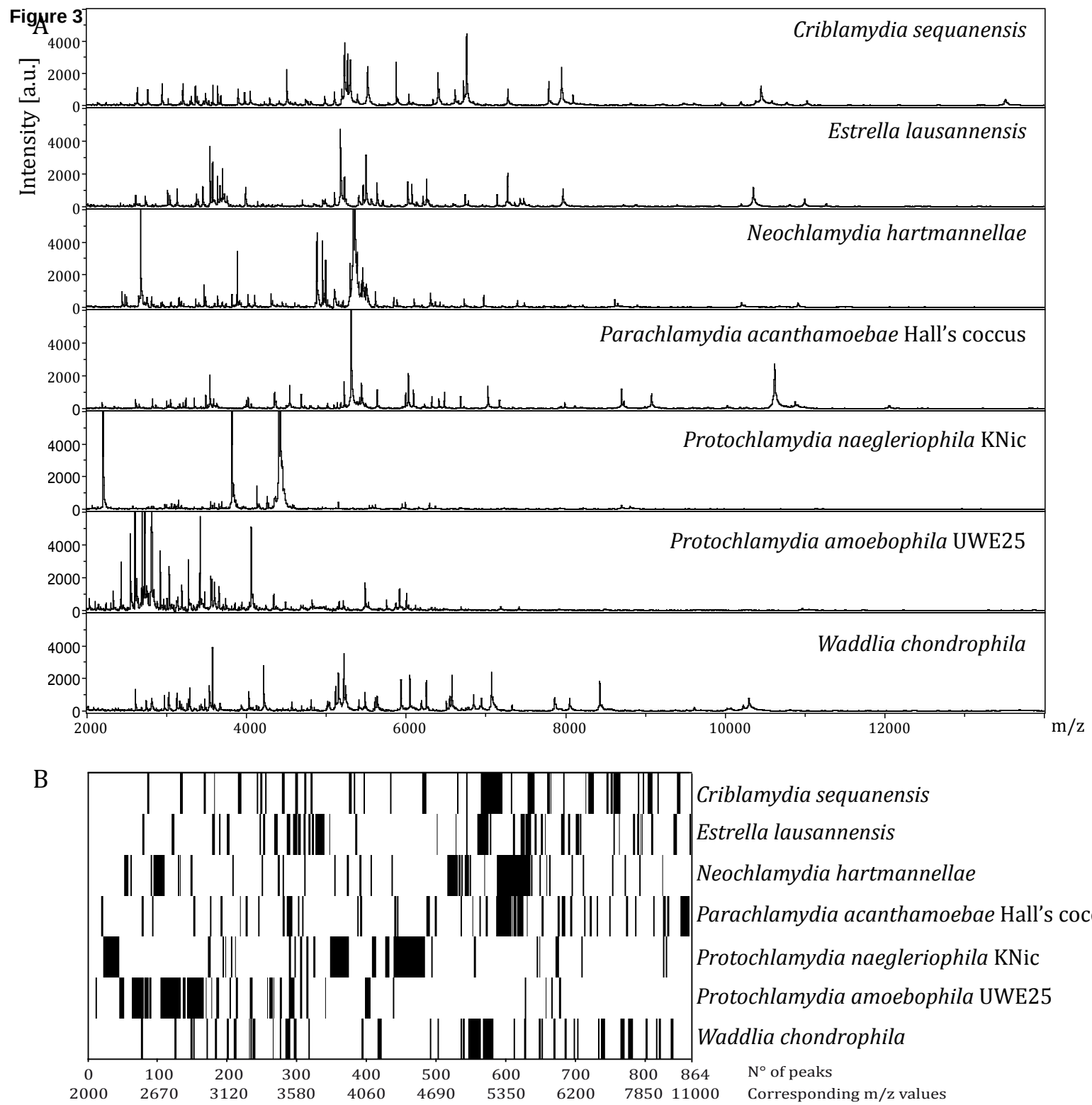

A
B
100
80
60
40
% sequence similarity with E. lausannensis
16S rDNA
23S rDNA
rpoB
secY
rpoA
gyrB
gyrA
topA
C. sequanensis
P. acanthamoebae
W. chondrophila
Pr. amoebophila UWE25
C. pneumoniae
C. abortus
C. muridarum
C. trachomatis
P. staleyi
100/100/78
100/100/79
99/91/73
100/100/68
100/100/96
100/100/100
100/100/100
C. sequanensis
E. lausannensis
W. chondrophila
P. acanthamoebae
Pr. amoebophila
C. abortus
C. pneumoniae
C. muridarum
C. trachomatis
P. staleyi
0.05

Figure 3


A
Intensity [a.u.]
Criblamydia sequanensis
Estrella lausannensis
Neochlamydia hartmannellae
Parachlamydia acanthamoebae Hall's coccus
Protochlamydia naegleriophila KNic
Protochlamydia amoebophila UWE25
Waddlia chondrophila
4000
2000
0
2000
4000
6000
8000
10000
12000
m/z

B
Criblamydia sequanensis
Estrella lausannensis
Neochlamydia hartmannellae
Parachlamydia acanthamoebae Hall's coccus
Protochlamydia naegleriophila KNic
Protochlamydia amoebophila UWE25
Waddlia chondrophila
0 100 200 300 400 500 600 700 800 864
2000 2670 3120 3580 4060 4690 5350 6200 7850 11000
N° of peaks
Corresponding m/z values

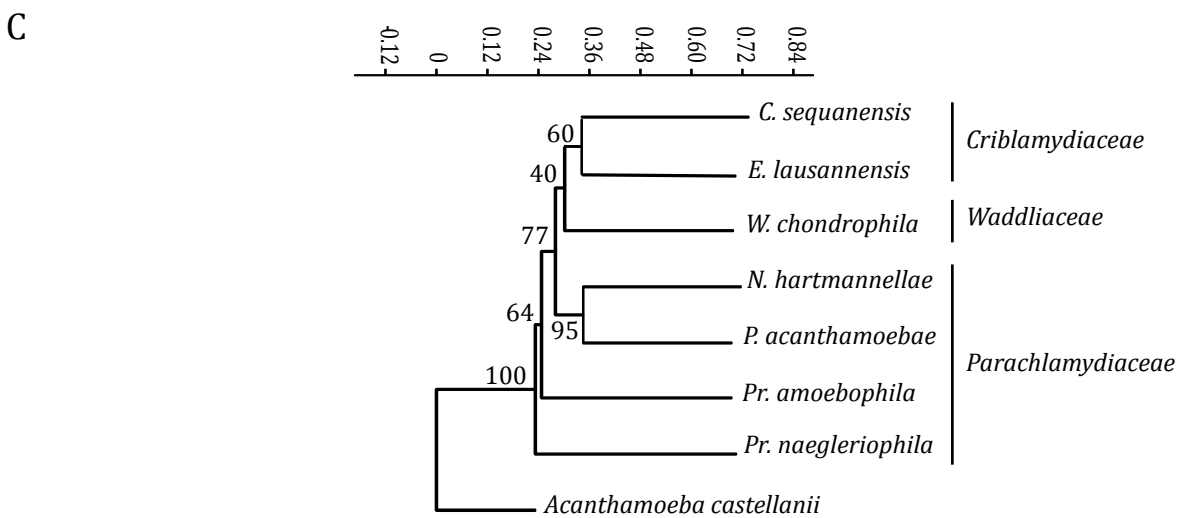

C
-0.12
0
0.12
0.24
0.36
0.48
0.60
0.72
0.84
C. sequanensis
E. lausannensis
W. chondrophila
N. hartmannellae
P. acanthamoebae
Pr. amoebophila
Pr. naegleriophila
Acanthamoeba castellanii
60
40
77
64
95
100
Criblamydiaceae
Waddliaceae
Parachlamydiaceae

Figure 4

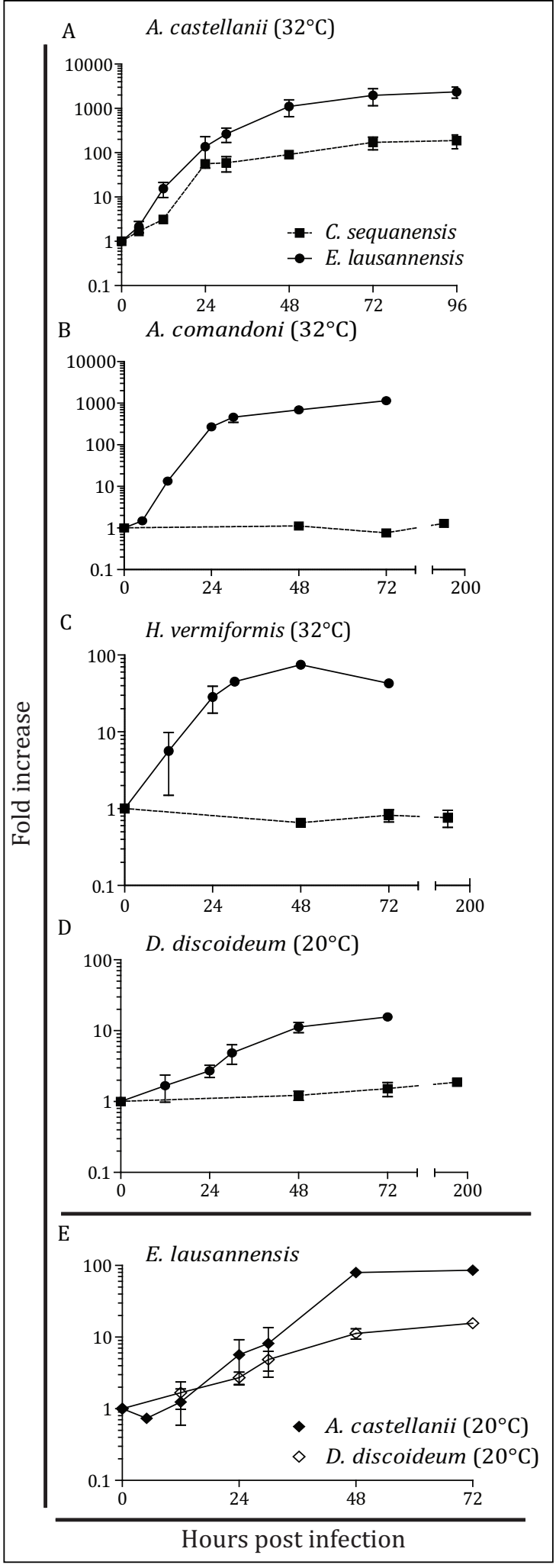
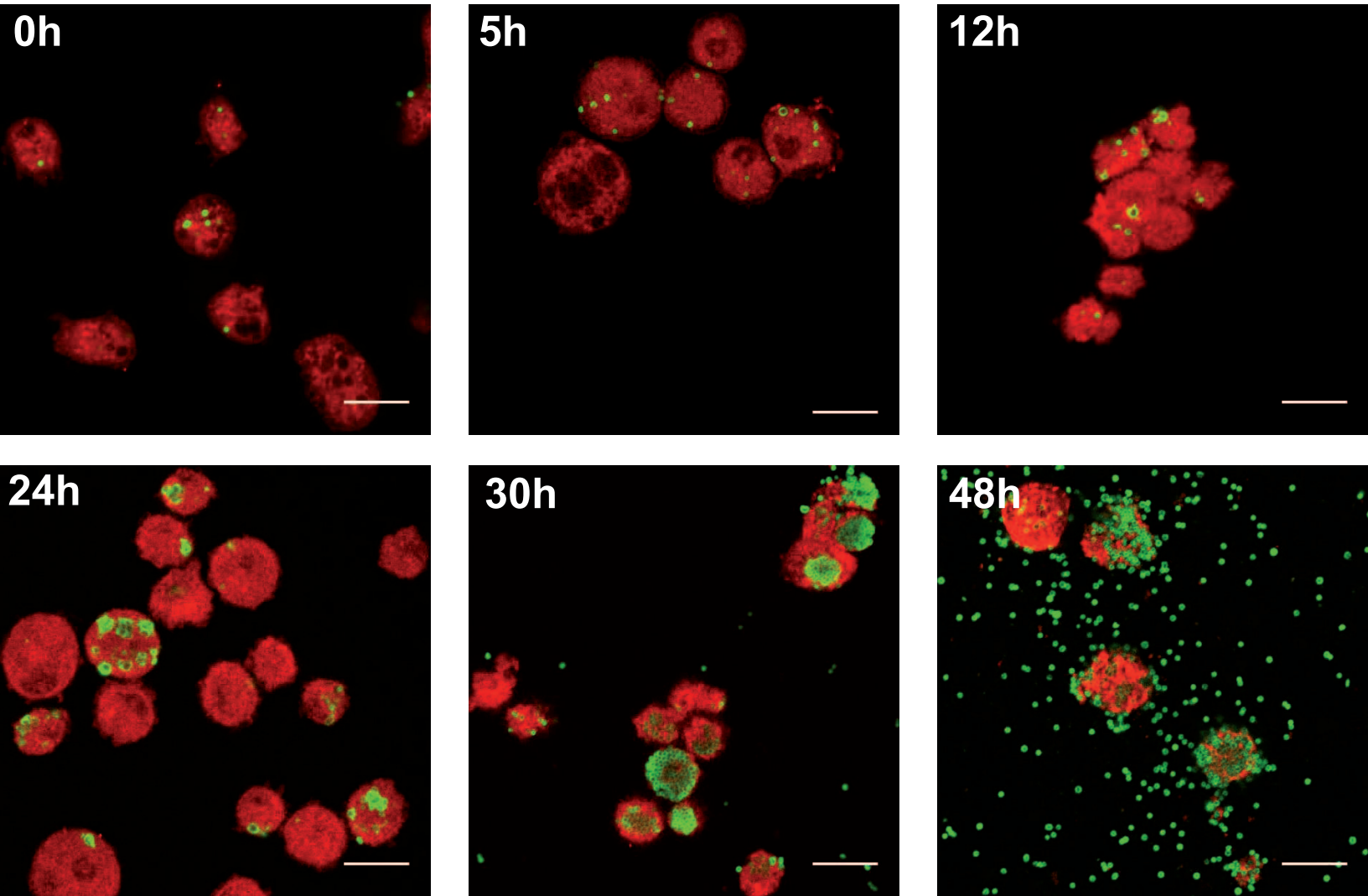

A
A. castellanii (32°C)
10000
1000
100
10
1
0.1
0
24
48
72
96
C. sequanensis
E. lausannensis
B
A. comandoni (32°C)
200
C
H. vermiformis (32°C)
D
D. discoideum (20°C)
E
E. lausannensis
A. castellanii (20°C)
D. discoideum (20°C)
Fold increase
Hours post infection

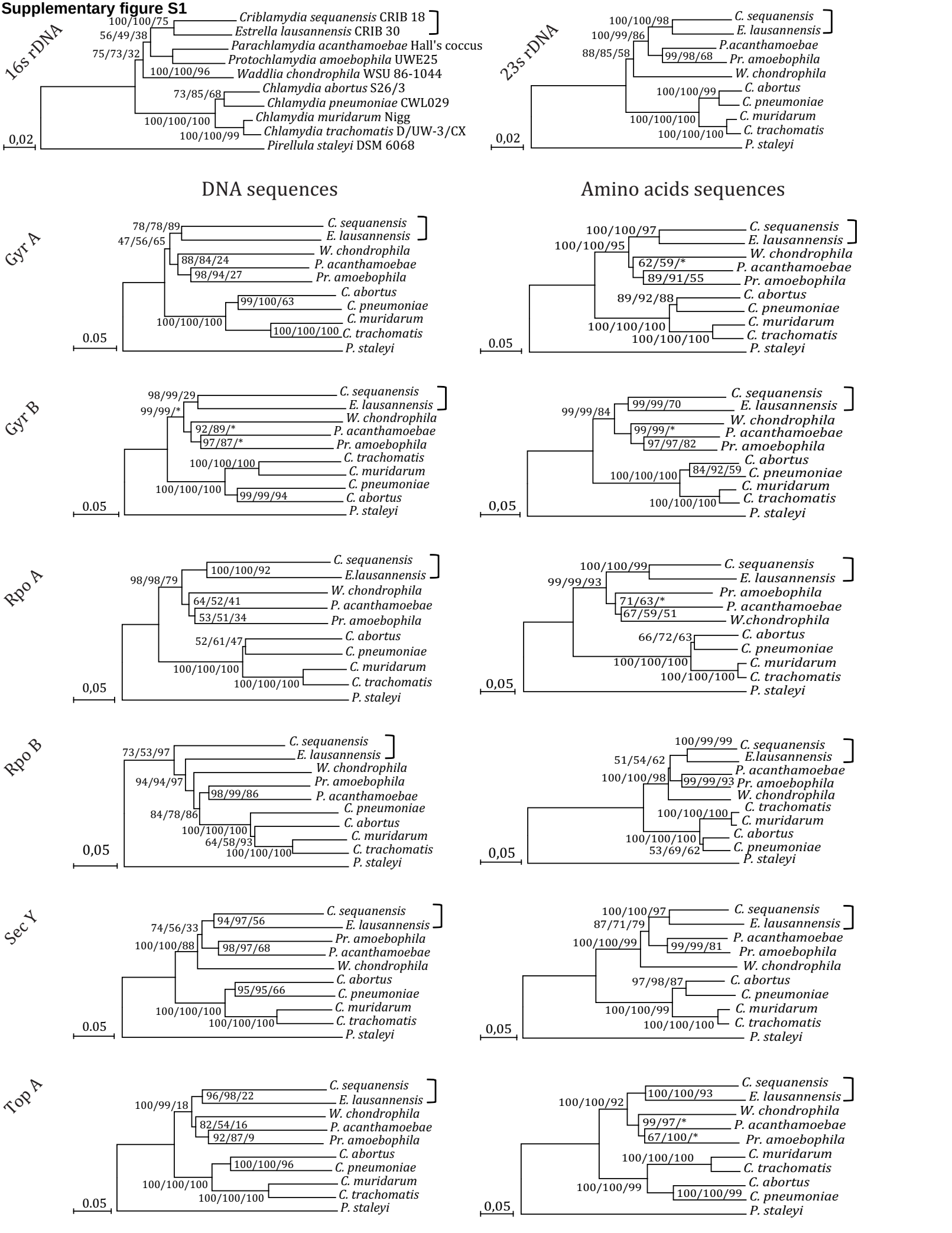
Figure 5



Legend of Supplementary Figure 1

Supplementary Figure 1: Phylogenetic analyses using the neighbor joining (NJ) (Saitou and Nei, 1987), minimum evolution (ME) (Kzhetsky and Nei, 1992) and maximum parsimony (MP) (Eck and Dayhoff, 1966) methods of the DNA sequences of the 16S rDNA, 23S rDNA, *gyrA* and *gyrB*, *rpoA* and *rpoB*, *secY* and *topA* encoding genes and of amino-acids sequences of the last 6 genes. Consensus phylogenetic NJ and p-distance trees model are shown. Percentages of the results of the bootstrap test (1000 replicates) are indicated next to the branches (NJ, ME, MP methods). The star indicates discordance for a ME or MP methods compared to NJ method. The bars represent the estimated evolutionary distance.

Supplementary figure S1



Supplementary table S1: Accession number of the genes used for phylogenetic analyses

Bacterial strains	Accession number
Chlamydia trachomatis strain D/UW-3/CX	NC_000117.1
Chlamydia pneumoniae strain CWL029	NC_000922.1
Chlamydia muridarum strain Nigg	NC_002620.2 (AE002160.2)
Chlamydia abortus strain S26/3	NC_004552.2 (CR848038.1)
Protochlamydia amoebophila strain UWE25	NC_005861.1
Parachlamydia acanthamoebae strain Hall's coccus	NZ_ACZE01000001.1 to NZ_ACZE01000095.1
Waddlia chondrophila strain WSU 86-1044	CP001928.1
Criblamydia sequanensis strain CRIB 18	DQ124300.1 and JN201876 to JN201882
Estrella lausannensis strain CRIB 30	EU074225.1 and JN201883 to JN201889
Pirellula staleyi strain DSM 6068	CP001848.1

Supplementary table S2: Similarity and distance indices (Dice index) between 7 members of the *Chlamydiales* order and the outgroup *Acanthamoeba castellanni*, based on MALDI TOF mass spectrometry on whole-cell proteins.

		1	2	3	4	5	6	7	8
1.	*A. castellanii*	1							
2.	*C. sequanensis*	0,0867	1						
3.	*E. lausannensis*	0,0200	0,2467	1					
4.	*N. hartmannellae*	0,0400	0,1867	0,1467	1				
5.	*P. acanthamoebae*	0,0800	0,1467	0,1800	0,2800	1			
6.	*Pr. naegleriophila*	0,0600	0,0333	0,0400	0,0400	0,0867	1		
7.	*Pr. amoebophila*	0,0733	0,0200	0,0800	0,1000	0,0800	0,0667	1	
8.	*W. chondrophila*	0,0600	0,1133	0,2533	0,1000	0,1533	0,0600	0,1467	1