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Insight in the biology of *Chlamydia*-related bacteria

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Abstract

The *Chlamydiales* order is composed of obligate intracellular bacteria and includes the *Chlamydiaceae* family and several family-level lineages called *Chlamydia*-related bacteria. In this review we will highlight the conserved and distinct biological features between these two groups. We will show how a better characterization of *Chlamydia*-related bacteria may increase our understanding on the *Chlamydiales* order evolution, and may help identifying new therapeutic targets to treat chlamydial infections.

Keywords: *Chlamydiae*; *Chlamydia*-related bacteria; Phylogeny; Chlamydial division

1. Introduction

The *Chlamydiales* order, composed of Gram-negative obligate intracellular bacteria, shares a unique biphasic developmental cycle. The order includes important pathogens of humans and animals. The order *Chlamydiales* includes the *Chlamydiaceae* family and several family-level lineages collectively called *Chlamydia*-related bacteria. Research performed during the last 20 years on these bacterial species has revealed a large diversity within the *Chlamydiales* order (Fig. 1). Among the described families of *Chlamydia*-related bacteria, we can find: (i) 5 genera of *Parachlamydiaceae* [1–3]; (ii) three species of the monophyletic *Waddliaceae* family [4–6]; (iii) four species of *Simkaniaceae* [7–10]; (iv) two species-level lineages within the *Rhabdochlamydiaceae* family [11,12]; (v) *Estrella* and *Criblamydia* genera within the *Criblamydiaceae* family [13,14]; (vi) the *Piscichlamydiaceae* family [15]; (vii) the *Clavichlamydiaceae* family [16]; and (viii) the *Parilichlamydiaceae* family [17].

Data indicate that most of the members of this order might be pathogenic for humans and/or animals [4,18–23,11,12,24,16]. The *Chlamydiaceae* are currently the best described family of the *Chlamydiales order* [25]. The *Chlamydiaceae* family is composed of 11 species including three major human and several animal pathogens.

Chlamydia trachomatis is the most common sexually transmitted infection leading to infertility, extrauterine pregnancy and miscarriages in females [26]. Furthermore, the infection can also be asymptomatic, being difficult to diagnose causing delayed treatment, thus potentially leading to serious complications. Invasive L serovars of *C. trachomatis* are leading to Lymphogranuloma venereum (LGV) that spread through lymphatic vessels to regional lymph nodes. Other *C. trachomatis* serovars (A to C) are causing trachoma, an infection of the inner surface of the eyelids, leading to blindness [27].

Chlamydia pneumoniae, another human pathogen, causes acute respiratory diseases of the upper and lower respiratory tract, such as pneumonia [23] and bronchitis [28]. It has also been recently identified as an agent of asthma exacerbation in children [29].

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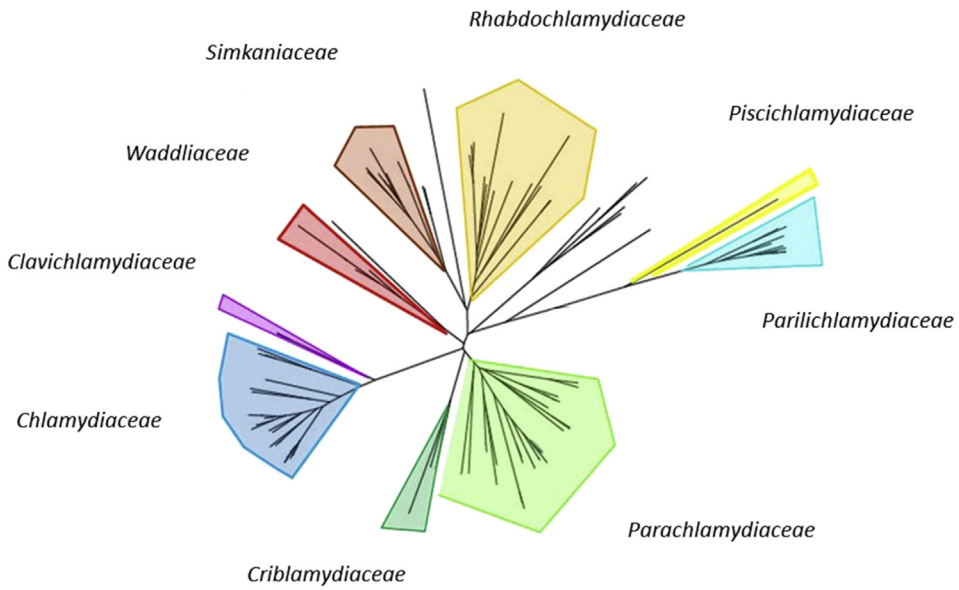

Fig. 1. *Chlamydiales* phylogeny based on the sequence of the 16S rRNA encoding genes. This unrooted tree was built using the neighbor-joining method. Each color highlights a different Family-level lineage.

Chlamydia psittaci is a leading cause of zoonotic avian chlamydiosis and mainly causes respiratory infections followed by diverse symptoms affecting different organs, such as heart, liver and gastro-intestinal tract. In some cases the infection may even have lethal outcome [24]. Recently, the zoonotic risk associated to exposure to horses was documented [30].

Furthermore, different species of the *Chlamydiaceae* family can also infect warm- and cold-blooded animals such as: rodents (*Chlamydia muridarum*), amphibians, reptiles and marsupials (*C. pneumoniae*), cattle (*C. abortus*) and koalas (*C. pecorum*). While some chlamydial species are specific to their host, others, such as *C. abortus* and *C. psittaci*, can cross the species barrier and cause zoonotic diseases.

In this review we would like to detail the recent discoveries in the chlamydial field that help to shed light on the *Chlamydiales* evolution. By comparing *Chlamydiaceae* and *Chlamydia*-related bacteria we intend to discuss conserved and specific mechanisms among them. We will also discuss the advantages and disadvantages of using *Chlamydiaceae* or *Chlamydia*-related bacteria as a model to study chlamydial biology and how comparative studies of the different members of the *Chlamydiales* order might provide new insight on the biology of chlamydia.

2. Molecular biology and genomics

In this part, we will describe the important discoveries done recently in the *Chlamydiales* field concerning the intracellular bacteria themselves, mainly thank to new genomics data and molecular tools. We will thus compare *Chlamydiaceae* and *Chlamydia*-related bacteria, especially regarding their genome composition, their life cycle, their division mechanisms and how these processes are tightly regulated by transcription factors.

2.1. Genome

In order to compare *Chlamydiaceae* and *Chlamydia*-related bacteria, it is essential to first investigate differences and similarities in their genomes. The first complete genome of *C. trachomatis* was sequenced in 1998 [31]. The genome included a single 1,042,519 bp circular chromosome and an extrachromosomal 7493 bp plasmid. In total, the average size of the genes was about 1050 bp with 894 gene-coding sequences. The *C. pneumoniae* genome published in 1999 contained 1,230,230 bp with approximately 1052 protein coding genes. Whole genome analysis of *C. pneumoniae* revealed 214-protein coding sequences not present in *C. trachomatis*, and most without homologues to previously described sequences [32]. Contrarily to *C. trachomatis*, most *C. pneumoniae* strains do not carry plasmids.

During the last years the genomes of several *Chlamydia*-related bacteria have been sequenced and published including those of *Protochlamydia amoebophila* [33]; *Parachlamydia acanthamoebae* [21]; *Waddlia chondrophila* [34]; *Simkania negevensis* [35]; *Criblamydia sequanensis* [36]; *Neochlamydia* spp. [37]; *Estrella lausannensis* [38]; *Rubidus massiliensis* [39]; *Protochlamydia phocaeensis* [40] and *Ca.* Similichlamydia epinephelii [41]. The genomes of *Chlamydiaceae* spp. exhibit a high level of synteny, whereas little synteny was shown among the different *Chlamydia*-related bacteria, partially due to the diversity of Family-level lineages present in this heterogeneous group [31,35]. Moreover, most known *Chlamydia*-related bacteria present a notably larger genome than the *Chlamydiaceae* genome. For instance the genome sizes of *P. amoebophila* (2.4 Mbp) and *W. chondrophila* (2.1 Mbp) are approximately twice larger than the ones of *Chlamydiaceae*, particularly of *C. trachomatis* (1.0 Mbp) and *C. pneumoniae* (1.2 Mbp) [34,35]. The only current exception is the recently described *Ca.* Similichlamydia epinephelii,

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which genome is about 981,542 bp [41]. These differences might be consistent with the fact that *Chlamydia*-related bacteria are adapted to different environments and able to colonize diverse hosts. On the other hand, genome size might also be related to the fact that most known *Chlamydia*-related bacteria thrive in amoebae, which is considered a prokaryote-rich environment where genes exchange may take place [42]. Taken together, comparative genome analyses of species described so far showed several notable differences between each other and compared to *Chlamydiaceae* (Table 1).

Nevertheless, even if members of the order *Chlamydiales* exhibit important genomic differences they still show all a conserved high coding density. *Chlamydiales* were shown to share a relatively large number of genes likely involved in essential functions responsible for the *Chlamydia* unique developmental cycle [35]. Recently, the list of core genes was reduced to 312 protein families conserved in each of 37 compared genomes [43].

These core genes, conserved among all the order, can be used to determine the evolutionary relationships within the *Chlamydiales* order. The species phylogeny of *Chlamydiales* was investigated based on concatenated alignments of core protein sequences [44]. In that work, Pillonel et al. [44] identified a small set of phylogenetic markers allowing the reconstruction of robust phylogenies and the classification of newly sequenced genomes [44]. These sequences provide a much better resolution than 16S RNA (rRNA) gene sequences and have been adopted by the International Subcommittee for the taxonomy of *Chlamydiae* (Greub, IJSEM in press). Comparative analyses performed among the representatives of *Simkaniceae*, *Waddliaceae* and *Parachlamydiaceae* families identified 171 clusters of orthologous proteins shared by these three families and not present in the *Chlamydiaceae*. Half of them were hypothetical proteins of unknown function and the other half were proteins involved in central metabolic processes. These proteins might be important in supporting the biosynthetic diversity in response to environmental changes in contrast to *Chlamydiaceae* [35].

2.2. Developmental cycle and morphology

Chlamydiales share a unique biphasic developmental cycle with two morphologically distinct forms: extracellular, infectious, metabolically inactive elementary bodies (EB) and intracellular, non-infectious metabolically active reticulate bodies (RB). EBs are able to invade the host cells through phagocytosis or endocytosis and therefore are engulfed in early endosome. However, the endosome membrane is being modified by secreted effectors, inhibiting fusion of the endosome with lysosome and resulting in a vacuole called an inclusion. Within the inclusion, EBs differentiate into RBs and start to divide [45]. After several cycles of replication, RBs re-differentiate into EBs and exit the cell via exocytosis or cell lysis [46]. After exiting the cell, EBs can then start a new infection cycle. All *Chlamydia*-related bacteria described so far have developmental cycles similar to *Chlamydiaceae*. Usually *Chlamydiaceae* are observed as small (0.3–0.35 μm) coccoid EBs and larger (0.5–2.0 μm) RBs [47]. However, minor differences were observed in morphology and physiology of developmental stages of *Chlamydia*-related bacteria [48]. For instance, developmental stages of *W. chondrophila*, *S. negevensis*, *P. acanthamoebae*, *P. amoebophila* and *Neochlamydia hartmannellae* are similar to those of *Chlamydiaceae*, whereas some members of *Clavichlamydiaceae*, *Rhabdochlamydiaceae* and *Criblamydiaceae* families have morphologically different developmental stages. In "*Candidatus* Clavochlamydia salmonicola" the EBs were elongated and contained a head and tail region [16]. *Rhabdochlamydia* spp. EBs were shown to have rod shapes, with a five-layered cell wall [12,11] whereas *C. sequanensis* and *E. lausannensis* EBs commonly exhibit a star-like morphology [13,49,14]. These star shapes might be linked to the fixatives and buffers used during the sample preparation and might reflect real difference of underlying membrane protein composition revealed by the Fixatives used [49]. All these data highlight the morphological diversity of the chlamydial infectious stage (EBs).

2.3. Persistence and ABs

It has been demonstrated that besides infectious and non-infectious developmental stages *Chlamydiales* can also enter persistent viable non-dividing non infectious form called aberrant bodies (ABs). ABs are characterized by enlargement of RBs due to an apparent continuous DNA replication without division. This stage was recently referred to as the

Table 1
Major discoveries made by studying the different *Chlamydia*-related bacteria. Non exhaustive list.

Discovery	*Chlamydia*-related bacteria	References
Evidence of long history of energy parasitism	*Parachlamydia acanthamoebae*	Greub et al., Appl Environ Microbiol (2003).
Electron transport (NAD/ATP) transporter	*Protochlamydia amoebophila*	Haferkamp et al., Nature (2004).
Type 3 secretion system	*Parachlamydia acanthamoebae* in natural host *A. castellanii*	Croxatto et al., Pathog Dis (2013).
Conjugative DNA transfer system (T4SS)	*Protochlamydia amoebophila*	Greub et al., BMC Microbiol (2004).
MreB and RodZ (division proteins)	*Waddlia chondrophila*	Jacquier et al., Nat Commun (2014).
Peptidoglycan sacculus	*Protochlamydia amoebophila*	Pilhofer et al., Nat Commun (2013).
Aminopeptidases and peptidoglycan remodeling	*Waddlia chondrophila*	Frandi et al., Nat Commun (2014).
Catalase	*P. acanthamoebae, Waddlia chondrophila*	Rusconi et al., J Bacteriol (2013).
CRISPR system	*Protochlamydia naegleriophila*	Bertelli et al., Genome Biol Evol (2016).

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stress response, in which the bacteria become enlarged and polyploid [50]. Diverse stimuli can induce formation of aberrant bodies. For instance, addition of β-lactams such as penicillin and clavulanic acid [51], other antibiotics such as phosphomycin [52], depletion of nutrients such as amino acids, iron or glucose [53], treatment with interferon (IFN-gamma) [53], as well as co-infection of the host with herpes or other viruses can induce the formation of ABs [54]. *C. pneumoniae* ABs express DNA replication genes, but not cytokinesis genes [55]. A similar process is observed during *C. trachomatis* persistence stage: approximately 16 copies of the genome were accumulated in penicillin treated *C. trachomatis* [56]. These changes are reversible and re-differentiation of the ABs to RBs is induced upon removal of the growth inhibitors allowing the completion of the developmental cycle and finally release of infectious EBs. Persistent stages have also been observed in *W. chondrophila* [57]. Since *Chlamydiales* are able to cause persistent infections, there might be a link between chronic infections and aberrant bodies. Further studies are needed to investigate the molecular mechanisms involved in the formation of the enlarged abnormal ABs and to precise their clinical importance. Indeed, a better understanding of the mechanisms at play in such persistence might improve diagnostics of chronic chlamydial infections and might help improve their treatment.

2.4. Chlamydial cell surface

There are significant differences in outer membrane composition between *Chlamydiaceae* and *Chlamydia*-related bacteria. Thus, the analysis of the chlamydial outer membrane proteins showed strong difference in the protein composition [34,58] (Table 2). The *Chlamydiaceae* outer membrane contains mainly two highly antigenic components: the genus-specific lipopolysaccharide (LPS) antigen and the major outer membrane protein (MOMP). MOMP is crucial for pathogenicity, adhesion and invasion. In contrast to *Chlamydiaceae*, that encode a single MOMP, 35 MOMP-like proteins were observed in *S. negevensis* [35] and 11 in *W. chondrophila* [34]. The role of all these MOMP-like proteins still remains unclear [58]. It is possible, that expression of MOMP-like proteins might change dependent on the conditions and host specificities. Conversely, while *P. acanthamoebae* and *P. amoebophila* lack MOMPs [59], *P. amoebophila* seems to functionally replace them with structurally similar porin proteins (Pom family) [59]. Pom family consist of four proteins and has no characterized homologues in other bacteria sequenced so far [60]. The Pom family proteins were suggested to function as porins and interact with OmcA and OmcB to form the COMC of *P. amoebophila*.

The polymorphic outer membrane proteins (Pmps) represent another important family of surface proteins present in *Chlamydiaceae*. Pmps are autotransporters associated with virulence, being involved in the adhesion to the host cell. Pmps are also present in *Chlamydia*-related bacteria such as *S. negevensis* and *W. chondrophila*. Thus, the *S. negevensis* genome encodes three PmpB homologues [58] whereas *W. chondrophila* encodes only a single putative Pmp-like protein [61]. This *Waddlia* Pmp-like autotransporter as well as two members of *Waddlia* OmpA protein family were shown to act as adhesins [61]. The diversity of outer membrane proteins and adhesins encoded by the different members of the *Chlamydiales* order might thus reflect their diverse host range [35,62].

2.5. Type III secretion system

A complete gene set for the type 3 secretion system (T3SS) is encoded in the *C. trachomatis* genome [31]. The T3SS is a needle-like injection mechanism which secretes effector proteins in the host cell, allowing the bacteria to modulate the host cell function. Bioinformatic analyses indicate that most of the T3SS components are conserved among all the *Chlamydia*-related bacteria and *Chlamydiaceae* [35,34]. Conversely, effector proteins are much less conserved and are much more species specific. A large part of the effector proteins are translocated to the inclusion membrane (Inc). Only three putative Inc proteins are conserved among all known *Chlamydiae*, while about 120 putative Inc proteins were identified among *Parachlamydia* (n = 38); *Simkania* (n = 41) and *Waddlia* (n = 41) [35]. The divergence of Inc proteins repertoire among *Chlamydiales* seems to reflect the specificity of their role during infection of diverse host cell types [35].

Table 2
Common features and differences between *Chlamydiaceae* and *Chlamydia*-related bacteria.

	PG sacculus	Plasmid	Catalase	MOMP (OmpA)	MOMP-like proteins	PmP	Pom protein family	OmcA	OmcB	T3SS	T4SS
Chlamydia trachomatis	▲	Δ*	Δ	▲	Δ	▲	Δ	▲	▲	▲	Δ
Chlamydia pneumoniae	?	Δ	Δ	▲	Δ	▲	Δ	▲	▲	▲	Δ
Chlamydia psittaci	?	▲	Δ	▲	Δ	▲	Δ	▲	▲	▲	Δ
Parachlamydia acanthamoebae	?	Δ	▲	Δ	Δ	Δ	▲	Δ	▲	▲	▲
Protochlamydia amoebophila	▲	Δ	Δ	Δ	Δ	Δ	▲	Δ	▲	▲	▲
Waddlia chondrophila	?	▲	▲	Δ	▲	▲	Δ	Δ	▲	▲	Δ
Simkania negevensis	?	▲	Δ	Δ	▲	▲	Δ	Δ	Δ	▲	▲
Estrella lausannensis	?	▲	▲	Δ	▲	▲	Δ	Δ	▲	▲	Δ
Criblamydia sequanensis	?	▲	▲	Δ	▲	▲	Δ	Δ	▲	▲	Δ
Rhadbochlamydia helveticae	?	▲	?	Δ	▲	▲	Δ	Δ	Δ	▲	Δ

▲ – present; Δ – absent; ? – n.d.; * – some strains might lack.

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2.6. Metabolism

Obligate intracellular lifestyle of *Chlamydiae* also has an influence on their physiology and metabolism. Comparative genomic analysis showed highly similar metabolic capacities among the *Chlamydiaceae* family members. Despite their small genome size, all *Chlamydiaceae* encode the enzymatic capacity to generate ATP via substrate-level phosphorylation [63]. Further analysis of the genomic data revealed, that *Chlamydiaceae* lack genes for the biosynthesis of D-glucose-6-phosphate and thus have to import it from the host cell cytosol [31,32]. The TCA cycle was also shown to be incomplete in *Chlamydiaceae* due to the absence of specific enzymes [31,32]. Moreover, a minimal respiratory chain was encoded by chlamydial genomes, which might be important in case of reduced oxygen conditions [63]. Genomic analysis also revealed in *Chlamydiaceae* a complete pentose phosphate pathway, required for regeneration of NADPH and synthesis of pentosephosphates and a complete pathway for gluconeogenesis. *Chlamydiaceae* are also able to synthesize and degrade the energy storage compound glycogen [31,32,63]. According to genome data, *Chlamydiaceae* are withdrawing ATP from the host cell cytosol using an ADP/ATP translocase and are also capable of independent ATP synthesis. This allows *Chlamydiaceae* to adapt to availability of energy source, using either ATP or glucose-6-phosphate.

This capacity is also present in *Chlamydia*-related bacteria. Thus, *Chlamydia*-related bacteria encode ATP/ADP translocase, similarly as *Chlamydiaceae*, and they have a higher capacity for host-independent production of ATP via oxidative phosphorylation [63]. Contrary to *Chlamydiaceae*, all *Chlamydia*-related bacteria also encode a glucokinase, which enables production of D-glucose-6-phosphate from D-glucose by their own [33–35,63]. Interestingly, *Chlamydia*-related bacteria encode a complete TCA cycle [33–35,63]. *W. chondrophila* and *P. acanthamoebae*, in contrast to all other *Chlamydiae*, encode a glyoxylate bypass, enabling them to produce glucose from fatty acids [34,35]. The exact role of this particularity has still to be investigated [63].

Chlamydia-related bacteria have enhanced biosynthetic capabilities compared to *Chlamydiaceae*. However, most of the amino acids, vitamins, cofactors and nucleotides pathways seem still to be abbreviated [63]. Same *Chlamydiales* import NAD and NADP from the host cell, but using slightly different mechanisms. *C. trachomatis* uses the translocase Npt1 (also called Tlc1 or Ntt1) and another non defined transporter. In contrast, *P. amoebophila* was shown to import NAD by a more specialized nucleotide transporter (Ntt4) and may then convert NAD to NADP using its own NAD kinase [63]. All other *Chlamydia*-related bacteria were shown to possess even higher biosynthetic potential, including the capacity to produce themselves NAD [34,35]. Another important difference in metabolic activity of *Chlamydia*-related bacteria compared to *Chlamydiaceae* is the biosynthesis of menaquinone, isoprenoids, and glycogen. *Chlamydiaceae* were formerly believed to be unable to synthesize menaquinone and they had to import ubiquinone from the host cell instead [31]. Most *Chlamydia*-related bacteria sequenced so far encode the proteins responsible for the synthesis of menaquinone by the chorismate pathway [35]. Recently, genes coding for a new metabolic pathway (the futalosine pathway) have been described in *Chlamydiaceae* genomes. This pathway enables the synthesis of menaquinone from chorismate via futalosine [63]. Taken together, these results show that *Chlamydia*-related bacteria apparently possess a significantly increased and highly variable metabolic potential, compared to *Chlamydiaceae* [34]. High metabolic capacity was thus proposed to be linked to their more diverse ecology [33,35,63,34].

2.7. Transcription and regulation

As described above, *Chlamydiales* possess a biphasic life cycle that needs to be tightly regulated. In order to control transitions between the different stages, regulation at the transcription initiation level is the most probable strategy used, regulation being mainly performed by one or several master (global) transcriptional factors (TFs) [64]. Free-living bacteria genomes usually encode hundreds of TFs. The number of TFs can vary depending on the bacteria species, life style, pathogenicity and many other factors [65,66]. Being obligate intracellular bacteria, *Chlamydiae* encode limited number of TFs in their reduced genomes [31,35], what makes them an interesting model to study the minimal machinery required for transcription regulation during intracellular growth [66]. At least 10 conserved TFs were predicted among *Chlamydiae* [67,34]. Eight of these TFs (AtoC, ChxR, DksA, Euo, HrcA, PhoU, YebC, and YtgR) are conserved among all the order. Two other (YbjN and NrdR) are conserved in nearly all *Chlamydiae*, being absent in only one chlamydial genome. Two other TFs (DcrA and GrgA) are specific for the *Chlamydiaceae* [65]. *W. chondrophila* was used as a model to investigate conservation of TFs in the *Chlamydiales* order. Using ChIP-Seq analysis, it was shown that the Euo protein of *W. chondrophila* binds to 109 putative targets which represent about 5% of the potential total transcriptome [66]. Notably, Euo protein was found to regulate conserved developmentally-regulated genes responsible for vertical genome transmission (cytokinesis and DNA replication) and genome plasticity (transposases) [66]. Euo might thus serve as a master TF that regulates developmental transcription.

3. Cell biology

3.1. Chlamydiales *cell wall*

Chlamydiales possess a cell envelope composed of two lipid bilayers, the inner membrane and the outer membrane, that anchor the chlamydial outer membrane proteins (OMPs). Interestingly, *Chlamydiaceae* do not possess a classical peptidoglycan sacculus (PG) [68]. This sacculus is apparently functionally replaced in *Chlamydiaceae* EBs by a stable structure containing OmcA and OmcB. Indeed, many chlamydial envelope proteins were shown to be disulfide cross-linked and might thus serve as structural support in EBs,

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functionally replacing a PG sacculus. These disulfide cross-linked proteins are not found in the osmotically fragile RBs [69], which is consistent with the fact that RBs are found only in vacuoles in which osmolarity is tightly controlled.

The peptidoglycan (PG) is a peptide cross-linked glycan polymer forming a mesh-like layer. PG is essential for giving a structural strength, maintaining the bacteria shape and is involved in bacteria division. *Chlamydiales* were initially thought to lack PG, until recent findings showing that certain members of *Chlamydiaceae* and *Chlamydia*-related-bacteria possess PG. A recently developed bacterial PG labeling technique allowed the detection of PG in *C. trachom*atis [70]. Another study, showed the presence of a PG-like structure in *P. amoebophila*. Interestingly, the PG structure seems different between *Chlamydiaceae* and *Chlamydia*-related bacteria. While PG in *P. amoebophila* forms a typical, cell-encompassing sacculus, *C. trachomatis* PG forms only a ring-like band at bacteria mid-cell in dividing RBs [71]. The ring-like PG structure of *C. trachomatis* of ≤140 nm width is formed immediately after the previous cell division and follows cell constriction at the division septum [72]. Septal PG was also detected in several other *Chlamydiaceae* [72].

3.2. Chlamydiales *division*

Chlamydiales cell division mechanism is atypical since it occurs in the absence of a sequence homologue of FtsZ, the main organizer of bacterial division. Nevertheless, *Chlamydiales* generally divide by binary fission [73]. Recently, polarized cell division was observed during the first divisions of *C. trachomatis* RBs, in a similar budding process as a subset of Planctomycetes that also lack FtsZ [74]. The exact mechanism and driving force of chlamydial division is not known yet. Localization of MreB actin homologue and its regulator RodZ to the chlamydial division septum has been recently shown in *W. chondrophila* [52,75−77]. Moreover, the importance of MreB for chlamydial division has been shown in *C. trachomatis* [78], *C. pneumoniae* and *W. chondrophila* [52] where MreB inhibitors were shown to block chlamydial proliferation. *W. chondrophila* genome also encodes for the enzyme NlpD which is able to remodel the PG and localizes at the division septum [76]. Pal protein was described as peptidoglycan-binding protein that also localizes to the chlamydial division septum with other components of the Pal−Tol complex. The Pal−Tol complex members are important for the outer membrane invagination during division [75]. Description of differences in PG structure and division mechanisms in different members of the *Chlamydiales* order will help to build a complete image of the evolution of chlamydial division and will help identifying new drug targets.

3.3. Intracellular trafficking

Chlamydiales are known as successful pathogens invading wide range of cells, residing and proliferating within a membrane-bound vacuole called inclusion. It has been shown that the intracellular trafficking differs between *Chlamydiaceae* and *Chlamydia*-related bacteria (Table 3).

Intracellular trafficking of *C. trachomatis* was observed in infected epithelial cells. Infection with *C. trachomatis* induced a dispersion of the Golgi apparatus. Small elements of the disrupted Golgi were associated with the inclusion membrane, apparently sustaining the lipid acquisition and the inclusion maturation [79].

In contrast to *C. trachomatis* no recruitment of Golgi apparatus was observed during *W. chondrophila* infection. *W. chondrophila* was associated first with mitochondria and later on with endoplasmic reticulum (ER) in infected human macrophages [80]. Presumably, the colocalization with the host cell mitochondria and ER improves nutrients delivery (including ATP and lipids) and allows modulation of host immunity.

Similar to *W. chondrophila*, *S. negevensis* was shown to interact with the host cell ER in infected amoebae and epithelial cells [81,82]. On the other hand, *S. negevensis* containing vacuole were associated with mitochondria in infected epithelial cells [83]. Some co-localization with the ER was observed at 48 h p.i., where *S. negevensis* instead of replication within a single vacuole, possibly forms a vacuolar network inside the ER [82]. Presumably, this allows bacteria to interrupt the ER stress signaling pathway. Moreover, at early time points *S. negevensis* bacteria particles were detected in the perinuclear region, which was not the case for *W. chondrophila* and *C. trachomatis* [82].

Amoebae symbiont *P. acanthamoebae* possess a radically different intracellular trafficking. *P. acanthamoebae* infection of macrophages leads to their lysis at 8 h p.i. Interestingly, during that time there was no colocalization of *P. acanthamoebae* with ER and Golgi apparatus, but with markers of late endocytic compartments. *P. acanthamoebae* inhibits fusion of

Table 3
Intracellular traffic of various species of *Chlamydia*-related bacteria and *Chlamydiaceae*. Please note that each Family-level lineage developed its own strategy to manipulate the host cell and generate a favorable replication niche.

Discovery	*Chlamydiales*	References
Recruitment of Golgi apparatus	*Chlamydia trachomatis*	Heuer D et al., Nature (2009).
Mitochondria and ER recruitment during infection	*Waddlia chondrophila*	Croxatto et al., Microbiology (2010).
ER recruitment and Golgi disruption	*Simkania negevensis*	Vouga et al., Pathog Dis (2017); Mehlitz et al., Cell Microbiol (2014).
Control of vacuole biogenesis during infection with reduced acidification and reduced maturation of the endocytic pathway	*Parachlamydia acanthamoebae*	Greub et al., Cell Microbiol (2005).

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late endosome with lysosomes (no accumulation of Cathepsin D, a lysosomal hydrolase) and subsequent destruction by macrophages [84], despite partial acidification of the *Parachlamydia*-containing vacuole.

A better understanding of differences and similarities in intracellular trafficking among the *Chlamydiales* members (Table 3) would also help to understand the chlamydial evolution and shade light on different survival strategies chosen by different order members.

4. Conclusion

Chlamydia-related bacteria could serve as an eligible model to better understand chlamydial biology and evolution despite some distinct features differentiating the members of *Chlamydiaceae* from *Chlamydia*-related bacteria. The differences in the genome size and in intracellular trafficking should be taken into account while making comparisons. For instance, the genome size of *Chlamydiaceae* and *Chlamydia*-related bacteria fluctuates largely depending on the ecology and lifestyle of the bacteria. Nevertheless, despite the difference in the genome size, the lengths and proportion of the coding regions are rather conserved except for the *P. amoebophila*, which encodes more than 50 large leucine-rich proteins [85].

Furthermore, intracellular trafficking also differs between the members of *Chlamydiaceae* and *Chlamydia*-related bacteria. *W. chondrophila* was shown to be associated with mitochondria and endoplasmic reticulum contrary to other *Chlamydiales* [80]. *Chlamydia trachomatis* seems to recruit the Golgi apparatus during endocytic trafficking [79], which differs from *S. negevensis* where Golgi apparatus was not shown to be recruited [79,82]. With discovery of new families and new species lineages, we expect that further different intracellular trafficking or novel unusual characteristics might be revealed.

With some circumspection, we suspect that *Chlamydia*-related bacteria could allow us to improve our knowledge on the *Chlamydiaceae*. Cell biology and molecular studies of *Chlamydia*-related bacteria should help improve the understanding of the *Chlamydiales* evolutionary history, shading light on the evolution of the host specificity of *Chlamydia trachomatis*, which is known to be a strict human pathogen [25]. Until now poorly described, *Chlamydia*-related bacteria prove to have pathogenic potential and their pathogenicity and host–pathogen interactions studies are to be excelled.

Conflict of interest

The authors have no conflict of interest.

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