Cells within cells: Rickettsiales and the obligate intracellular bacterial lifestyle

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Abstract:

The Rickettsiales are a group of obligate intracellular vector-borne Gram-negative bacteria that include many organisms of clinical and agricultural importance, including *Anaplasma* spp, *Ehrlichia chaffeensis*, *Wolbachia*, *Rickettsia* spp, and *Orientia tsutsugamushi*. This review provides an overview of the current state of knowledge of the biology of these organisms and their interactions with host cells, with a particular focus on pathogenic species or those that are otherwise important for human health. This includes a description of rickettsial genomics, bacterial cell biology, the intracellular lifestyles of Rickettsiales and the mechanisms by which they induce and evade the innate immune response.

Keywords: obligate intracellular bacteria, host-pathogen cell biology, bacterial virulence, bacterial development, neglected and emerging pathogens.

Introduction:

Obligate intracellular bacteria are a group of highly specialized organisms that have become so effective at living within eukaryotic cells that they have lost the genes that would allow them to live outside their cellular hosts There are two major groups of obligate intracellular bacteria that cause human disease: the Chlamydiales and the Rickettsiales. The Gram-negative alpha-proteobacteria Rickettsiales, which are the focus of this review, are a family of vector-borne species that include non-pathogens, animal pathogens and human pathogens. Seven major genera and at least 87 species have been described within the order and they exhibit a high degree of diversity in cell structure, vector preference, host cell type preference, pathogenicity and infection cycle. For example, some of the organisms generate a membrane-bound vacuole within which to replicate inside the host cell, whilst others escape from the endolysosomal pathway and replicate directly in the host cell cytoplasm. This natural diversity makes them a powerful group of organisms for the comparative study of factors that determine host-pathogen interactions, whilst their streamlined genomes make them valuable model systems for understanding minimal requirements for complex machineries involved in bacterial growth and division.

The order Rickettsiales is comprised of two families: the Anaplasmataceae, which includes species *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Neorickettsia sennetsu* and *Wolbachia* (supergroups A-H)[1], and the Rickettsiaceae, which includes species *Orientia tsutsugamushi* and *Rickettsia* spp. Bacteria in the genus *Rickettsia* are classified as being in the spotted fever group (SFG; *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia parkeri*), typhus group (TG; *Rickettsia prowazekii*, *Rickettsia typhi*), transitional group (TrG; *Rickettsia felis*, *Rickettsia akari*) and the non-pathogenic ancestral group (AG; *Rickettsia canadensis*, *Rickettsia bellii*)[2,3]. All are found in specific invertebrate hosts (ticks, mites, insects, trematodes, nematodes and others), and many can cause disease in humans or animals when infected arthropods feed on mammalian hosts. Rickettsial diseases are typically zoonotic due to the animal reservoirs that maintain pathogen populations

in the wild. The phylogenetic relationships between selected important species is shown in Fig. 1. and an overview of the diseases, vectors, reservoirs, host cells and geographical distributions is shown in Table 1.

The relationship between Rickettsiales and their invertebrate hosts can be parasitic, symbiotic or commensal depending on whether the infection confers a fitness cost or benefit, and the infection can be vertically transmitted from mother to offspring and thus sustained within a host population over multiple generations, or horizontally acquired through feeding on infected animals during the larval or nymph stage where it can be retained through to the adult stage but not transmitted to subsequent generations[4–6]. *Wolbachia* is a widespread endosymbiont of insects and arthropods that can be transmitted both vertically and horizontally in the case of the reproductive parasite strains such as *Wolbachia pipientis* endosymbiont of *Drosophila melanogaster* (wMel), or strictly vertically transmitted in the case of the non-parasitic mutualist *Wolbachia* endosymbiont of *Brugia malayi* (wBm)[6]. *Orientia tsutsugamushi* is found in the ovaries and salivary glands of mites and is primarily vertically transmitted[7]. Horizontal acquisition from other mites or infected animals can occur through cofeeding but bacteria acquired in this way are not efficiently transmitted to the next generation[8,9]. Some *Rickettsia* species can be vertically transmitted[10,11] whilst others rely on horizontal transmission. *Anaplasma phagocytophilum, Anaplasma marginale* and *Ehrlichia chaffeensis* are all maintained in animal reservoirs and transferred to ticks through horizontal transmission[12].

Rickettsiales species cause disease in humans or animals when (i) the preferred invertebrate host feeds on mammals in such a way as to transmit pathogens and (ii) the interaction between replicating bacteria and the mammalian immune system results in bacterial growth and tissue damage. With the exception of *Rickettsia prowazekii*, humans are dead end hosts and the bacterium does not return to the environmental reservoir. Bacteria are inoculated into the blood or skin of humans through the saliva or feces of feeding arthropods and insects, and rickettsial pathogens are disseminated through the body via the blood and/or lymphatic system. The tropism to mammalian cells varies between species and includes endothelial cells

(*Rickettsia* spp., *Orientia tsutsugamushi*)[13–17], dendritic cells (*Orientia tsutsugamushi*)[18], monocytes/macrophages (*Orientia tsutsugamushi, Ehrlichia chaffeensis*)[18,19] and neutrophils (*Anaplasma phagocytophilum*)[20]. Symptoms begin 5-14 days post inoculation and rickettsial diseases are characterized by headache, fever, myalgia and sometimes a rash, as well as an eschar in scrub typhus and some spotted fever group rickettsial diseases[14]. Rickettsial species are not susceptible to many classes of antibiotics and doxycycline is the preferred drug for treatment of rickettsial diseases[7,21,22]. Whilst disease severity is variable, all the infections can be life threatening and there are currently no approved vaccines for any human rickettsial disease[23–26].

The intracellular lifecycles of Anaplasmataceae and Rickettsiaceae are fundamentally different from each other (Fig. 2). Anaplasmataceae reside within a membrane-bound vacuole in the infected host cell and *Anaplasma marginale, Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* differentiate between a replicative intracellular form called reticulate cells, and an infectious extracellular form called dense-core cells. Rickettsiaceae, in contrast, escape from the endolysosomal pathway shortly after entry and complete their growth and replication directly in the host cell cytoplasm. Following replication *O. tsutsugamushi* buds out of infected cells prior to infecting new cells[27]. *Rickettsia* spp. move directly into adjacent cells within a monolayer, and distinct developmental forms during the course of the cellular infection cycle, equivalent to the reticulate and dense-core forms in Anaplasmataceae, have not been described for any *Rickettsia* species.

This review describes the key features of the best studied Rickettsiales species that are pathogenic to humans or animals, or otherwise important for human health (*Anaplasma phagocytophilum, Anaplasma marginale*, *Ehrlichia chaffeensis*, *Wolbachia*, *Orientia tsutsugamushi,* SFG and TG *Rickettsia* spp.) and the molecular basis of their interactions with mammalian host cells. In the case of *Wolbachia,* that does not directly infect mammalian cells, the interactions with invertebrate cells is described. Excellent recent reviews on specific organisms can be found here[19,28] [4,15,29–34]. The current review does not cover the

interactions between Rickettsiales and their invertebrate hosts, and readers are referred to the following reviews on this topic [5,6,35,36].

Rickettsial genomes

As obligate intracellular bacteria, Rickettsiales genomes are physically isolated from other bacteria and experience small effective population sizes. The consequence of this is that novel genes are rarely introduced into the population, and mutations lead to gene loss and pseudogenization[37]. This reductive genome evolution has resulted in small Rickettsiales genomes, with sizes ranging from 0.8 Mb to 2.5 Mbp and a predicted core genome across the Rickettsiales of ~420 genes[38] and a GC content of 27-50% (Table 1). Differences in genome size reflect differences in the number of repeats, non-coding DNA and pseudogenes, with the 1.11 Mbp genome of *R. prowazekii* having 0.3% of its genome composed of repetitive elements compared with 49% in the 2.5 Mbp genome of *Orientia tsutsugamushi* (strain Karp).

The Rickettsiales include organisms that range from symbiont to pathogen, making them ideal models for studying drivers of pathogenicity in bacteria using comparative genomics approaches (reviewed in [4,39,40 41]). Reductive evolution, driven by loss-of-function mutations and subsequent gene loss, is positively associated with pathogenicity in Rickettsiales, with more virulent species generally having smaller genomes than avirulent relatives[42,43] as seen in *R. prowazekii*, *R. typhi*, *A. marginale* and *E. chaffeensis*. As an example, this has been shown by comparing the highly virulent *R. prowazekii* with less virulent strains including *R. conorii* [44] and *R. africae* [45]. This can be explained by a loss of control mechanisms that regulate host cell entry, replication, and transmission leading to increased cellular damage during these processes and subsequent virulence to the host[40,41,45,46]. However, reductive evolution is not the sole driver of virulence and Rickettsiales also encode genes that are positively associated with virulence. For example, the comparison of virulent *R. rickettsii* with less virulent *R. rickettsii* strains, and an avirulent relative *R. peacockii*[42,43] led to the identification of a number of potential virulence factors, whilst the identification of

four genes specific to typhus group rickettsiae, which have the smallest genomes in the Rickettsiaceae, suggested a specific role for these genes in virulence[2,47].

Mobile genetic elements are present in the genomes of some Rickettsiales species and in some cases this has driven the proliferation of repetitive DNA and gene pseudogenization. The evolution of *Wolbachia* genomes has been shaped by the presence of bacteriophages and insertion sequence (IS) elements[48], whilst conjugative transfer elements are found in Rickettsiaceae[42,49]. One particular conjugative transfer element, the Rickettsiales Amplified Genetic Element (RAGE), is rampant in some Rickettsiaceae genomes including *Orientia tsutsugamushi* and *Rickettsia buchneri* (formerly *Rickettsia* endosymbiont of *Ixodes scapularis*) [49,5051–53] and present in single and/or degraded copies in some other rickettsial genomes[49]. Mobile genetic elements are not present in *Anaplasma* or *Ehrlichia.* Extrachromosomal plasmids have been described in some *Rickettsia* species[54 3,55] and these have facilitated the development of genetic tools in Rickettsiales (see box 1)[3,56].

Bacterial cell biology

Cell membrane and cell wall

Gram-negative bacteria typically have a cell wall composed of peptidoglycan (an extensive polysaccharide-peptide mesh), and an outer membrane in which the outer leaflet is comprised of lipopolysaccharide (LPS), (a membrane embedded sugar molecule). Peptidoglycan and LPS are both potent stimulators of the innate immune system in both mammals and invertebrates and many Rickettsiales species have been shown to reduce or lack these normally essential components of the cell wall and cell membrane. Whilst LPS has been found in species from the *Rickettsia* genus[57–59], genes for its biosynthesis are absent in *Orientia tsutsugamushi* and Anaplasmataceae species[60,61]. *Rickettsia* spp. are also the only Rickettsiales predicted to synthesize a complete, classical peptidoglycan cell wall[62,63]. *Orientia tsutsugamushi, Wolbachia* and *Anaplasma marginale* possess a subset of peptidoglycan biosynthesis genes and are thought to build a less abundant peptidoglycan-like structure[60,64–66], whilst *Anaplasma phagocytophilum* and *Ehrlichia* spp. lack

genes for peptidoglycan biosynthesis. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* are unusual amongst bacteria in having cholesterol-rich membranes, which likely confer some rigidity in the absence of peptidoglycan and LPS. The bacteria lack the biosynthetic capability to synthesize cholesterol and scavenge it from host cells[67,61,68.] The osmotically protective intracellular niche likely enables survival in the absence of these otherwise essential bacterial components, but questions remain as to the differential selective pressures that led to these differences in cell wall and cell membrane structures across the Rickettsiales[60].

Bacterial surface proteins

Proteins embedded on the outer surface of the outer membrane of Rickettsiales bacteria play an important role in host-pathogen interactions because these structures are in direct contact with the outside environment, and this is particularly important in those organisms that lack LPS which normally shields the outer membrane surfaces of bacteria. Consequently, outer membrane proteins play important roles in adhesion to host cells, in interactions with cytoplasmic components of host cells (in cytoplasm-dwelling Rickettsiaceae), and in stimulating and evading an immune response. The immunogenic nature of many Rickettsiales surface proteins has made them strong potential vaccine candidates. However, they are under selective pressure to evade immune recognition, and thus some surface proteins exhibit substantial variation either between strains and/or during the course of an infection (Table 2). This limits their suitability for developing widely effective vaccines.

The predominant abundant and immunogenic outer membrane proteins in *Ehrlichia chaffeensis, Anaplasma marginale*, *Anaplasma phagocytophilum* and *Wolbachia* are porin proteins from the OMP-1/MSP2/P44 superfamily (pfam01617). These are composed of eight transmembrane beta barrel sheets, which are generally highly conserved, and a number of surface exposed loops containing hypervariable domains. Variations in the hypervariable and immunogenic domains enable immune evasion, and Anaplasmataceae have evolved different strategies to introduce variation in these hypervariable domains. In *Anaplasma* spp.

the hypervariable regions in MSP2 undergo recombination during the course of an infection, contributing to immune evasion[34,69–71]. In *A. phagocytophilum* there are ~100 *msp2(p44)* paralogs (strain HZ) and one expression site, and the bacterium uses RecF-mediated gene conversion to vary gene expression[72–74]. *Anaplasma marginale* similarly uses recombination to generation antigenic variation of MSP2 but the number of donor pseudogenes is much smaller (5-7 in strain St. Maries) and segmental recombination is used to generate a mosaic gene, thus increasing variation[75,76]. *Anaplasma marginale* also switches expression between MSP2 and another OMP-1/MSP2/P44 family protein MSP3, which is also polymorphic, conferring further antigenic variation. The MSP2 homolog in *Ehrlichia chaffeensis* is P28. *Ehrlichia* spp. encode a tandem series of 17-22 *p28* genes, mostly with functional promoters. Antigenic variation is achieved through differential expression of intact gene copies[77]. The MSP2 homolog in *Wolbachia* is the *Wolbachia* Surface Protein (WSP). The hypervariable regions of WSP exhibit strain to strain variation driven by mutation and intra- and intergenic recombination[78]. Antigenic variation is generally thought to be driven by response to the adaptive immune response, and since this is lacking in invertebrates the presence of antigenic variation in WSP suggests a different selective pressure experienced during adaptation to specific invertebrate hosts.

The Anaplasmataceae encode other major surface antigens and these include tandem repeat protein 32 (TRP32), TRP47 and TRP120 in *Ehrlichia chaffeensis*, MSP1a/MSP1b, MSP4, MSP5 and OmpA in *Anaplasma marginale*, and MSP4, HSP70, AipA, OmpA and Asp14 in *Anaplasma phagocytophilum* (Table 2). Some of these play a role in attachment and entry into host cells as described below. In contrast to the hypervariable surface proteins described above, proteins involved in attachment to host cells, or other host cell interactions, tend to have highly conserved domains in order to preserve the protein-protein binding site.

The Rickettsiaceae lack OMP-1/MSP2/P44 homologs, but express multiple surface cell antigen (Sca) autotransporter family proteins (type V secretion systems) in their outer membrane. These proteins, which

are widespread in pathogenic bacteria, are composed of a transmembrane beta barrel domain and a soluble extracellular passenger domain. The passenger domain is sometimes cleaved and secreted into the extracellular space[79,80]. *Rickettsia* spp. encode a broadly conserved set of five autotransporter proteins, Sca0 (rOmpA), Sca1, Sca2, Sca4 and Sca5 (rOmpB), although TG rickettsia lack ScaO/rOmpA[4,81], and a further 12 less conserved families across the *Rickettsia*[81]. *Orientia tsutsugamushi* encodes four conserved autotransporter proteins ScaA, ScaC, ScaD and ScaE[82], which are unrelated to the Sca proteins of *Rickettsia,* and an additional two, ScaB and ScaF, that are not widely conserved across strains[203]. Rickettsiaceae autotransporter proteins are involved attachment and entry into host cells and, in the case of some *Rickettsia* spp., actin polymerization. In addition to Sca family proteins, GroEL and DnaK are found at high levels on the surface of different Rickettsiales[83–88] although their roles in host cell interactions are not well described.

The major surface antigen of *Orientia tsutsugamushi* is TSA56, a strongly immunogenic protein that exhibits substantial variation in its 4 hypervariable regions that is driven by high mutation rates and intragenic recombination[89,90]. Recombination between strains may occur in mites coinfected with multiple genotypes, or during cofeeding of multiple infected mites on one rodent, and the process is likely mediated by the conjugative transfer systems and recombination and repair machinery encoded by *Orientia tsutsugamushi*, although this has not been shown experimentally. TSA22 and TSA47 are the other immunogenic surface proteins of *Orientia tsutsugamushi*[91]. TSA22 has no known function nor homolog. TSA47 is highly conserved between strains, contains serine protease and PDZ domains, and is involved in budding out of host cells[92,89,83–88].

Bacterial gene regulation

Gene expression in Rickettsiales species needs to be regulated to reflect the different growth conditions between mammals and invertebrates, and to support the differentiation between reticulate and dense-core cells in Anaplasmataceae. Two component systems are used by bacteria to sense and respond to changes in their environment. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* each encode three predicted

histidine kinase/response regulator pairs[93] and a similar number is found in the genomes of other Rickettsiales. Developmental differentiation in Anaplasmataceae resembles that of *Chlamydiae* and *Coxiella* spp. but the bacterial homologs of eukaryotic histone H1 used by those organisms are not found in Anaplasmataceae[94]. Rickettsiales encode only two predicted sigma factors, the housekeeping RpoD (σ^{70}) and alternate heat shock sigma factor RpoH (σ^{32})[95], which may be important in regulating the response to temperature changes between ectothermic arthropods and warm-blooded mammals. Less than ten transcription factors are predicted, although complete Rickettsiales genomes encode between 88 and 536 hypothetical proteins (Table 1), some of which may encode currently undiscovered transcription factors or other regulatory elements. The transcription factor CtrA, which is a well described regulator of bacterial differentiation in the free living alpha proteobacterium *Caulobacter crescentus*, is found across the Rickettsiales and has been characterized in *Ehrlichia chaffeensis*[96,97]. The (p)ppGpp synthetase/hydrolase SpoT regulates dormancy and stress response in many bacteria, but whilst *Rickettsia* spp. possess multiple partially degraded paralogs within their genomes, *Orientia tsutsugamushi* is the only Rickettsiales known to encode a full-length *spoT* gene. Toxin antitoxin systems are used by bacteria to regulate the stress response, and whilst a number of these have been identified in genomes from the *Rickettsia* genus it is unknown whether they are functional[2,98,99]. Transcription of small RNAs has been demonstrated in *Rickettsia*[100–102] and *Orientia*[103], and in *Wolbachia* small RNAs regulate the expression of both bacteria and host genes[104]. Gene expression is negatively regulated by antisense transcription in the RAGE element of *Orientia tsutsugamushi*[103].

The intracellular lifecycle of Rickettsiales

Binding and invasion

Rickettsiales enter into mammalian host cells using a “zipper-like” process of induced endocytosis, in those organisms in which this process has been studied. This process occurs within minutes of host cell contact and involves one or several adhesins on the bacterial surface interacting with one or several receptors on

the host cells (Fig. 3) This triggers a signaling cascade within the host cells which leads to clathrin- and/or caveolin-mediated internalization that is dependent on actin remodeling. Type III secretion systems which are used for the "trigger-like" internalization of many other intracellular bacteria are not found in Rickettsiales.

In *Rickettsia* spp. the surface protein Sca5 (rOmpB) binds to Ku70 which is embedded in cholesterol-rich lipid rafts and induces bacterial entry in an actin, c-Cbl, clathrin and caveolin 2-dependent mechanism[105]. In SFG rickettsiae Sca0 (rOmpA) is an additional adhesin which interacts with integrin α2ß1and fibroblast growth factor receptor [106], and Sca1 and Sca2 have been shown to play a role in adhesion (Sca1) and adhesion and invasion (Sca2) of *Rickettsia conorii* to host cells[107, 108]. Host cell exchange protein directly activated by cyclic AMP (Epac) has been shown to play a crucial role in internalization and pathogenicity of SFG *Rickettsia australis,* although the exact mechanism by which Epac facilitates bacterial entry is unknown[109]. Actin reorganization is an important process in the internalization of bacteria and an RNAi screen of entry of *Rickettsia parkeri* into *Drosophila melanogaster* S2R+ cells identified a role for Arp2/3, the WAVE complex and Rho family GTPases Rac1 and Rac2, with the involvement of Arp 2/3 being most conserved in mammalian cells [110]. This may be mediated by secreted RickA, which activates Arp2/3 during actin-based motility, but this has not been demonstrated. TG rickettsiae secrete a type IV secretion system effector RalF[111,112], which is a guanine nucleotide exchange factor of ADP-ribosylation factors (Arf-GEF), that induces Arf6 activation resulting in actin remodeling and bacterial internalization[111,112]. In *Orientia tsutsugamushi*, the abundant surface protein TSA56 acts as an adhesin and invasin[113], whilst the autotransporter ScaC also mediates attachment but not invasion[82]. Fibronectin and Syndecan-4 act as host receptors, and binding triggers an integrin-mediated signaling cascade resulting in clathrin-mediated internalization[114].

In *Anaplasma phagocytophilum* a number of adhesins have been described including OmpA, AipA[115] and Asp14[116], with OmpA interacting with alpha2,3-sialic acid residues on sialyl Lewis x-capped P-selectin

glycoprotein ligand 1 (PSGL1)[117] and alpha2,3-sialic acid residues on undetermined glycoproteins[116] and Asp14 interacting with protein disulfide isomerase[118]. Entry is dependent on lipid rafts, glycosylphosphatidylinositol (GPI)-anchored proteins (GAPs) and flotillin 1 and colocalizes with caveolin-1[119]. Clathrin is not required. OmpA is also an adhesin in *A. marginale*[120] although the mechanisms of entry are less well understood. Entry of *Ehrlichia chaffeensis* into the human acute leukemia cell lines (THP-1) is dependent on host cell lipid rafts and GPI-anchored proteins[119]. EtpE Is the best characterized adhesin in *Ehrlichia chaffeensis*. It interacts with GPI-anchored DNAse X[121] and induces bacterial entry via N-WASP dependent actin polymerization[122]. Tandem repeat protein TRP120 (glycoprotein 120) is also an *Ehrlichia chaffeensis* adhesin that induces bacterial entry via activation of Wnt signalling[123], whilst OmpA has been proposed to play a role in entry because it is differentially expressed on DC cells and because an antibody against OmpA inhibits entry of bacteria into THP-1 cells[96] .

Wolbachia differs from other Rickettsiales because it can remain in host cells through the host cell division cycle, rather than exiting infected cells and then infecting naïve host cells[124]. However, Wolbachia has been shown to survive outside of host cells[125], and entry of free Wolbachia into new host cells in a clathrin- and dynamin-dependent manner was recently demonstrated[126], both of which are required for effective horizontal transfer to new hosts.

Cytoplasmic-dwelling rickettsiae

Rickettsiaceae escape from the endolysosomal pathway less than 30 minutes after attachment and internalization and undergo replication directly in the host cell cytoplasm. *Rickettsia* spp. and *Orientia tsutsugamushi* can be cultured in a range of endothelial, epithelial, fibroblast and macrophage cells, and commonly used host cells include monkey epithelial Vero cells, human epithelial HeLa, mouse fibroblast L929 cells and baby hamster kidney (BHK-21) cells. Disruption of the endosomal membrane is required for rickettsiae to enter the host cytosol, and this is likely achieved by the activity of phospholipase D (*pld*) that is present in *Rickettsia* spp. and *Orientia tsutsugamushi* [127], with the possible involvement of hemolysin

C (*tlyC*) in *Rickettsia* spp. Phospholipase D enzymatic activity has been demonstrated in *R. typhi*[128,129]. Rickettsiales generally lack flagella (although these have been described in the *Midichloria,* a non-pathogenic divergent lineage of the Rickettsiales[130,131]), and motility of cytoplasmic bacteria is dependent on the host cell cytoskeleton. Actin-based motility has been observed in SFG rickettsiae *Rickettsia rickettsii, Rickettsia conorii* and *Rickettsia parkeri*[132]. Two forms of actin-based motility are used: one driven by a Wiskott-Aldrich syndrome protein (WASP) called RickA on the bacterial surface that activates the host Arp 2/3 complex and drives actin polymerization into short, curved tails, and another driven by Sca2 that functionally mimics host formins and drives the formation of long, straight actin tails[133–135]. *Rickettsia prowazekii* does not exhibit actin-based motility and the other TG rickettsia, *Rickettsia typhi,* generates much smaller actin tails than SFG rickettsia that confer limited motility[132]. In contrast to the *Rickettsia, Orientia tsutsugamushi* uses minus end directed dynein-dependent microtubule motility to move to the perinuclear region following entry into the cytoplasm, although the bacterial effectors that mediate this process are unknown[136].

Unlike the Anaplasmataceae, Rickettsiaceae do not generally differentiate into distinct forms during infection of a single cell. However, spotted fever group Rickettsia transition into a dormant, non-infectious state in starving ticks, and these are reactivated upon tick feeding prior to or during infection into a susceptible host[137,138]. This reactivation is associated with changes in gene expression, in particular Type IV secretion system genes and OmpB[139], both of which are associated with entry into and manipulation of mammalian host cells. *Rickettsia felis* has also been shown to differentiate into elongated and short forms[140]. Together these indicate that the Rickettsiaceae may have mechanisms to modulate gene expression to exist in distinct subpopulations, although these are not as well described as the developmental differentiation observed in Anaplasmataceae.

Bacterial differentiation and remodeling the parasitic vacuole

All studied Anaplasmataceae species remain within membrane bound vacuoles during their intracellular lifecycle[20,34,141,142]. With the exception of *Wolbachia*, this involves a differentiation from the infecting dense core (DC) form in early endosomes, to the replicative reticulate cell (RC) form within the mature pathogen-containing vacuole (PV) (Fig. 2)[20,34,141,142]. Bacterial differentiation describes the process whereby genetically identical cells differentiate into distinct subpopulations, which differ in morphology and/or other phenotypic characteristics as driven by regulated changes in gene expression[143]. PVs containing multiple RC bacteria are called morulae because of the resemblance to mulberries[24]. Pathogen-driven vacuole maturation involves blocking lysosome fusion and redirecting host nutrients to the PV, and these processes are directed by secreted bacterial effector proteins[20,34,141,142]. Vacuole biogenesis and expansion imposes a substantial burden on host cells through increased requirement for lipid membranes [68].

Anaplasma phagocytophilum has mostly been studied in human promyelocytic HL-60 cells, *Ixodes scapularis* embryonic ISE6 cells and primate RF/6A cells[20,142,144]. The *Anaplasma phagocytophilum* vacuole (ApV) does not acidify, and exploits the autophagy machinery, whose induction promotes bacterial growth[145]. Similar to autophagosomes the ApV has a double lipid bilayer and associates with LC3, Beclin-1, ATG8 and ATG6 but these autophagosome-like vacuoles do not fuse with lysosomes. It also recruits Rab GTPases typical of recycling endosomes and of mediating ER to Golgi trafficking[146]. The ApV associates with the endoplasmic reticulum (ER) and Golgi apparatus[147,148] enabling it to scavenge amino acids and lipids for nutrition. *Anaplasma marginale* is typically grown in ISE6 and RF/6A cells and its vacuole (AmV) also does not acidify, does not fuse with lysosomes, and interacts with the Golgi and ER[17,141,148]. In contrast with the ApV, however, the AmV does not exploit autophagy and retains markers of the early endosome (Rab5), late endosome and recycling endosome[148,149]. *Ehrlichia chaffeensis* is most commonly cultured in dog macrophage DH82 cells, although it can also be propagated in HL-60 cells, RF/6A and various tick cell lines[150–152]. The *Ehrlichia chaffeensis* vacuole (EcV) has characteristics of both early[150] and late endosomes, and is acidified to pH 5.2[152] suggesting a different mechanism of vacuolar

survival from *Anaplasma* spp. The *Wolbachia* vacuole (WbV) is associated with the ER[153] and Golgi-derived vesicles[154].

Nutrient acquisition and metabolism

Obligate intracellular bacteria are nutritional parasites that depend on host cells for carbon sources and metabolic intermediates. They have highly degraded metabolic pathways and an expanded repertoire of importers to take up nutrients from host cells. Chlamydiales and Rickettsiales (except Anaplasmataceae) are unique amongst bacteria in encoding an ATP/ADP symporter that allows to them scavenge ATP directly from host cells, although Rickettsiales are also able to do oxidative phosphorylation and thus generate their own ATP. Analysis of the genomes of Rickettsiales shows variation in the extent of degradation of different metabolic pathways[38,155,156] with the Rickettsiaceae being generally more degraded than the Anaplasmataceae, perhaps reflecting a greater access to host cell metabolites in the cytoplasm compared with the vacuole. Rickettsiales lack the ability to carry out glycolysis or gluconeogenesis and have a limited ability to synthesize amino acids. Rickettsiaceae further lack an ability to synthesize nucleotides or carry out the pentose phosphate pathway, whilst these pathways are generally present in Anaplasmataceae. The TCA cycle is complete in Anaplasmataceae and *Rickettsia* spp. but *Orientia tsutsugamushi* lacks full length copies of the first three enzymes including a functional pyruvate dehydrogenase complex. Pathways for *de novo* synthesis of vitamins and cofactors are largely absent in Rickettsiaceae but partially present in Anaplasmataceae. Both Rickettsiaceae and Anaplasmataceae have largely intact lipid biosynthesis pathways but may rely on host derived lipids or lipid intermediates to make up for deficiencies in the biosynthesis pathways, as has been shown for *Ehrlichia chaffeensis*[68]. Rickettsiales likely primarily utilize host-derived amino acids and TCA intermediates as carbon sources[38,155,156]. In support of this, *Wolbachia* has been shown to require an intact endoplasmic reticulum associated protein degradation (ERAD) pathway as a source of amino acids[157], whilst *Orientia tsutsugamushi* actively manipulates the activity of this pathway via secreted Ank4 effector protein[158]. Similarly, *Anaplasma marginale* and *Anaplasma phagocytophilum* both interact closely with the ER[148] and *Rickettsia rickettsii* targets the endoplasmic

reticulum via a secreted effector protein RARP2[159] although a direct role in nutrient scavenging has not been shown in these organisms.

Bacterial replication and host cell exit

Rickettsiales replicate free in the cytoplasm or in vacuoles by binary fission and then use a range of strategies to exit infected host cells. SFG rickettsiae replicate to about 30-50 bacteria dispersed throughout the cytoplasm and occasionally in the nucleus, and this level of replication causes substantial damage to host cell ultrastructure beginning around 48 hours post infection. Bacteria move directly into adjacent cells using actin-mediated polymerization, in a process facilitated by Sca4 altering the force dynamics of the cell membrane[160], or they exit through eventual lysis of damaged host cells[161]. TG rickettsiae, by contrast, replicate to 1000 bacteria per cell or more but do not cause substantial damage to host cells. At very late time points (96-120 hours post infection) host cell lysis occurs, releasing the intracellular bacteria[162]. *Orientia tsutsugamushi* moves to the perinuclear region after infection and replicates as a microcolony beginning around 24-48 hours post infection. Bacteria can replicate to a very high level of 1000 or more bacteria per cell, depending on the bacterial strain, and host cells can survive intact up to 7 days or longer. Bacterial exit begins around 4 days post infection, with individual bacteria moving to the edge of infected cells and budding out as enveloped bacteria in a lipid raft-dependent process[92]. *Ehrlichia chaffeensis*, *Anaplasma marginale* and *Anaplasma phagocytophilum* transition from the DC to RC several hours post infection then bacterial replication occurs over around 48 hours up to a level of up to several hundred per vacuole. Unlike some other vacuole-residing bacterial, individual vacuoles of Anaplasmataceae do not fuse with one another. RC bacteria transition back to mature DC forms, completing the cycle around 72 hours post infection[28]. *Ehrlichia chaffeensis* uses two mechanisms to exit cells. At early stages of infection, bacteria traffic directly between adjacent cells in an actin-dependent mechanism via filopodia[163]. At later stages of infection, host cell rupture adjacent to morulae causes extrusion of bacterial cells[163]. In the case of *Anaplasma* spp., release of membrane-bound bacterial vacuoles by exocytosis have been described[164,165] although lytic release of heavily infected cells also causes bacterial release.

Manipulating the host response

Secretion systems and effector proteins

A major strategy that intracellular bacteria use to manipulate host cell activity is the secretion of effector proteins that bind to host cell machinery and modulate its function to benefit the bacterium. Mechanisms of action for rickettsial secreted effectors include binding to host cell DNA or chromatin and modulating gene expression (nucleomodulins), binding to host proteins and interfering with signaling pathways, modifying the activity of host proteins through post-translational modifications such as ubiquitination, and interacting with phosphatidylinositol signaling through lipid binding. Effector proteins typically interact with host cell proteins by structurally mimicking protein-protein interactions, and thus they can be identified by the presence of domains normally found in eukaryotic cells. Ankyrin repeat contain proteins (Anks) are a large family of eukaryotic proteins that contain 1-34 copies of a ~33 amino acid Ank repeat that is the most common protein-protein interaction motif in nature[166]. Anks are commonly found in the genomes of pathogenic bacteria and viruses and are widely distributed amongst the Rickettsiales. A range of mechanisms have been ascribed to Ank proteins in *Anaplasma, Ehrlichia, Wolbachia, Rickettsia* and *Orientia* including disrupting organelles, modifying host gene expression, inhibiting apoptosis, subverting autophagy, and interfering with vesicular trafficking[159,167–174]. Tetratricopeptide repeat proteins (TPR) contain a 34 amino acid repeat present in a tandem array of 3-16 motifs, and are also well-known domains of effector proteins[175]. TPR proteins have been found in *R. felis* and *Orientia tsutsugamushi* where they have been shown to inhibit eukaryotic translation[176]. *Ehrlichia chaffeensis* encodes a number of tandem repeat proteins (TRPs) that, in addition to their roles as surface proteins described above, are secreted into host cells and interact with a range of host pathways[177–179]. TRP120 is the best characterized TRP in *Ehrlichia chaffeensis* and induces host SUMOylation[180] and ubiquitination activity[181] which it uses to modify host and bacterial proteins including polycomb group proteins[182] and tumor suppressor FBW7 protein[183]. Through this activity and that of other secreted effectors it leads to changes in host gene expression, both through interacting with signaling pathways and direct modification of host epigenetic

machinery, and these changes in host cell gene expression promote its intracellular survival. *Ehrlichia chaffeensis* also secretes effector protein Etf-2 that binds to RAB5 and prevents GTPase activity thus delaying endosome maturation and enabling formation of the EcV[184]. Other secreted effector proteins include the TG *Rickettsia* Arf-GEF RalF, that is involved in bacterial entry, and phoshatidylinositol 3-kinase effector Risk1 that is involved in autophagy evasion. A deubiquitylase from *Orientia tsutsugamushi*, otDUB, has been characterized *in vitro* although its role during infection is unknown[185]. Two effector proteins have been characterized in *Wolbachia*, TomO[186] and WalE[187], whilst a number of others have been predicted using bioinformatics pipelines and exogenous expression approaches[188 189]. In addition to these characterized effector proteins, there are likely to be many additional secreted effectors in the Rickettsiales. These can be predicted *in silico* using dedicated algorithms for the identification of putative new effectors[190,191] or identified using bacterial two-hybrid screens using components of the secretion apparatus as bait (e.g.[111]).

Rickettsiales encode five known protein secretion systems that allow cytoplasmic proteins to be brought to the cell surface or extracellular space[99]. A type IV secretion system (T4SS) is widespread amongst the Rickettsiales. These are membrane spanning protein secretion machineries that transfer proteins, DNA or nucleoproteins across Gram positive and Gram negative envelopes[192]. There are at least three classes of T4SS, and the Rickettsiales possess a system called *rvh* that encodes 12 scaffold genes (*virB1-B11, virD4*) homologous to the archetypal *vir* system encoded by the pTi plasmid of *Agrobacterium tumefaciens*[155] although it lacks the minor pilin subunit *virB5* that is required for pilus formation but not substrate transfer. In addition to *rvh*, the RAGE mobile genetic element that is found in *Orientia tsutsugamushi* and some *Rickettsia* spp. contains a second T4SS that is homologous to the conjugative F plasmid T4SS of *E. coli*. The RAGE T4SS encodes 15 *tra/trb* genes that form a T4SS scaffold but lacks components involved in the regulation of conjugation. A number of *rvh* substrates, including Sec7 domain containing proteins, a phosphatidylinositol 3-kinase effector and some Ank proteins, have been identified in *Rickettsia*

rickettsii[159], *Rickettsia typhi*[111,112,159,193], *Wolbachia*[188], *Anaplasma marginale*[194], *Anaplasma phagocytophilum*[195–199] and *Ehrlichia chaffeensis*[198,200].

In addition to the T4SS, Rickettsiales encode T1SS (TolC), twin-arginine translocation (Tat) systems, the Sec-TolC pathway and T5SS (autotransporter proteins). The T1SS is a chaperone-dependent secretion system that spans both membranes of the Gram-negative envelope and is comprised of three proteins: an inner membrane ABC transporter, a periplasmic adaptor protein and an outer membrane efflux pump (TolC). The T1SS typically secretes proteins, ions, drugs and polysaccharides. The Sec translocon is an inner membrane complex that transports unfolded proteins across the membrane. TolC is also used in a Sec-dependent mechanism. T1SS/Sec-TolC has been shown to be involved in the secretion of Ank proteins in *Rickettsia typhi*[173] and *Orientia tsutsugamushi*[201] and tandem repeat proteins (TRPs) in *Ehrlichia chaffeensis*[202]. The Tat pathway is used to secrete folded proteins and although Tat systems have been identified across Rickettsiales, very few predicted substrates have been identified suggesting that this is not a major system in these organisms[99]. Finally, the T5SS uses the Sec translocon to transport outer membrane proteins that have a ß-barrel autotransporter domain and a surface exposed passenger domain, which is sometimes cleaved off and released into the extracellular space. Sca autotransporter proteins in *Rickettsia* and *Orientia* are used in attachment and entry into host cells as well as actin polymerization as described in the sections above.

Immune recognition and subversion of innate immunity

Pathogenic Rickettsiales cause vascular infections in humans, targeting primarily the endothelium (Rickettsiaceae) and circulating leucocytes (Anaplasmataceae). Interactions with these target cells leads to the secretion of proinflammatory chemokines and cytokines, resulting in endothelial activation and the infiltration of CD4+/CD8+ T cells, Natural killer cells and monocytes/macrophages. Overactivation of this immune response can cause tissue damage and this is an important driver of pathogenicity in rickettsial diseases. The interplay between the invading pathogen and the cellular innate immune system of the target

cell determines whether the pathogen is rapidly cleared, or whether the bacterium replicates and disseminates causing damage to host cells and inducing a stronger and sometimes damaging inflammatory response. Whilst Rickettsiales have primarily evolved as arthropod endosymbionts, and not human pathogens, similarities in the pathways involved in pathogen detection and destruction in eukaryotic cells means that infection sometimes leads to a pathogenic outcome during mammalian infection. The major mechanisms by which Rickettsiales subvert eukaryotic cellular defense mechanisms are described in the section below and shown in an overview in Fig. 4.

Intracellular Rickettsiales are sensed by cytosolic and membrane bound pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs). These include Toll-like receptors TLR2 across Rickettsiales[79,204–207] and TLR4 in some species but not *Anaplasma phagocytophilum*[79,204–207]. The nucleotide-binding oligomerization domain proteins NOD1 and NOD2 recognize peptidoglycan fragments and have been shown to be activated in response to *Orientia*[208] and, possibly, *Wolbachia*[208–210] but this has not been tested in *Rickettsia.* In new hosts, *Wolbachia* (*w*AlbB) is recognized by Toll and IMD and also induces expression of peptidoglycan recognition protein (PGRP)-LE[211]. However, in co-evolved associations *Wolbachia* does not induce an immune response including the release of antimicrobial peptides, reflecting adaptation by the bacterium and/or host[212]. TLR3 recognizes cytosolic dsRNA and was recently found to be upregulated during infection with *Orientia tsutsugamushi* suggesting a possible role in bacterial detection[103]. Cyclic GMP-AMP synthase/stimulator of interferon genes (cGAS/STING) recognizes cytosolic DNA in *Rickettsia parkeri*[213] and this may be involved in other Rickettsiales. In addition to these receptors that recognize intracellular bacteria directly, antibody-coated *Ehrlichia chaffeensis* is recognized by the cytosolic receptor TRIM21 which targets the bacteria for degradation[214].

Rickettsiales-activated PRRs signal through adaptor molecules including MyD88 and Toll-receptor-associated activator of interferon (TRIF) to induce NF-kB, ERK1/2, p38 MAPK and JNK pathways leading to upregulation of expression of interferons and proinflammatory cytokines/chemokines that trigger

activation of the immune system. PRR activation also leads to the activation of a multiprotein complex called the inflammasome that induces IL-1ß and IL-18 as well as apoptotic and pyroptotic cell death. Rickettsial infection triggers an antiviral type I proinflammatory response, although the exact signaling pathways and cytokine profiles vary by pathogen and cell type. Rickettsiales have evolved to tolerate this response by repressing cytokine expression[215,216] and modulating inflammasome activation[213,217–219]. Wnt and Notch pathways play a role in regulating activation of the innate immune response, and this is exploited by *Ehrlichia chaffeensis* which uses secreted TRP120 to activate Notch, leading to downregulation of TLR2/4 expression [220].

The inflammatory response leads to oxidative stress through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and this is highly toxic to intracellular bacteria. This response is particularly pronounced in those bacteria that live inside macrophages and neutrophils, and both *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* actively inhibit the formation of reactive oxygen species in host cells[200,221–223 224 225], although endothelial cells infected with rickettsia also induce a bactericidal cytokine/chemokine induced RNS response[226]. In addition to having antimicrobial activity, oxidative stress can contribute to pathogenesis through causing tissue damage, and the antioxidant alpha-lipoic acid has been shown to be protective in *Rickettsia rickettsii* and *Rickettsia conorii* infections[226–228]. *Wolbachia* can induce both ROS and antioxidative mechanisms, and this may contribute to redox regulation in coevolved associations between endosymbiont and host[229].

Intracellular bacteria are vulnerable to detection and destruction by autophagy, in which bacteria are recognized by the host ubiquitin machinery leading to polyubiquitination of the bacterial surface and subsequent recruitment of the autophagy machinery that encases bacteria in a double membrane and targets them to the lysosome for degradation[230]. This process is particularly active in macrophage cells[231]. Bacteria adapted to cytoplasmic growth have had to evolve mechanisms to evade or exploit this system, and *Rickettsia* spp. and *Orientia* have been shown both to induce autophagy and to escape it. Autophagy was

shown to promote the growth of SFG *Rickettsia australis* in macrophages[232], and *R. typhi* in HeLa cells[193] In the case of *R. typhi*, the bacteria induce autophagosome induction but inhibit its maturation, and this process is mediated by the secreted effector Risk1. Conversely, OmpB allows *Rickettsia parkeri* to evade autophagy in macrophages by shielding bacteria from the ubiquitination machinery[233]. *Orientia tsutsugamushi* has been shown to induce autophagy upon internalization, but then evade it by an unknown mechanism[234,235]. *Anaplasma phagocytophilum* repurposes the autophagy machinery to build its vacuole, as described above and *Ehrlichia chaffeensis* also benefits from autophagy by using it to provide nutrients for bacterial growth in the EcV. In contrast *Wolbachia* does not exploit the autophagy process, and levels of intracellular bacteria are controlled by it as shown by increased bacterial growth in the presence of autophagy inhibitors. This difference with *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* is likely explained by the mutualistic relationship of most *Wolbachia* species, whose survival is dependent on a healthy host that requires an intact autophagy system[236].

Cells that have been infected by pathogens frequently induce apoptosis as a means of pathogen clearance. Rickettsial pathogens have evolved to inhibit apoptosis in a range of cell types including *Rickettsia* spp. in endothelial cells[237–239], *Ehrlichia chaffeensis* in monocytes[200] and *Ehrlichia ewingii* and *Anaplasma phagocytophilum* in neutrophils[240,241]. The latter is particularly important because neutrophils naturally have a very short life span and the antiapoptotic activity it required to allow time for bacterial replication. *Orientia tsutsugamushi* has been shown to have both pro-apoptotic and anti-apoptotic activity depending on the bacteria and host strains and the stage of infection[242–244]. Similarly, *Wolbachia* has been shown to both inhibit and induce apoptosis in hosts, with protective inhibition of apoptosis seen in obligate mutualistic relationships and damaging induction of apoptosis observed in pathogenic relationships[236].

Conclusions and future directions

Studying the interactions between Rickettsiales and their host cells is important for two reasons. First, it helps us to better understand these medically and agriculturally important organisms and thus develop

appropriate interventions. Therapeutic treatment options for rickettsial infections are limited and there are no licensed human vaccines available, and the development of both requires a better understanding of bacterial growth and pathogenesis. Second, it uncovers fundamental biology about both prokaryotic and eukaryotic cells. Obligate intracellular bacteria and viruses have evolved an intimate relationship with their eukaryotic hosts over millennia of coevolution, and a detailed study of the mechanisms by which these cellular pathogens exploit host cell machinery illuminates mechanisms of that host cell machinery, for example actin polymerization and autophagy. Furthermore, obligate intracellular bacteria have reduced and streamlined genomes, retaining only those genes essential for their infection and growth. A study of those minimal machineries reveals the core requirements for systems that are more complex in other bacterial species. For example, a study of the peptidoglycan biosynthesis machinery in obligate intracellular bacteria revealed the minimal gene set required to build a peptidoglycan-like structure[60]. The field of rickettsial cell biology has made great advances in the time since the first rickettsial genome was sequenced[245]. Genetic tools are now being developed and future advances in genetic technology as well as other cell biology tools such as fluorescence microscopy (see box 1) will support a deeper understanding of the biology of these important and fascinating bacterial species.

Box 1. Tools for working with Rickettsiales. Genetic tools for working with Rickettsiales are limited in availability compared with other intracellular bacteria *Chlamydia trachomatis* and *Coxiella burnetii* although important improvements have been made over the past decade. These are summarized in a recent comprehensive review[246]. Obligate intracellular bacteria are difficult to genetically modify because they generally need to be isolated from host cells for transformation which risks damaging the bacteria. In addition, the intracellular lifecycle means that it is not possible to generate mutations in genes required for internalization or intracellular growth. In spite of these challenges, genetic modification has been successful in *Rickettsia* spp., *Anaplasma* spp. and *Ehrlichia chaffeensis.* There are no reports of genetic modification in *Orientia tsutsugamushi* or *Wolbachia.* Transformation has been achieved using electroporation[247–251,151] and polyamidoamine dendrimers[252]. Protein overexpression has been achieved in *Rickettsia* spp. using

590 shuttle vectors derived from plasmids from *Rickettsia amblyommatis*[253–256]. Random mutagenesis has been
591 achieved in *Rickettsia*, *Anaplasma phagocytophilum*, *Anaplasma marginale* and *Ehrlichia chaffeensis* using
592 a *Himar 1* mariner transposon system[135,249,250,257,258,151 259] and recently in *R. conorii* using a *kkaebi* mini
593 transposon system[59], whilst targeted mutagenesis has been achieved in *Ehrlichia chaffeensis* and *Rickettsia*
594 using allelic exchange[250,260] and mobile group II introns[250,261]. Transient knockdowns using peptide nucleic
595 acid technology has also been described in *Rickettsia* and *Anaplasma marginale*[245].
596 Where genetic tools are available, overexpression of fluorescence proteins has been used for imaging
597 intracellular Rickettsiales in cultured cells and animals[5,252,256,262–266]. Given the limited availability of genetic
598 tools, however, alternative approaches for fluorescence microscopy are required. Giemsa staining is
599 traditionally used to image bacteria in tissue sections and clinical samples[267], whilst immunofluorescence
600 microscopy is widely used to study fixed samples of Rickettsiales in cultured cells and animal models
601 although this suffers the limitation that antibodies need to be custom generated for particular organisms and
602 strains. A clickable methionine probe that specifically labels bacteria that undergoing protein synthesis has
603 been used to label intracellular Rickettsiales species and identify individual cells that are metabolically
604 active[268]. Commercially available fluorescent probes have been used to carry out live cell imaging of
605 *Orientia tsutsugamushi*,[269] *Wolbachia*[270 271,272] and *Ehrlichia chaffeensis*[273] whilst fluorescence *in situ*
606 hybridization (FISH) has been used to label *Wolbachia* and *Rickettsia* in fixed cells and tissues[274,275 276 277].

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