Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Orientia and *Rickettsia*: different flowers from the same garden

Joseph J. Gillespie[1] and Jeanne Salje[2]

Recent discoveries of basal extracellular Rickettsiales have illuminated divergent evolutionary paths to host dependency in later-evolving lineages. Family Rickettsiaceae, primarily comprised of numerous protist- and invertebrate-associated species, also includes human pathogens from two genera, *Orientia* and *Rickettsia*. Once considered sister taxa, these bacteria form distinct lineages with newly appreciated lifestyles and morphological traits. Contrasting other rickettsial human pathogens in Family Anaplasmataceae, *Orientia* and *Rickettsia* species do not reside in host-derived vacuoles and lack glycolytic potential. With only a few described mechanisms, strategies for commandeering host glycolysis to support cytosolic growth remain to be discovered. While regulatory systems for this unique mode of intracellular parasitism are unclear, conjugative transposons unique to *Orientia* and *Rickettsia* species provide insights that are critical for determining how these obligate intracellular pathogens overtake eukaryotic cytosol.

Addresses
[1] Department of Microbiology and Immunology, School of Medicine, University of Maryland Baltimore, USA
[2] Department of Biochemistry, Department of Pathology, and Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Corresponding authors:
Gillespie, Joseph J (jgillespie@som.umaryland.edu),
Salje, Jeanne (jss53@cam.ac.uk)

Current Opinion in Microbiology 2023, **74**:102318

This review comes from a themed issue on **Cell Regulation**

Edited by **Erin Goley** and **Peter Chien**

Available online xxxx

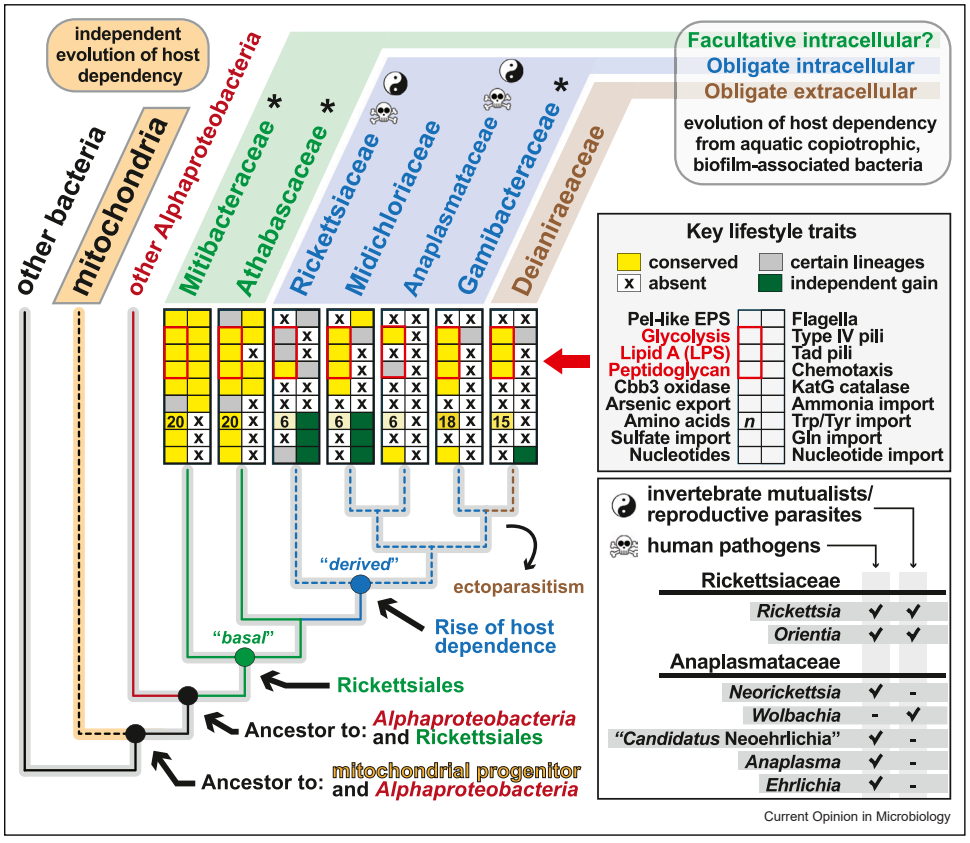
https://doi.org/10.1016/j.mib.2023.102318

Rickettsiales and the emergence of host dependency

Historically, Rickettsiales (*Alphaproteobacteria*) were acknowledged to encompass a diverse assemblage of obligate intracellular bacteria (IB), widely considered a sister lineage to the mitochondria progenitor [1,2]. Pioneering work on rickettsial genomes identified decreased genome size and pseudogenization of genes within many metabolic pathways, conceptualized as 'reductive genome evolution', behind addiction to the eukaryotic cytosol [3–8]. This observation held for two decades as hundreds of diverse Rickettsiales genomes were sequenced. However, recent phylogenetic analysis of deep marine metagenomes illustrated that mitochondria likely originated outside of all described *Alphaproteobacteria* [9], and phylogenomic description of certain novel genomes established two basal rickettsial lineages, Mitibacteraceae and Athabascaceae, with features indicating an extracellular lifestyle not dependent on eukaryotic hosts [10]. These findings culminated a growing trend in discovering mostly aquatic, protist-associated rickettsial species with traits more characteristic of free-living and facultative IB but absent from the numerous genomes of well-characterized invertebrate- and animal-associated rickettsial species [11–16]. Importantly, the current Rickettsiales phylogenetic framework allows for assessing the evolutionary trajectories within five derived lineages for structural and metabolic innovations that emerged from transitions to host dependency (Figure 1).

Notable rickettsial species with human health relevance, that is, those found in blood-feeding arthropods, occur in Anaplasmataceae (genera *Neorickettsia*, *Wolbachia*, '*Candidatus* Neoehrlichia', *Anaplasma*, and *Ehrlichia*) and Rickettsiaceae (genera *Orientia* and *Rickettsia*). Much of the literature dealing with the genomics, bacterial cell biology, intracellular lifestyles, and interactions with host immune responses for these select rickettsiae has been reviewed in contemporary synopses [17,18]. Very recent advances on the mechanisms of pathogenesis [19–38] and interactions with host immune systems [39–47] continue to refine knowledge on rickettsial biology. We focus here on how bacterial growth and metabolism relate to cell envelope architecture and intracellular lifestyle, highlighting *Orientia* and *Rickettsia* species as underappreciated scavengers of host cell glycolysis (Figure 1). We further identify knowledge gaps for how these bacteria regulate their growth in the intracellular environment and discuss the possible role of conjugative transposons in this process.

Figure 1



Phylogeny and metabolic diversity of Rickettsiales.
The tree summarizes phylogeny estimations reported by Schön et al. [10], with asterisks denoting the newly proposed families Mitibacteraceae, Athabascaceae, and Gamibacteraceae. Skull-and-crossbones: lineages harboring known human pathogens; yin-yang: lineages harboring invertebrate mutualists and/or reproductive parasites (see white inset for breakdown). Dashed branches indicate independent evolution of host dependency in the mitochondrial progenitor and the five derived Rickettsiales families. Gray inset: key lifestyle traits of rickettsial families identified from comparative genomics analysis, with red emphasizing glycolysis and glycolysis-derived glycoconjugates. Redrawn from our original work [48].

Orientia and *Rickettsia* species: o sister, where art though?

Orientia causes the human mite-borne disease scrub typhus. The genus is dominated by one species, *Orientia tsutsugamushi*, which is prevalent in Asia. Two additional species have recently been described, *Orientia chuto* isolated from a patient in Dubai [49] and '*Candidatus* Orientia Chiloensis' found in Chile [50]. There is greater species diversity within genus *Rickettsia*, which contains both pathogenic and nonpathogenic species that are broadly classified into five groups: Bellii Group, Transitional Group, Typhus Group (TG), Tamurae/Ixodes Group, and Spotted Fever Group (SFG) [51]. While most described *Rickettsia* species are tick-borne, others are transmitted by body lice, fleas, and mites. Both *Rickettsia* and *Orientia* infections present clinically with headache, fever, and rash, although additional clinical symptoms are species-specific, such as the presence of a necrotic lesion at the site of infection, which is only present in scrub typhus and some SFG rickettsial infections.

Although it was historically classified within genus *Rickettsia*, *Orientia tsutsugamushi* became recognized as a phylogenetically divergent sister taxon to *Rickettsia* species [52]. More recently, analysis of genome sequences from environmental Rickettsiaceae species (i.e. those from protists, apicomplexans, diplomonads, crustaceans, and insects) has illuminated basal lineages of Rickettsiaceae that substantially inform on the divergence of *Orientia* and *Rickettsia* lineages from other Rickettsiales [11,53–57]. Furthermore, phylogenetic analysis of genome sequences from novel genera '*Candidatus* Sarmatiella' (paramecium symbiont) [58] and '*Candidatus* Megaira' (symbionts of algae and ciliates) [14,59] indicates *Orientia* and *Rickettsia* species are not monophyletic, that is, they lack common ancestry within Rickettsiaceae. Fortifying this, a long-standing recognized basal lineage of *Rickettsia* termed 'Torix

Group', which is highly diverse and widely present in nonblood-feeding arthropods [60–64], was recently classified as a new genus, '*Candidatus* Tisiphia', in a study that identified many new provisional *Rickettsia* species from metagenomic analyses of diverse arthropods [65]. This revolutionized Rickettsiaceae tree now provides a framework to assess the emergence of unique traits, as well as the convergence of common traits, for these diverse genera.

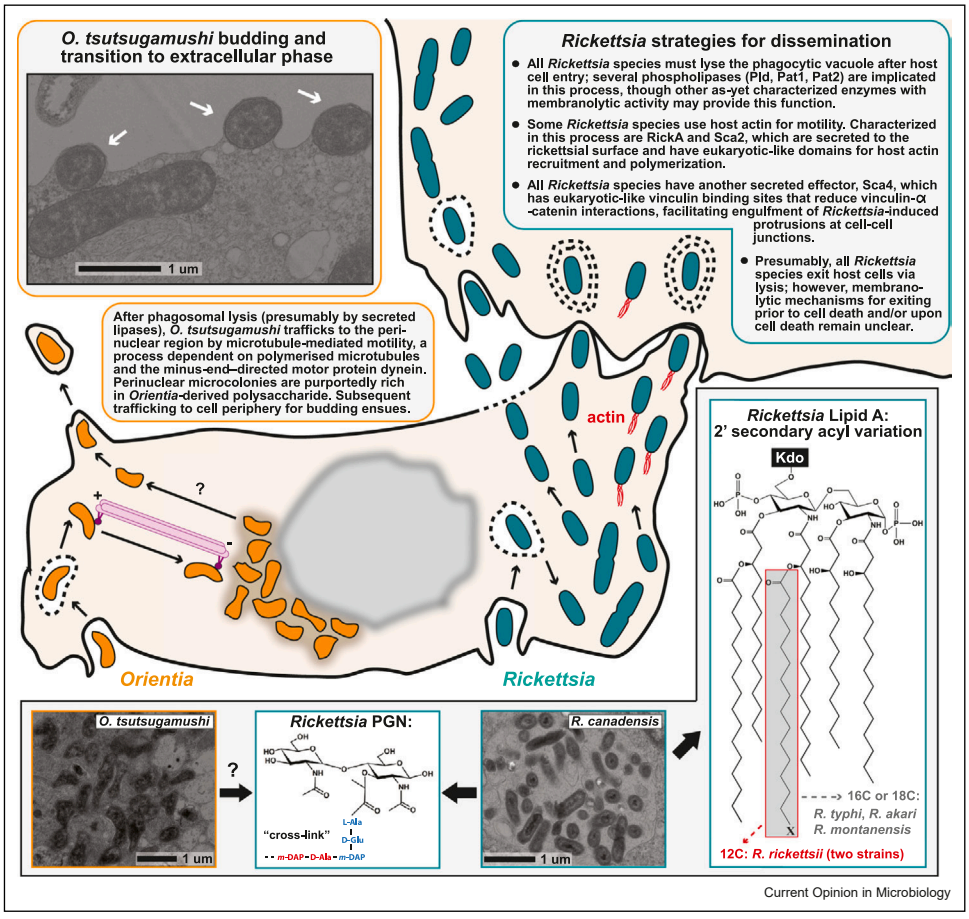
Orientia and *Rickettsia* are divergent in morphology and intracellular lifestyle

The greater phylogenetic divergence between *Orientia* and *Rickettsia* species is evinced by stark differences in gene content and genome architecture [66,67]. Some of these differences, in turn, corroborate long-established phenotypic traits that distinguish *O. tsutsugamushi* from *Rickettsia* species, namely a cell envelope lacking lipopolysaccharide (LPS) [68] and a viral-like budding mechanism of host cell exit (as opposed to host cell lysis and/or intercellular spread in *Rickettsia* spp.) [69] (Figure 2).

Recently appreciated divergent cell envelope morphologies

The presence of peptidoglycan (PGN) synthesis genes in sequenced *O. tsutsugamushi* genomes [70–72] confounded previously observed PGN-less *O. tsutsugamushi* cells. This puzzle was eventually solved by demonstration of D-alanine-labeled fluorescent structures in *O. tsutsugamushi* cells and their susceptibility to drugs targeting PGN biosynthesis, although the level of PGN is

Figure 2



Orientia and *Rickettsia* species have divergent lifestyles and cell envelope morphologies.
The divergent mechanisms utilized by *O. tsutsugamushi* (orange) and rickettsiae (teal) for host cell entry have been thoroughly described elsewhere [121,123,124]. While both microbes escape the endolysosomal pathway by lysing their phagocytic vacuoles and evade autophagy [32,40,101–107], their cytosolic behavior differs markedly elsewise. Purple, minus-end-directed dynein-dependent microtubule motility. EM image at the top shows several *O. tsutsugamushi* budding out from a host L929 cell (white arrows). Box at the bottom and left: EM images of *O. tsutsugamushi* showing highly pleiomorphic forms during cytosolic growth in a L929 cell and R. canadensis showing coccobacilli replicating in a host cell. The recently determined structure identified by nano-LC/MS for *R. typhi* PGN is shown [82] and presumed to be similar for *O. tsutsugamushi* PGN. Right, summary of *Rickettsia* lipid-A structures determined from growth in Vero cells: three (*R. typhi*, *R. akari*, and *R. montanensis*) have longer (C16/C18) 2' acyl chains (gray) compared with R. rickettsii strains Sheila Smith and Iowa (shorter C12 2' acyl chain, red) [98].

low and not sufficient to confer cell rigidity [73]. Further comparison of *O. tsutsugamushi* with Rickettsiales species harboring similar PGN biosynthesis gene profiles (*Wolbachia pipientis* and *Anaplasma marginale*), those lacking any PGN synthesis genes (*Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*), or *Rickettsia canadensis* (complete PGN synthesis pathway and observable cell wall) revealed that *O. tsutsugamushi*, *A. marginale*, and *W. pipientis* synthesize a PGN-like cell wall with minimal abundance and rigidity, yet visible after growth in media containing D-Ala clickable probes [74]. These minimal PGN-like cell walls were also detectable by host PGN receptor Nucleotide Binding Oligomerization Domain Containing 1 (NOD1) and were sensitive to drugs targeting muropeptide synthesis. Furthermore, along with three other divergent bacterial lineages (intracellular species in the *Gammaproteobacteria*, Planctomycetes–Verrucomicrobia–Chlamydiae superphylum, and Actinobacteria), species demonstrated or predicted to harbor minimal PGN-like cell walls collectively lack class-A penicillin-binding proteins (aPBPs) but contain class-B penicillin-binding proteins (bPBPs) in complex with shape, elongation, division, and sporulation (SEDS) proteins. aPBPs are bifunctional enzymes usually essential in free-living bacteria that possess both glycosyltransferase and transpeptidase activity. They are traditionally thought to be the main drivers of incorporation of lipid-II precursors into an elongating PGN sacculus, howeve,r it is now known that a complex of bPBP and SEDS proteins can carry out the same enzymatic activity [75,76]. Given that the majority of PGN-synthesizing bacteria carry both aPBP and bPBP–SEDS machineries, these collective data indicate minimal PGN-like cell walls resulting from a loss of aPBP are an evolutionary recurring feature of the intracellular lifestyle [74]. The importance and role of minimal PGN-like cell walls in certain obligate IB are unknown, but may reflect a compromise between retaining machinery required for cell division while minimizing immune response activation.

The absence of PGN or presence of minimal PGN-like cell wall in *O. tsutsugamushi* and certain Anaplasmataceae contrasts the more complete biosynthesis of PGN in *Rickettsia* species. Corroborating prior studies that utilized electron microscopy and biochemical assays to show the *Rickettsia* cell envelope contains canonical Gram-negative bacterial PGN [77–81], a recent report used Liquid chromatography–mass spectrometry (LC/MS) to generate PGN structure for numerous muropeptide configurations, including D,D cross-linked subunits [82]. This work confirmed the presence of *meso*-diaminopimelic acid (DAP) and purified entire PGN sacculi from *Rickettsia* cells, illustrating that *Rickettsia* species indeed build a typical Gram-negative bacterial PGN sacculus. Furthermore, relative to genomes of species synthesizing minimal PGN-like cell walls, *Rickettsia* genomes harbor a more complete set of PGN remodeling and turnover enzymes that is more characteristic of bacteria elaborating a murein sacculus [82,83].

Rickettsia species are also the lone Rickettsiales of human health significance that synthesize LPS, a large glycolipid composed of three structural domains: membrane-embedded lipid A, the core oligosaccharide, and surface-exposed O-antigen. Humans infected with either TG or SFG rickettsiae elicit cross-reactive antibodies that recognize LPS purified from certain TG species or SFG Rickettsiae [84–94]. While some of these foundational studies determined certain biochemical properties of the *Rickettsia* O-antigen, a later study showed that a mutant for a polysaccharide synthesis operon (*pso*) of *Rickettsia conorii* diminished S-layer formation and rendered bacteria less pathogenic yet insensitive to bactericidal antibodies targeting O-antigen [95]. The *pso* is highly variable in gene content across rickettsiae, suggesting the carbohydrate composition of O-antigen and possibly core oligosaccharide is variable at the species level. Remarkably, the structure for *R. typhi* lipid A, which was shown to elaborate longer acyl chains than the highly proinflammatory structure of *E. coli* lipid A [96,97], was representative for a diverse range of *Rickettsia* species with the notable exception of *R. rickettsii*, which produces acyl chain lengths similar to *E. coli* [98] (Figure 2). Considering the specificity (i.e. lipid-A acyl chain length, phosphate modification, etc.) of LPS detection by vertebrate immune responses [99,100], revealing the significance of *Rickettsia* LPS compositional and immunological variation is critical for understanding rickettsial-induced host immune responses.

Hijacking host cytoskeleton and the emergence of *Orientia* as shapeshifters

Diverging from Anaplasmataceae pathogens, which replicate within bacterial-modified vacuoles of phagosome origin, *O. tsutsugamushi* and *Rickettsia* species exit the endolysosomal pathway shortly after entry by lysing their phagosomes (Figure 2). Both microbes evade destruction by autophagosomes and may benefit from nutrients accumulated from these degradative structures [32,40,101–107]. Remarkably, *O. tsutsugamushi* utilizes an unknown mechanism to commandeer minus-end-directed dynein-dependent microtubule motility to traffic to perinuclear regions [108]. Replication of highly pleiomorphic bacteria occurs in 'biofilm-like' microcolonies that produce a polysaccharide of unknown composition [109]. Several '*Rickettsia*-like' glycosyltransferases and sugar hydrolases encoded in *Orientia* genomes could participate in synthesizing such carbohydrate moieties.

The factors triggering migration of *O. tsutsugamushi* to the cell periphery are unknown, though recent

progress has been made in characterizing the transition to an extracellular environment [110]. Extracellular bacteria (EB) that have budded out of host cells (Figure 2), represent a developmental stage distinct from IB. EB are largely round in morphology and comprise the majority of the budded vesicle. Furthermore, relative to IBs, EBs contain less PGN and are more sensitive to osmotic pressure, taking on characteristics more common to vacuolar rickettsial species. Curiously, while both IB (purified from host cells) and EB (purified from bud membranes) are infectious, IB entry into host cells is sensitive to inhibitors of both clathrin-mediated endocytosis and macropinocytosis, while entry of EBs is only sensitive to a macropinocytosis inhibitor.

No such developmental stages have been described for *Rickettsia* species, as bacteria are mostly rod-like and replicate throughout the host cytosol (Figure 2). Some species utilize surface-localized effectors, that is, RickA and/or Sca2, to recruit and polymerize host actin for intracellular motility [111–114]. While these effectors may contribute to intercellular spread, the more conserved effector Sca4 probably plays a greater role in this process, as it reduces mechanotransduction at cell–cell junctions [115,116]. Still, TG rickettsiae (and most likely other species) are known to disseminate via cell lysis, though the bacterial or host-induced mechanism remains unknown. Several phospholipases have been characterized for different roles during infection [32,117–119], yet, while having potential for membranolytic activity, none has been directly shown to lyse phagosome or plasma membranes during rickettsial infection. Unlike genus *Orientia*, where only recently have a few new species been described [49,50,120], genus *Rickettsia* houses dozens of recognized and provisional (*Candidatus*) species, as well as unnamed organisms assigned to the genus, most of which have complete or nearly complete genome assemblies. Correlating the distribution of dozens of characterized surface proteins and secreted effectors with the observed phenotypic traits of these numerous species (host range, cell tropism, etc.) within a phylogenomic context [121,122] is imperative for defining the limits of the *Rickettsia* lifestyle and whether or not different developmental stages occur.

Thick as thieves: remarkable metabolic parasitism

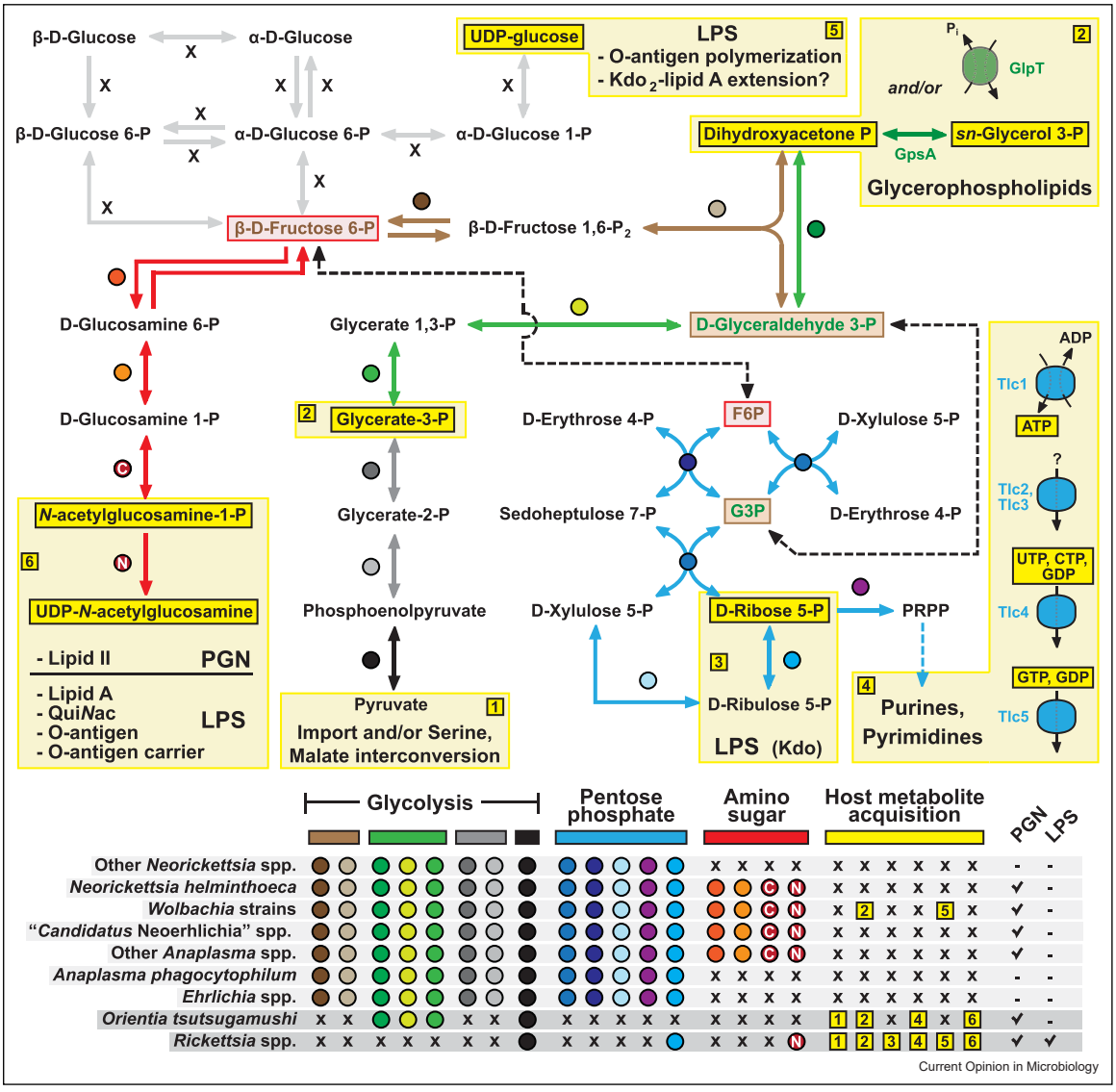
Genome sequencing has divulged key characteristics that distinguish *Orientia* and *Rickettsia* genomes from other Rickettsiales, including a greater reliance on host metabolites and the presence of a promiscuous conjugative transposon termed Rickettsiales Amplified Genetic Element (RAGE) [71,125].

Convergent evolution of glycolytic parasitism

Prior metabolomic reconstructions illuminated incomplete or absent glycolytic pathways in *Orientia* or *Rickettsia* genomes, respectively [8,67]. It was hypothesized that the presence of an adenosine triphosphate (ATP) importer facilitated the erosion of glycolytic enzymes [5]. However, the electron transport systems should suffice to supply the majority of ATP in these bacteria; further, complete (*Rickettsia*) or nearly complete (*Orientia*) tricarboxylic acid cycle (TCA) cycles, in conjunction with pyruvate dehydrogenase complexes, would sufficiently operate with host-acquired pyruvate and/or amino acids converted to pyruvate [126]. A different perspective views acquired ATP as essential for efficient DNA and RNA metabolism, along with other ribonucleotides pilfered from the host using five conserved and substrate-specific transporters [127], but ancillary for energy. Such parasitism made the pentose phosphate pathway and glycolytic arm generating fructose-6-P (F6P) expendable in *Orientia* and *Rickettsia* genomes (Figure 3). The inability to synthesize F6P prevents the generation of amino sugars, which these bacteria must acquire for the biosynthesis of cell envelope glycoconjugates (PGN and/or LPS) by mechanisms that have yet to be described. Notably, the bacteria have evolved different strategies for generating glycerophospholipids: *Orientia* species generate dihydroxyacetone P (DHAP) by predicted glycerate-3-P import, while *Rickettsia* species directly import both DHAP and glycerol-3-P [128,129]. The remaining differences between these microbes pertain to *Rickettsia* acquisition of host metabolites for biosynthesis of lipid A (ribose-5-P) and core and outer polysaccharide (Uridine diphosphate (UDP)-glucose) of LPS.

Remarkably, the revised phylogenetic framework for Rickettsiales indicates that *Orientia* species and all Anaplasmataceae species independently lost the capacity for LPS biosynthesis (Figures 1; 3). Furthermore, the collective rickettsial species synthesizing minimal PGN also converged on this cell envelope architecture numerous times, possibly indicating a common strategy that evolved within the context of the vertebrate or arthropod immune system. Considering osmoregulatory demands for intracellular colonization, *Orientia* species are rather remarkable for uniquely minimizing their cell envelope while residing vacuole-free in host cytosol. Osmotic protection in the absence of a complete PGN cell wall may be bolstered by the previously described extensive cross-linking of the abundant outer membrane protein TSA56 [73]. Moreover, this reveals how odd *Rickettsia* species are among Rickettsiales, as they are the lone group of metazoan pathogens to synthesize a canonical Gram-negative cell envelope rich in ligands of vertebrate immune receptors such as TLR2, TLR4, NOD1, and Caspase-11.

Figure 3

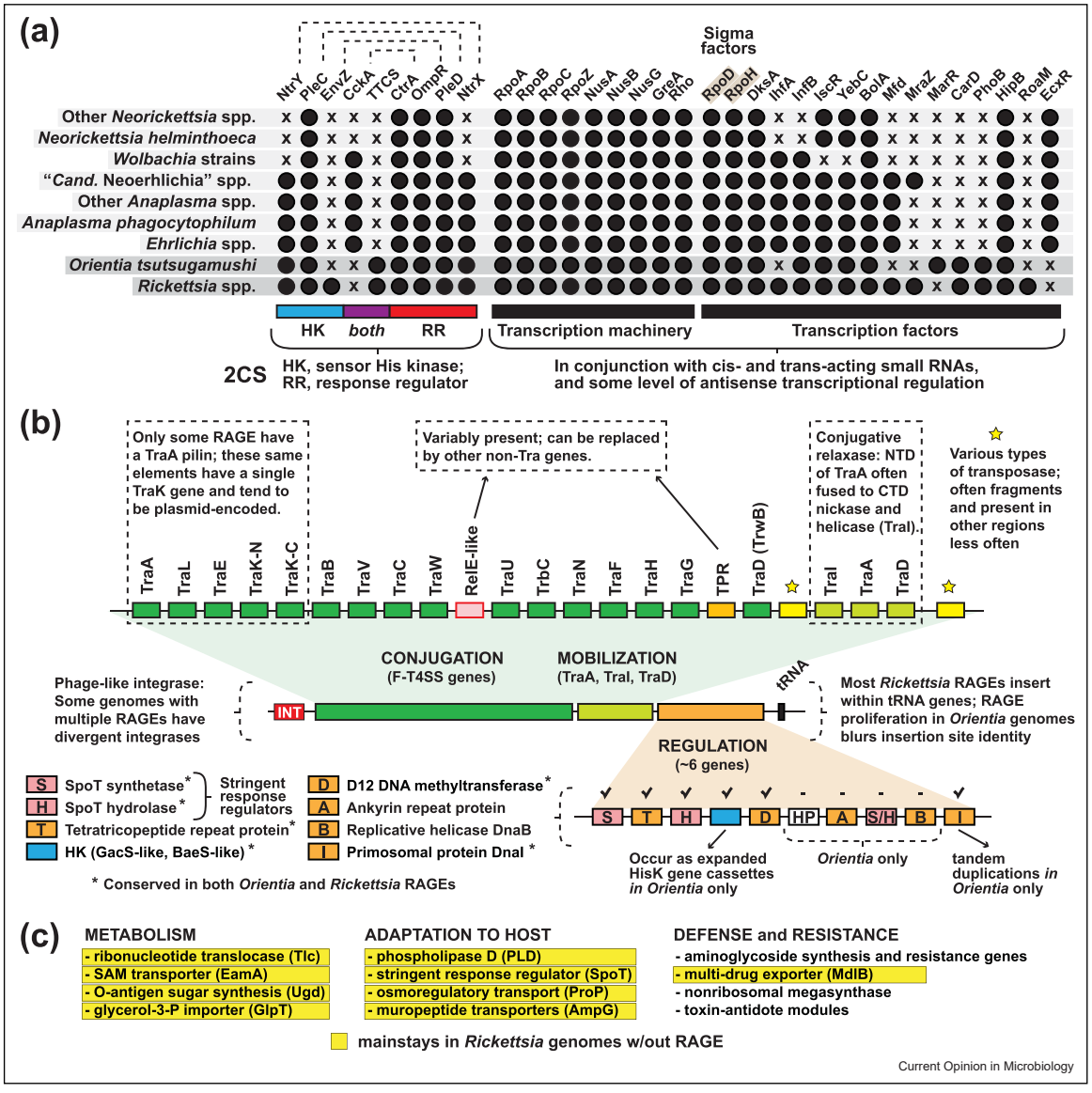


Orientia and *Rickettsia* species hijack host cell glycolysis to complement their incomplete metabolic circuitry.
Pathways for glycolysis, pentose phosphate metabolism, and amino sugar synthesis [130]. Enzymes are depicted by colored circles, which are listed below for rickettsial species encoding them (Xs denote absence of genes). Yellow highlighting depicts each point of the host metabolic network where rickettsial species acquire host metabolites, with numbers below showing pilfering mechanisms across taxa. For *Rickettsia* species, ribonucleotide [127], DHAP/glycerol-3-P [128,129], and UDP-glucose [131] import have been characterized. Other mechanisms are predicted from comparative metabolome reconstruction [126]. Wolbachiae encode enzymes for UDP-glucose metabolism and glycerol-3-P import that are products of lateral gene transfer between WO phage and Rickettsia plasmids [125].

Regulation in the intracellular environment

Whether cytosolic or vacuolar, rickettsial species require mechanisms to sense the intracellular environment, that is, pH/salinity, nutrient availability, reactive oxygen species, and density of cytosol/vacuole occupied by self or possibly other congener intracellular species (quorum sensing). Further, species capable of replicating in multiple hosts (e.g. protist, invertebrate, and vertebrate) and particularly those transmitted to vertebrates from blood-feeding invertebrates must utilize ways to distinguish between and adapt to divergent intracellular environments. Transcriptional programs seem to be controlled in part by two-component systems (2CS), for which several are encoded in rickettsial genomes [132] (Figure 4a). The distribution of sigma factors and transcriptional machinery is strictly conserved between *Orientia* and *Rickettsia*, with limited differences in 2CS and transcription. 2CSs have been best characterized in *E. chaffeensis*, which differentially utilizes three 2CSs, His kinases NtrY, PleC, and CckA that presumably signal to

Figure 4



Synopsis for genes involved in adapting to the intracellular environment. **(a)** Conservation of 2CS, transcription machinery, and transcription factor genes in select rickettsial taxa. sodium solute transporter-2CS chimeric protein (TTCS), sodium solute transporter–2CS chimeric protein. **(b)** General schema of RAGEs found in many *Rickettsia* genomes and proliferated in all *Orientia* genomes. Modified from previous reports [125,145]. Cargo genes (not shown) are typically found at numerous sites within T4SS, mobilization, or regulatory regions. **(c)** Broad functional categories for certain cargo genes found within *Rickettsia* RAGEs. Most of these genes (highlighting) are found in other regions of *Rickettsia* chromosomes and encode functions tailored to the intracellular lifestyle.

NtrX, PleD, and CtrA response regulators, to block lysosome fusion to the bacteria-enclosed vacuole [133], protect bacteria from invasion-induced host oxidative stress [134], and coordinate import of Pro and Gln from the host cytosol [135]. These and several other genes encoding His kinases and response regulators are differentially conserved across Rickettsiales species (Figure 4a), indicating variation in platforms for sensing the intracellular environment and signaling pathways for gene regulation. It is worth noting that these genes have been identified by sequence homology and the precise pairing of 2CS - components as well as their activity requires experimental verification.

While transcriptional machinery is highly conserved in Rickettsiales genomes, a survey (not exhaustive) of transcription factors indicates considerable variation in genetic regulation (Figure 4a). Two sigma factors, the housekeeping RpoD (σ70) and alternate heat shock RpoH (σ32), are highly conserved and proposed for

certain species to regulate transitions between invertebrate and vertebrate hosts [136]. While such host-specific transcriptional patterns have been shown in numerous studies on rickettsial species, exciting research on *R. conorii* indicates that transcription start sites for 75 genes are different in distinct host environments and that host background dictates 6S RNA transcription levels [137]. Most other transcription factors are variably present across Rickettsiales genomes and, in conjunction with 2CS profiles, indicate that regulatory programs are genus- (and possibly species-) specific. Furthermore, small RNAs likely play an important role in genome regulation but, thus far, have only been described for a few *Rickettsia* species and *Wolbachia* strains [138–141].

RAGE against the dying genome

While generally considered an artifact of transcription, antisense transcriptional regulation of protein synthesis may be utilized by Rickettsiales to preclude pseudogene products. Evidence for such a strategy exists for *O. tsutsugamushi*, which has a highly repetitive genome riddled with fragmented RAGEs that encode mostly pseudogenes [70,71]. Utilizing proteomics in conjunction with transcriptomics, *O. tsutsugamushi* genes expressing detectable proteins had a significantly lower antisense–sense transcript read ratio relative to genes for which protein expression was undetectable [142]. As the latter were predominantly RAGE genes, antisense RNA expression was posited to inhibit translation of the proliferated genes of RAGE fragments that are no longer functional. Why this strategy for purging nonfunctional RAGEs exists in *Orientia* genomes but not *Rickettsia* genomes, wherein RAGE degradation occurs without rampant proliferation/recombination, remains unknown.

Like other integrative and conjugative elements, complete RAGEs can be discretely partitioned into genes for conjugation, mobilization, and regulation (Figure 4b). For *Rickettsia* genomes, integrases predominantly direct insertion within tRNA genes, with many scars from RAGE insertion observable near tRNA genes even in the absence of annotated RAGE genes [125]. Few complete RAGEs are present in public assemblies, yet analyses of *R. buchneri* within deer tick populations indicate that RAGEs are highly dynamic, even within bacteria from a single tick [143]. Cargo genes, or those piggybacking on RAGEs at indiscriminate insertion sites, have a plethora of different functions but can be distinguished from genes within the regulation region by being present in *Rickettsia* genomes that lack RAGE altogether (Figure 4c). This observation led to the hypothesis that RAGEs function in shuttling genes important for intracellular survival, both replenishing genomes for critical genes that have pseudogenized and equipping genomes with new genes that provide novel traits with selective advantages [125]. Supporting this assertion, many genes for osmoregulation, stringent response, and metabolite synthesis and transport are found on different RAGEs yet always present (and more often than not) duplicated within other chromosomal regions.

For *Orientia* genomes, reconstructing complete RAGEs from the proliferated fragments has proven laborious, yet better assessments of evolutionary processes and cargo genes are now achievable with improved assemblies generated from long-read sequencing [144]. There is an expanded repertoire of RAGE-associated cargo genes compared with *Rickettsia* RAGEs, likely resulting from the explosive proliferation of these mobile elements in *Orientia* genomes. While all complete *Orientia* genomes published to date encode at least 70 discrete RAGE elements, these are mostly partially or heavily degraded. It is not yet known whether intact RAGEs still exist in any *Orientia* genomes, nor whether they remain mobilizable potentially using machinery assembled from multiple partially degraded RAGEs distributed across the genome.

Conclusion/future directions

We highlight here several underappreciated aspects of the biology of *Orientia* and *Rickettsia* species, two lineages with distinct ancestries within Rickettsiaceae. The ever-growing rickettsial diversity will continue to shed light on how such differing strategies for intracellular parasitism evolved in the context of host dependency. Correlating divergent cell envelope architectures, intracellular lifestyles, and host/cellular tropism with sensing/signaling pathways and transcriptional control is an understudied, yet essential aspect of future research on these remarkably clever intracellular pathogens of substantial human health importance.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data Availability

No data was used for the research described in the article.

Acknowledgements

We acknowledge the funding support from the National Institutes of Health, USA, R21 AI156762 and R21 AI166832 (to J.J.G.) and Wellcome Trust Senior Research Fellowship 224277/Z/21/Z (to J.S.). We are grateful to Edward Bonder from the Department of Biological Sciences, Rutgers University, Newark, New Jersey, USA, for electron microscopy imaging.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Viklund J, Ettema TJG, Andersson SGE: **Independent genome reduction and phylogenetic reclassification of the oceanic SAR11 clade**. *Mol Biol Evol* 2012, **29**:599-615.

2. Gillespie JJ, Nordberg EK, Azad AA, Sobral BW: **Phylogeny and comparative genomics: the shifting landscape in the genomics era**. In *Intracellular Pathogens II: Rickettsiales*. Edited by Azad AF, Palmer GH. American Society of Microbiology; 2012:84-141.

3. Andersson SGE, Zomorodipour A, Andersson JO, Sicheritz-Pontén T, Alsmark UCM, Podowski RM, Näslund AK, Eriksson A-SS, Winkler HH, Kurland CG: **The genome sequence of Rickettsia prowazekii and the origin of mitochondria.** *Nature* 1998, **396**:133-140.

4. Andersson JO, Andersson SG: **Insights into the evolutionary process of genome degradation**. *Curr Opin Genet Dev* 1999, **9**:664-671.

5. Fuxelius H-HH, Darby A, Min C-KK, Cho N-HH, Andersson SGE: **The genomic and metabolic diversity of Rickettsia**. *Res Microbiol* 2007, **158**:745-753.

6. Andersson JO, Andersson SG: **Genome degradation is an ongoing process in Rickettsia**. *Mol Biol Evol* 1999, **16**:1178-1191.

7. Darby AC, Cho N-H, Fuxelius H-H, Westberg J, Andersson SGE: **Intracellular pathogens go extreme: genome evolution in the Rickettsiales**. *Trends Genet* 2007, **23**:511-520.

8. Fuxelius H-H, Darby AC, Cho N-H, Andersson SG: **Visualization of pseudogenes in intracellular bacteria reveals the different tracks to gene destruction**. *Genome Biol* 2008, **9**:1-15 (2008 92).

9. • Martijn J, Vosseberg J, Guy L, Offre P, Ettema TJG: **Deep mitochondrial origin outside the sampled alphaproteobacteria**. *Nat* 2018, **557**:101-105 (2018 5577703).
Novel alphaproteobacterial lineages were identified from analysis of deep-ocean MAGs, with phylogeny estimation robustly placing the mitochondrial ancestor outside of all recognized *Alphaproteobacteria*, including Rickettsiales.

10. •• Schön ME, Martijn J, Vosseberg J, Köstlbacher S, Ettema TJG: **The evolutionary origin of host association in the Rickettsiales**. *Nat Microbiol* 2022, **7**:1189-1199 (2022 78).
Analysis of deep-ocean MAGs led to the discovery of novel lineages of Rickettsiales, with two new basal families, Mitibacteraceae and Athabascaceae, possessing genomic features characteristic of EB. This showed for the first time an origin for host dependency within the Rickettsiales that is independent of the mitochondrial ancestor.

11. Martijn J, Schulz F, Zaremba-Niedzwiedzka K, Viklund J, Stepanauskas R, Andersson SGE, Horn M, Guy L, Ettema TJG: **Single-cell genomics of a rare environmental alphaproteobacterium provides unique insights into Rickettsiaceae evolution**. *ISME J* 2015, **9**:2373-2385.

12. Schulz F, Martijn J, Wascher F, Lagkouvardos I, Kostanjšek R, Ettema TJG, Horn M: **A Rickettsiales symbiont of amoebae with ancient features**. *Environ Microbiol* 2016, **18**:2326-2342.

13. • Castelli M, Sabaneyeva E, Lanzoni O, Lebedeva N, Floriano AM, Gaiarsa S, Benken K, Modeo L, Bandi C, Potekhin A, *et al.*: **Deianiraea, an extracellular bacterium associated with the ciliate Paramecium, suggests an alternative scenario for the evolution of Rickettsiales**. *ISME J* 2019, **13**:2280-2294, https://doi.org/10.1038/s41396-019-0433-9.
The first identification of extracellular Rickettsiales, which are shown to grow and replicate extracellularly on their ciliate hosts and synthesize nearly all amino acids. A new family, Deianiraeaceae, is proposed based on phylogenomics analysis and environmental diversity.

14. Lanzoni O, Sabaneyeva E, Modeo L, Castelli M, Lebedeva N, Verni F, Schrallhammer M, Potekhin A, Petroni G: **Diversity and environmental distribution of the cosmopolitan endosymbiont "Candidatus Megaira"**. *Sci Rep* 2019, **9**:1179.

15. Sassera D, Lo N, Epis S, D'Auria G, Montagna M, Comandatore F, Horner D, Peretó J, Luciano AM, Franciosi F, *et al.*: **Phylogenomic evidence for the presence of a flagellum and cbb(3) oxidase in the free-living mitochondrial ancestor**. *Mol Biol Evol* 2011, **28**:3285-3296.

16. Giannotti D, Boscaro V, Husnik F, Vannini C, Keeling PJ: **The "Other" Rickettsiales: an overview of the family "Candidatus Midichloriaceae"**. *Appl Environ Microbiol* 2022, **88**:e0243221.

17. Salje J: **Cells within cells: Rickettsiales and the obligate intracellular bacterial lifestyle**. *Nat Rev Microbiol* 2021, **19**:375-390 (2021 196).

18. Kaur R, Shropshire JD, Cross KL, Leigh B, Mansueto AJ, Stewart V, Bordenstein SR, Bordenstein SR: **Living in the endosymbiotic world of Wolbachia: a centennial review**. *Cell Host Microbe* 2021, **29**:879-893.

19. Turco J: **Involvement of pore formation and osmotic lysis in the rapid killing of gamma interferon-pretreated C166 endothelial cells by Rickettsia prowazekii**. *Trop Med Infect Dis* 2022, **7**:163.

20. Matos AL, Curto P, Simões I: **Moonlighting in Rickettsiales: expanding virulence landscape**. *Trop Med Infect Dis* 2022, **7**:32.

21. Kim Y, Wang J, Clemens EG, Grab DJ, Dumler JS: **Anaplasma phagocytophilum Ankyrin A Protein (AnkA) enters the nucleus using an importin-β-, RanGTP-dependent mechanism**. *Front Cell Infect Microbiol* 2022, **12**:828605.

22. Zhu B, McBride JW: **Alpha enolase 1 ubiquitination and degradation mediated by ehrlichia chaffeensis trp120 disrupts glycolytic flux and promotes infection**. *Pathogens* 2021, **10**:962.

23. Rogan MR, Patterson LL, Byerly CD, Luo T, Paessler S, Veljkovic V, Quade B, McBride JW: **Ehrlichia chaffeensis TRP120 Is a Wnt ligand mimetic that interacts with Wnt receptors and contains a novel repetitive short linear motif that activates Wnt signaling**. *mSphere* 2021, **6**:e00216-21.

24. Byerly CD, Mitra S, Patterson LNL, Pittner NA, Velayutham TS, Paessler S, Veljkovic V, McBride JW: **Ehrlichia SLiM ligand mimetic activates Hedgehog signaling to engage a BCL-2 anti-apoptotic cellular program**. *PLoS Pathog* 2022, **18**:e1010345.

25. Patterson LNL, Velayutham TS, Byerly CD, Bui DC, Patel J, Veljkovic V, Paessler S, McBride JW: **Ehrlichia SLiM ligand mimetic activates notch signaling in human monocytes**. *MBio* 2022, **13**:e0007622.

26. Wang JY, Zhu B, Patterson LNL, Rogan MR, Kibler CE, McBride JW: **Ehrlichia chaffeensis TRP120-mediated ubiquitination and proteasomal degradation of tumor suppressor FBW7 increases oncoprotein stability and promotes infection**. *PLoS Pathog* 2020, **16**:e1008541.

27. Nguyen YTH, Kim C, Kim Y, Jeon K, Kim HII, Ha NY, Cho NH: **The Orientia tsutsugamushi ScaB autotransporter protein is required for adhesion and invasion of mammalian cells**. *Front Microbiol* 2021, **12**:626298.

28. Huang W, Xiong Q, Lin M, Rikihisa Y: **Anaplasma phagocytophilum hijacks flotillin and NPC1 complex to acquire intracellular cholesterol for proliferation, which can be inhibited with ezetimibe**. *MBio* 2021, **12**:e0229921.

29. Yan Q, Zhang W, Lin M, Teymournejad O, Budachetri K, Lakritz J, Rikihisa Y: **Iron robbery by intracellular pathogen via bacterial effector-induced ferritinophagy**. *Proc Natl Acad Sci USA* 2021, **118**:e2026598118.

30. Li R, Ma Z, Zheng W, Wang Z, Yi J, Xiao Y, Wang Y, Chen C: **Multiomics analyses reveals Anaplasma phagocytophilum Ats-1 induces anti-apoptosis and energy metabolism by upregulating the respiratory chain-mPTP axis in eukaryotic mitochondria**. *BMC Microbiol* (1) 2022, **22**:271.

31. •• Cull B, Burkhardt NY, Wang XR, Thorpe CJ, Oliver JD, Kurtti TJ, Munderloh UG: **The Ixodes scapularis symbiont Rickettsia buchneri inhibits growth of pathogenic Rickettsiaceae in tick cells: implications for vector competence**. *Front Vet Sci* 2022, **8**:748427, https://doi.org/10.3389/fvets.2021.748427.
The prevalent endosymbiont of the black-legged tick, *R. buchneri*, is shown to inhibit the coinfection in tick cells of the pathogen *R. parkeri*. Evidence for a bactericidal factor in *R. buchneri*-infected tick cell lysate indicates a mechanism whereby this nonpathogen precludes *I. scapularis* infection by other *Rickettsia* species.

32. Borgo GM, Burke TP, Tran CJ, Lo NTN, Engström P, Welch MD: **A patatin-like phospholipase mediates Rickettsia parkeri**

escape from host membranes. *Nat Commun* 2022, **13**:1-15 (2022 131).

33. Sanderlin AG, Hanna RE, Lamason RL: **The Ankyrin repeat protein RARP-1 is a periplasmic factor that supports Rickettsia parkeri growth and host cell invasion**. *J Bacteriol* 2022, **204**:e0018222.

34. Adcox HE, Berk JM, Hochstrasser M, Carlyon JA: **Orientia tsutsugamushi OtDUB is expressed and interacts with adaptor protein complexes during infection**. *Infect Immun* 2022, **90**:e0046922, https://doi.org/10.1128/IAI.00469-22

35. Berk JM, Lee MJ, Zhang M, Lim C, Hochstrasser M: **OtDUB from the human pathogen Orientia tsutsugamushi modulates host membrane trafficking by multiple mechanisms**. *Mol Cell Biol* 2022, **42**:e0007122.

36. Gupta S, Hunt JR, Adcox HE, Andersen SE, Gumpf JJ, Green RS, Beyer AR, Evans SM, VieBrock L, Read CB, *et al.*: **Functional characterization of non-Ankyrin repeat domains of Orientia tsutsugamushi Ank effectors reveals their importance for molecular pathogenesis**. *Infect Immun* 2022, **90**:e0062821.

37. Wangsanut T, Brann KR, Adcox HE, Carlyon JA: **Orientia tsutsugamushi modulates cellular levels of NF-κB inhibitor p105**. *PLoS Negl Trop Dis* 2021, **15**:e0009339.

38. Adcox HE, Hatke AL, Andersen SE, Gupta S, Otto NB, Marconi RT, Carlyon JA, Weber MM: **Orientia tsutsugamushi nucleomodulin Ank13 exploits the RaDAR nuclear import pathway to modulate host cell transcription**. *MBio* 2021, **12**:e0181621.

39. Fisher J, Card G, Liang Y, Trent B, Rosenzweig H, Soong L: **Orientia tsutsugamushi selectively stimulates the C-type lectin receptor Mincle and type 1-skewed proinflammatory immune responses**. *PLoS Pathog* 2021, **17**:e1009782.

40. Engström P, Burke TP, Tran CJ, Iavarone AT, Welch MD: **Lysine methylation shields an intracellular pathogen from ubiquitylation and autophagy**. *Sci Adv* 2021, **7**:eabg2517.

41. Jordan JM, Woods ME, Soong L, Walker DH: **Rickettsiae stimulate dendritic cells through toll-like receptor 4, leading to enhanced NK cell activation in vivo**. *J Infect Dis* 2009, **199**:236-242.

42. Mendell NL, Xu G, Shelite TR, Bouyer DH, Walker DH: **A murine model of waning scrub typhus cross-protection between heterologous strains of Orientia tsutsugamushi**. *Pathogens* 2022, **11**:512.

43. Liang Y, Fisher J, Gonzales C, Trent B, Card G, Sun J, Tumanov AV, Soong L: **Distinct role of TNFR1 and TNFR2 in protective immunity against Orientia tsutsugamushi infection in mice**. *Front Immunol* 2022, **13**:867924, https://doi.org/10.3389/fimmu.2022.867924

44. Petermann M, Orfanos Z, Sellau J, Gharaibeh M, Lotter H, Fleischer B, Keller C: **CCR2 deficiency impairs Ly6Clo and Ly6Chi monocyte responses in Orientia tsutsugamushi infection**. *Front Immunol* 2021, **12**:670219.

45. Luce-Fedrow A, Chattopadhyay S, Chan TC, Pearson G, Patton JB, Richards AL: **Comparison of lethal and nonlethal mouse models of Orientia tsutsugamushi infection reveals T-cell population-associated cytokine signatures correlated with lethality and protection**. *Trop Med Infect Dis* 2021, **6**:121.

46. Inthawong M, Sunyakumthorn P, Wongwairot S, Anantatat T, Dunachie SJ, Im-Erbsin R, Jones JW, Mason CJ, Lugo LA, Blacksell SD, *et al.*: **A time-course comparative clinical and immune response evaluation study between the human pathogenic Orientia tsutsugamushi strains: Karp and Gilliam in a rhesus macaque (Macaca mulatta) model**. *PLoS Negl Trop Dis* 2022, **16**:e0010611.

47. Kang SJ, Park KJ, Jin HM, Cho YN, Oh TH, Kim SE, Kim UJ, Park KH, Jung SI, Kim TO, *et al.*: **Circulating plasmacytoid and conventional dendritic cells are numerically and functionally deficient in patients with scrub typhus**. *Front Immunol* 2021, **12**:700755.

48. Verhoeve VI, Gillespie JJ: **Origin of rickettsial host dependency unravelled**. *Nat Microbiol* 2022, **7**:1110-1111 (2022 78).

49. Izzard L, Fuller A, Blacksell SD, Paris DH, Richards AL, Aukkanit N, Nguyen C, Jiang J, Fenwick S, Day NPJ, *et al.*: **Isolation of a novel *Orientia* species (*O. chuto* sp. nov.) from a patient infected in Dubai**. *J Clin Microbiol* 2010, **48**:4404-4409.

50. Abarca K, Martínez-Valdebenito C, Angulo J, Jiang J, Farris CM, Richards AL, Acosta-Jamett G, Weitzel T: **Molecular description of a novel Orientia species causing scrub typhus in Chile**. *Emerg Infect Dis* 2020, **26**:2148-2156.

51. Verhoeve VI, Fauntleroy TD, Risteen RG, Driscoll TP, Gillespie JJ: **Cryptic genes for interbacterial antagonism distinguish Rickettsia species infecting blacklegged ticks from other Rickettsia pathogens**. *Front Cell Infect Microbiol* 2022, **12**:880813, https://doi.org/10.3389/fcimb.2022.880813

52. Tamura A, Ohashi N, Urakami H, Miyamura S: **Classification of Rickettsia tsutsugamushi in a new genus, Orientia gen. nov., as Orientia tsutsugamushi comb. nov**. *Int J Syst Bacteriol* 1995, **45**:589-591.

53. Muñoz-Gómez SA, Hess S, Burger G, Franz Lang B, Susko E, Slamovits CH, Roger AJ: **An updated phylogeny of the alphaproteobacteria reveals that the parasitic rickettsiales and holosporales have independent origins**. *Elife* 2019, **8**:e42535.

54. Yurchenko T, Ševčíková T, Přibyl P, El Karkouri K, Klimeš V, Amaral R, Zbránková V, Kim E, Raoult D, Santos LMA, *et al.*: **A gene transfer event suggests a long-term partnership between eustigmatophyte algae and a novel lineage of endosymbiotic bacteria**. *ISME J* 2018, **12**:2163-2175.

55. Hunter ES, Paight C, Lane CE: **Metabolic contributions of an alphaproteobacterial endosymbiont in the apicomplexan Cardiosporidium cionae**. *Front Microbiol* 2020, **11**:580719.

56. Tashyreva D, Prokopchuk G, Votýpka J, Yabuki A, Horák A, Lukeš J: **Life cycle, ultrastructure, and phylogeny of new diplonemids and their endosymbiotic bacteria**. *MBio* 2018, **9**:e02447-17.

57. Prokopchuk G, Tashyreva D, Yabuki A, Horák A, Masařová P, Lukeš J: **Morphological, ultrastructural, motility and evolutionary characterization of two new hemistasiidae species**. *Protist* 2019, **170**:259-282.

58. • Castelli M, Lanzoni O, Nardi T, Lometto S, Modeo L, Potekhin A, Sassera D, Petroni G: **'Candidatus Sarmatiella mevalonica' endosymbiont of the ciliate Paramecium provides insights on evolutionary plasticity among Rickettsiales**. *Environ Microbiol* 2021, **23**:1684-1701.
This ciliate-associated Rickettsiaceae species contains the mevalonate (MEV) pathway for isoprenoid biosynthesis, a first for Rickettsiales. While rare in bacteria, MEV is a common pathway in eukaryotes, indicating acquisition via lateral gene transfer and a possible mechanism for generating acetyl-CoA from acetoacetyl-CoA. Phylogeny estimation places '*Cand*. Sarmatiella mevalonica' between *Rickettsia* and *Orientia* clades, illustrating that these two genera are no longer sister taxa.

59. Schrallhammer M, Ferrantini F, Vannini C, Galati S, Schweikert M, Görtz H-D, Verni F, Petroni G: **"Candidatus Megaira polyxenophila" gen. nov., sp. nov.: considerations on evolutionary history, host range and shift of early divergent rickettsiae**. *PLoS One* 2013, **8**:e72581.

60. Park E, Poulin R: **Widespread Torix Rickettsia in New Zealand amphipods and the use of blocking primers to rescue host COI sequences**. *Sci Rep* 2020, **10**:1-13 (2020 101).

61. Pilgrim J, Ander M, Garros C, Baylis M, Hurst GDD, Siozios S: **Torix group Rickettsia are widespread in Culicoides biting midges (Diptera: Ceratopogonidae), reach high frequency and carry unique genomic features**. *Environ Microbiol* 2017, **19**:4238-4255.

62. Thongprem P, Davison HR, Thompson DJ, Lorenzo-Carballa MO, Hurst GDD: **Incidence and diversity of Torix Rickettsia–Odonata symbioses**. *Micro Ecol* 2020, **81**:203-212 (2020 811).

63. Wang H-L, Lei T, Wang X-W, Maruthi MN, Zhu D-T, Cameron SL, Rao Q, Shan H-W, Colvin J, Liu Y-Q, *et al.*: **A newly recorded Rickettsia of the Torix group is a recent intruder and an**

endosymbiont in the whitefly Bemisia tabaci. *Environ Microbiol* 2020, **22**:1207-1221.

64. Pilgrim J, Thongprem P, Davison HR, Siozios S, Baylis M, Zakharov EV, Ratnasingham S, deWaard JR, Macadam CR, Smith MA, *et al.*: **Torix Rickettsia are widespread in arthropods and reflect a neglected symbiosis**. *Gigascience* 2021, **10**:giab021.

65. Davison HR, Pilgrim J, Wybouw N, Parker J, Pirro S, Hunter-Barnett S, Campbell PM, Blow F, Darby AC, Hurst GDD, *et al.*: **Genomic diversity across the Rickettsia and 'Candidatus Megaira' genera and proposal of genus status for the Torix group**. *Nat Commun* 2022, **13**:1-14 (2022 131).

66. Eremeeva ME, Madan A, Shaw CD, Tang K, Dasch GA: **New perspectives on rickettsial evolution from new genome sequences of rickettsia, particularly R. canadensis, and Orientia tsutsugamushi**. *Ann N Y Acad Sci* 2005, **1063**:47-63.

67. Min C-K, Yang J-S, Kim S, Choi M-S, Kim I-S, Cho N-H: **Genome-based construction of the metabolic pathways of *Orientia tsutsugamushi* and comparative analysis within the rickettsiales order**. *Comp Funct Genom* 2008, **2008**:1-14.

68. Silverman DJ, Wisseman CL, Waddell A: **In vitro studies of Rickettsia-host cell interactions: ultrastructural study of Rickettsia prowazekii-infected chicken embryo fibroblasts**. *Infect Immun* 1980, **29**:778-790.

69. Rikihisa Y, Ito S: **Localization of electron-dense tracers during entry of Rickettsia tsutsugamushi into polymorphonuclear leukocytes**. *Infect Immun* 1980, **30**:231-243.

70. Cho N-H, Kim H-R, Lee J-H, Kim S-Y, Kim J, Cha S, Kim S-Y, Darby AC, Fuxelius H-H, Yin J, *et al.*: **The Orientia tsutsugamushi genome reveals massive proliferation of conjugative type IV secretion system and host cell interaction genes**. *Proc Natl Acad Sci* 2007, **104**:7981-7986.

71. Nakayama K, Yamashita A, Kurokawa K, Morimoto T, Ogawa M, Fukuhara M, Urakami H, Ohnishi M, Uchiyama I, Ogura Y, *et al.*: **The Whole-genome sequencing of the obligate intracellular bacterium Orientia tsutsugamushi revealed massive gene amplification during reductive genome evolution**. *DNA Res* 2008, **15**:185-199.

72. Nakayama K, Kurokawa K, Fukuhara M, Urakami H, Yamamoto S, Yamazaki K, Ogura Y, Ooka T, Hayashi T: **Genome comparison and phylogenetic analysis of Orientia tsutsugamushi strains**. *DNA Res* 2010, **17**:281-291.

73. Atwal S, Giengkam S, Chaemchuen S, Dorling J, Kosaisawe N, VanNieuwenhze M, Sampattavanich S, Schumann P, Salje J: **Evidence for a peptidoglycan-like structure in *Orientia tsutsugamushi***. *Mol Microbiol* 2017, **105**:440-452.

74. •• Atwal S, Chuenklin S, Bonder EM, Flores J, Gillespie JJ, Driscoll TP, Salje J: **Discovery of a diverse set of bacteria that build their cell walls without the canonical peptidoglycan polymerase apbp**. *MBio* 2021, **12**:e0134221.

Comparative genomics revealed that the normally essential PGN polymerase aPBP has been lost in multiple lineages of host-associated bacteria. Experiments demonstrated that this led to formation of a cell wall reduced in abundance and structural rigidity in three Rickettsiales species. The authors hypothesize that the loss of aPBP results in a minimal cell wall-like structure that is beneficial for obligate intracellular growth.

75. Cho H, Wivagg CN, Kapoor M, Barry Z, Rohs PDA, Suh H, Marto JA, Garner EC, Bernhardt TG: **Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously**. *Nat Microbiol* 2016, **1**:1-8 (2016 110).

76. Emami K, Guyet A, Kawai Y, Devi J, Wu LJ, Allenby N, Daniel RA, Errington J: **RodA as the missing glycosyltransferase in Bacillus subtilis and antibiotic discovery for the peptidoglycan polymerase pathway**. *Nat Microbiol* 2017, **2**:1-9 (2017 23).

77. Pang H, Winkler HH: **Analysis of the peptidoglycan of Rickettsia prowazekii**. *J Bacteriol* 1994, **176**:923-926.

78. Smith DK, Winkler HH: **Separation of inner and outer membranes of Rickettsia prowazeki and characterization of their polypeptide compositions**. *J Bacteriol* 1979, **137**:963-971.

79. Popov VL, Dyuisalieva RG, Smirnova NS, Tarasevich IV, Rybkin NN: **Ultrastructure of Rickettsia sibirica during interaction with the host cell**. *Acta Virol* 1986, **30**:494-498.

80. Silverman DJ, Wisseman CL: **Comparative ultrastructural study on the cell envelopes of Rickettsia prowazekii, Rickettsia rickettsii, and Rickettsia tsutsugamushi**. *Infect Immun* 1978, **21**:1020-1023.

81. Wisseman CL, Silverman DJ, Waddell A, Brown DT: **Penicillin-induced unstable intracellular formation of spheroplasts by rickettsiae**. *J Infect Dis* 1982, **146**:147-158.

82. Oyler BL, Salje J, Rennoll-Bankert KE, Verhoeve VI, Rahman MS, Skerry C, Azad AF, Goodlett DR, Gillespie JJ: **Evidence for canonical Gram-negative bacterial peptidoglycan structure and murein sacculi in Rickettsia pathogens**. *bioRxiv* 2022, https://doi.org/10.1101/2022.03.10.483817

83. Figueroa-Cuilan WM, Irazoki O, Feeley M, Smith E, Nguyen T, Cava F, Goley ED: **Quantitative analysis of morphogenesis and growth dynamics in an obligate intracellular bacterium**. *Mol Biol Cell* 2023, https://doi.org/10.1091/mbc.E23-01-0023 mbcE23010023.

84. Fumarola D, Munno I, Monno R, Miragliotta G: **Lipopolysaccharides from Rickettsiaceae: limulus endotoxin assay and pathogenetic mediators in rickettsiosis**. *Acta Virol* (2) 1980, **24**:155.

85. Teysseire N, Raoult D: **Comparison of Western immunoblotting and microimmunofluorescence for diagnosis of Mediterranean spotted fever**. *J Clin Microbiol* 1992, **30**:455-460.

86. Amano KI, Williams JC, Dasch GA: **Structural properties of lipopolysaccharides from Rickettsia typhi and Rickettsia prowazekii and their chemical similarity to the lipopolysaccharide from Proteus vulgaris OX19 used in the Weil-Felix test**. *Infect Immun* 1998, **66**:923-926.

87. Uchiyama T, Uchida T, Wen JW, Walker DH: **Demonstration of heat-labile and heat-stable epitopes of Rickettsia japonica on ultrathin sections**. *Lab Invest* 1994, **71**:432-437.

88. Uchiyama T, Zhao L, Yan Y, Uchida T: **Cross-reactivity of Rickettsia japonica and Rickettsia typhi demonstrated by immunofluorescence and Western immunoblotting**. *Microbiol Immunol* 1995, **39**:951-957.

89. Schramek S, Brezina R, Kazár J: **Some biological properties of an endotoxic lipopolysaccharide from the typhus group rickettsiae**. *Acta Virol* 1977, **21**:439-441.

90. Ziolkowski A, Shashkov AS, Swierzko AS, Senchenkova SN, Toukach FV, Cedzynski M, Amano KI, Kaca W, Knirel YA: **Structures of the O-antigens of Proteus bacilli belonging to OX group (serogroups O1-O3) used in Weil-Felix test**. *FEBS Lett* 1997, **411**:221-224.

91. Fodorová M, Vadovic P, Skultety L, Slaba K, Toman R: **Structural features of lipopolysaccharide from Rickettsia typhi: the causative agent of endemic typhus**. *Ann N Y Acad Sci* 2005, **1063**:259-260.

92. Peturova M, Vitiazeva V, Toman R: **Structural features of the O-antigen of Rickettsia typhi, the etiological agent of endemic typhus**. *Acta Virol* 2015, **59**:228-233.

93. Jordan JM, Woods ME, Olano J, Walker DH: **The absence of toll-like receptor 4 signaling in C3H/HeJ mice predisposes them to overwhelming rickettsial infection and decreased protective Th1 responses**. *Infect Immun* 2008, **76**:3717-3724.

94. Amano K, Fujita M, Suto T: **Chemical properties of lipopolysaccharides from spotted fever group rickettsiae and their common antigenicity with lipopolysaccharides from Proteus species**. *Infect Immun* 1993, **61**:4350-4355.

95. Kim HK, Premaratna R, Missiakas DM, Schneewind O: **Rickettsia conorii O antigen is the target of bactericidal Weil-Felix antibodies**. *Proc Natl Acad Sci USA* 2019, **116**:19659-19664.

96. Fodorová M, Vadovič P, Toman R: **Structural features of lipid A of Rickettsia typhi**. *Acta Virol* 2011, **55**:31-44.

97. Guillotte ML, Gillespie JJ, Chandler CE, Rahman MS, Ernst RK, Azad AF: ***Rickettsia* lipid A biosynthesis utilizes the late acyltransferase LpxJ for secondary fatty acid addition**. *J Bacteriol* 2018, **200**:e00334-18.

98. •• Guillotte ML, Chandler CE, Verhoeve VI, Gillespie JJ, Driscoll TP, Rahman MS, Ernst RK, Azad AF: **Lipid A structural divergence in Rickettsia pathogens**. *mSphere* 2021, **6**:e00184-21.
Lipid-A structures from three human pathogens (*R. akari*, *R. typhi*, and *R. rickettsii*) and a nonpathogen (*R. montanensis*) are hexa-acylated and bisphosphorylated; however, the secondary fatty acid palmitate (C16) or stearate (C18) on the hydroxypalmitate (C16-OH) at the 2' position in *R. akari*, *R. typhi*, and *R. montanensis* lipid A was found as a laurate (C12) in *R. rickettsii*. This result indicates rickettsiae vary in membrane structure and inflammatory potential in vertebrates.

99. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O: **The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex**. *Nature* 2009, **458**:1191-1195.

100. Miller SI, Ernst RK, Bader MW: **LPS, TLR4 and infectious disease diversity**. *Nat Rev Microbiol* 2005, **3**:36-46.

101. Voss OH, Gillespie JJ, Lehman SS, Rennoll SA, Beier-Sexton M, Rahman MS, Azad AF: **Risk1, a phosphatidylinositol 3-kinase effector, promotes rickettsia typhi intracellular survival**. *MBio* 2020, **11**:1-22.

102. Engström P, Burke TP, Mitchell G, Ingabire N, Mark KG, Golovkine G, Iavarone AT, Rape M, Cox JS, Welch MD: **Evasion of autophagy mediated by Rickettsia surface protein OmpB is critical for virulence**. *Nat Microbiol* 2019, **4**:2538-2551.

103. Bechelli J, Rumfield CS, Walker DH, Widen S, Khanipov K, Fang R: **Subversion of host innate immunity by Rickettsia australis via a modified autophagic response in macrophages**. *Front Immunol* 2021, **12**:638469, https://doi.org/10.3389/fimmu.2021.638469

104. Bechelli J, Vergara L, Smalley C, Buzhdygan TP, Bender S, Zhang W, Liu Y, Popov VL, Wang J, Garg N, *et al.*: **Atg5 supports Rickettsia australis infection in macrophages in vitro and in vivo**. *Infect Immun* 2019, **87**:e00651-18.

105. Uchiyama T, Kishi M, Ogawa M: **Restriction of the growth of a nonpathogenic spotted fever group rickettsia**. *FEMS Immunol Med Microbiol* 2012, **64**:42-47.

106. Ko Y, Choi JH, Ha NY, Kim IS, Cho NH, Choi MS: **Active escape of Orientia tsutsugamushi from cellular autophagy**. *Infect Immun* 2013, **81**:552-559.

107. Choi JH, Cheong TC, Ha NY, Ko Y, Cho CH, Jeon JH, So I, Kim IK, Choi MS, Kim IS, *et al.*: **Orientia tsutsugamushi subverts dendritic cell functions by escaping from autophagy and impairing their migration**. *PLoS Negl Trop Dis* 2013, **7**:e1981.

108. Kim SW, Ihn KS, Han SH, Seong SY, Kim IS, Choi MS: **Microtubule- and dynein-mediated movement of Orientia tsutsugamushi to the microtubule organizing center**. *Infect Immun* 2001, **69**:494-500.

109. Lee SM, Kim MK, Kim MJ, Kang JS: **Novel polysaccharide antigen of Orientia tsutsugamushi revealed by a monoclonal antibody**. *FEMS Microbiol Lett* 2009, **297**:95-100.

110. • Atwal S, Wongsantichon J, Giengkam S, Saharat K, Pittayasathornthun YJ, Chuenklin S, Wang LC, Chung T, Huh H, Lee SH, *et al.*: **The obligate intracellular bacterium Orientia tsutsugamushi differentiates into a developmentally distinct extracellular state**. *Nat Commun* 2022, **13**:1-12 (2022 131).
In this paper, it was shown that *Orientia* differentiates into an extracellular form that is distinct from its intracellular counterpart in shape, protein composition, metabolic activity, and peptidoglycan level. This was the first example of a developmental cycle in a cytoplasmic Rickettsiales.

111. Reed SCO, Lamason RL, Risca VI, Abernathy E, Welch MD: **Rickettsia actin-based motility occurs in distinct phases mediated by different actin nucleators**. *Curr Biol* 2014, **24**:98-103.

112. • Nock AM, Clark TR, Hackstadt T: **Regulator of actin-based motility (RoaM) downregulates actin tail formation by Rickettsia rickettsii and is negatively selected in mammalian cell culture**. *MBio* 2022, **13**:e0035322.
Identification of a factor, RoaM, in the rickettsial cytoplasm that negatively regulates actin-based motility in *R. rickettsii*. A *roaM* mutant increased actin tail formation relative to the wild-type strain but did not increase virulence in guinea pigs. *roaM* is often pseudogenized in highly passaged laboratory strains, indicating serial passage of rickettsial strains in cell culture may eliminate essential genes lacking environmental-selective pressure.

113. Kleba B, Clark TR, Lutter EI, Ellison DW, Hackstadt T: **Disruption of the Rickettsia rickettsii Sca2 autotransporter inhibits actin-based motility**. *Infect Immun* 2010, **78**:2240-2247.

114. Gouin E, Egile C, Dehoux P, Villiers V, Adams J, Gertler F, Li R, Cossart P: **The RickA protein of Rickettsia conorii activates the Arp2/3 complex**. *Nature* 2004, **427**:457-461.

115. Park H, Lee JH, Gouin E, Cossart P, Izard T: **The rickettsia surface cell antigen 4 applies mimicry to bind to and activate vinculin**. *J Biol Chem* 2011, **286**:35096-35103.

116. Lamason RL, Bastounis E, Kafai NM, Serrano R, del Álamo JC, Theriot JA, Welch MD: **Rickettsia Sca4 reduces vinculin-mediated intercellular tension to promote spread**. *Cell* 2016, **167**:670-683 e10..

117. Whitworth T, Popov VL, Yu XJ, Walker DH, Bouyer DH: **Expression of the Rickettsia prowazekii pld or tlyC gene in Salmonella enterica serovar typhimurium mediates phagosomal escape**. *Infect Immun* 2005, **73**:6668-6673.

118. Rahman MS, Ammerman NC, Sears KT, Ceraul SM, Azad AF: **Functional characterization of a phospholipase A2 homolog from Rickettsia typhi**. *J Bacteriol* 2010, **192**:3294-3303.

119. Rahman MS, Gillespie JJ, Kaur SJ, Sears KT, Ceraul SM, Beier-Sexton M, Azad AF: **Rickettsia typhi possesses phospholipase A2 enzymes that are involved in infection of host cells**. *PLoS Pathog* 2013, **9**:e1003399.

120. Weitzel T, Aylwin M, Martínez-Valdebenito C, Jiang J, Munita JM, Thompson L, Abarca K, Richards AL: **Imported scrub typhus: first case in South America and review of the literature**. *Trop Dis Travel Med Vaccin* (10) 2018, **4**, https://doi.org/10.1186/s40794-018-0070-8

121. Gillespie JJ, Kaur SJ, Sayeedur Rahman M, Rennoll-Bankert K, Sears KT, Beier-Sexton M, Azad AF: **Secretome of obligate intracellular Rickettsia**. *FEMS Microbiol Rev* 2015, **39**:47-80.

122. Verhoeve VI, Lehman SS, Driscoll TP, Beckmann JF, Gillespie JJ: **Metagenome diversity illuminates origins of pathogen effectors**. *bioRxiv* 2023, https://doi.org/10.1101/2023.02.26.530123

123. Ge Y, Rikihisa Y: **Subversion of host cell signaling by Orientia tsutsugamushi**. *Microbes Infect* 2011, **13**:638-648.

124. Rennoll-Bankert KE, Rahman MS, Gillespie JJ, Guillotte ML, Kaur SJ, Lehman SS, Beier-Sexton M, Azad AF: **Which way in? The RalF Arf-GEF orchestrates rickettsia host cell invasion**. *PLoS Pathog* 2015, **11**:e1005115.

125. Gillespie JJ, Joardar V, Williams KP, Driscoll TP, Hostetler JB, Nordberg E, Shukla M, Walenz B, Hill CA, Nene VM, *et al.*: **A Rickettsia genome overrun by mobile genetic elements provides insight into the acquisition of genes characteristic of an obligate intracellular lifestyle**. *J Bacteriol* 2012, **194**:376-394.

126. Driscoll TP, Verhoeve VI, Guillotte ML, Lehman SS, Rennoll SA, Beier-Sexton M, Rahman MS, Azad AF, Gillespie JJ: **Wholly *Rickettsia* ! reconstructed metabolic profile of the quintessential bacterial parasite of eukaryotic cells**. *MBio* 2017, **8**:e00859-17.

127. Audia JP, Winkler HH: **Study of the five Rickettsia prowazekii proteins annotated as ATP/ADP translocases (Tlc): only Tlc1 transports ATP/ADP, while Tlc4 and Tlc5 transport other ribonucleotides**. *J Bacteriol* 2006, **188**:6261-6268.

128. Frohlich KM, Roberts RAW, Housley NA, Audia JP: **Rickettsia prowazekii uses an sn-glycerol-3-phosphate dehydrogenase and a novel dihydroxyacetone phosphate transport system to supply triose phosphate for phospholipid biosynthesis**. *J Bacteriol* 2010, **192**:4281-4288.

129. Frohlich KM, Audia JP: **Dual mechanisms of metabolite acquisition by the obligate intracytosolic pathogen Rickettsia prowazekii reveal novel aspects of triose phosphate transport**. *J Bacteriol* 2013, **195**:3752-3760.

130. Kanehisa M, Sato Y, Morishima K: **BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences**. *J Mol Biol* 2016, **428**:726-731.

131. Winkler HH, Daugherty RM: **Acquisition of glucose by Rickettsia prowazekii through the nucleotide intermediate uridine 5'-diphosphoglucose**. *J Bacteriol* 1986, **167**:805-808.

132. Cheng Z, Kumagai Y, Lin M, Zhang C, Rikihisa Y: **Intra-leukocyte expression of two-component systems in Ehrlichia chaffeensis and Anaplasma phagocytophilum and effects of the histidine kinase inhibitor closantel**. *Cell Microbiol* 2006, **8**:1241-1252.

133. Kumagai Y, Cheng Z, Lin M, Rikihisa Y: **Biochemical activities of three pairs of Ehrlichia chaffeensis two-component regulatory system proteins involved in inhibition of lysosomal fusion**. *Infect Immun* 2006, **74**:5014-5022.

134. Yan J, Liang Q, Chai Z, Duan N, Li X, Liu Y, Yang N, Li M, Jin Y, Bai F, *et al*.: **Glutathione synthesis regulated by CtrA protects Ehrlichia chaffeensis from host cell oxidative stress**. *Front Microbiol* 2022, **13**:846488.

135. Cheng Z, Lin M, Rikihisa Y: **Ehrlichia chaffeensis proliferation begins with NtrY/NtrX and PutA/GlnA upregulation and CtrA degradation induced by proline and glutamine uptake**. *MBio* 2014, **5**:e02141.

136. Liu H, Von Ohlen T, Cheng C, Faburay B, Ganta RR: **Transcription of Ehrlichia chaffeensis genes is accomplished by RNA polymerase holoenzyme containing either Sigma 32 or Sigma 70**. *PLoS One* 2013, **8**:e81780.

137. Narra H, Sahni A, Alsing J, Schroeder C, Golovko G, Nia A, Fofanov Y, Khanipov K, Sahni S: **Comparative transcriptomic analysis of Rickettsia conorii during in vitro infection of human and tick host cells**. *BMC Genom* 2020, **21**:665, https://doi.org/10.1186/s12864-020-07077-w

138. Schroeder CLC, Narra HP, Rojas M, Sahni A, Patel J, Khanipov K, Wood TG, Fofanov Y, Sahni SK: **Bacterial small RNAs in the genus Rickettsia**. *BMC Genom* 2015, **16**:1-18 (2015 161).

139. Schroeder CLC, Narra HP, Sahni A, Rojas M, Khanipov K, Patel J, Shah R, Fofanov Y, Sahni SK: **Identification and characterization of novel small RNAs in Rickettsia prowazekii**. *Front Microbiol* 2016, **7**:859.

140. Narra HP, Schroeder CLC, Sahni A, Rojas M, Khanipov K, Fofanov Y, Sahni SK: **Small regulatory RNAs of Rickettsia conorii**. *Sci Rep* 2016, **6**:1-16 (2016 61).

141. Mayoral JG, Hussain M, Albert Joubert D, Iturbe-Ormaetxe I, O'Neill SL, Asgari S: **Wolbachia small noncoding RNAs and their role in cross-kingdom communications**. *Proc Natl Acad Sci USA* 2014, **111**:18721-18726.

142. • Mika-Gospodorz B, Giengkam S, Westermann AJ, Wongsantichon J, Kion-Crosby W, Chuenklin S, Wang LC, Sunyakumthorn P, Sobota RM, Subbian S, *et al*.: **Dual RNA-seq of Orientia tsutsugamushi informs on host-pathogen interactions for this neglected intracellular human pathogen**. *Nat Commun* 2020, **11**:1-14 (2020 111).

The first use of dual RNAseq in any Rickettsiales, this paper revealed transcript levels of two different strains of *Orientia tsutsugamushi* in cultured human unbilical vein endothelial cells (HUVECs). Differences in virulence in a mouse model correlated with differences in induction of host cell immune response genes. Antisense transcription was demonstrated predominantly in RAGE genes.

143. Hagen R, Verhoeve VI, Gillespie JJ, Driscoll TP: **Conjugative transposons and their cargo genes vary across natural populations of Rickettsia buchneri infecting the tick Ixodes scapularis**. *Genome Biol Evol* 2018, **10**:3218-3229.

144. •• Batty EM, Chaemchuen S, Blacksell S, Richards AL, Paris D, Bowden R, Chan C, Lachumanan R, Day N, Donnelly P, *et al*.: **Long-read whole genome sequencing and comparative analysis of six strains of the human pathogen Orientia tsutsugamushi**. *PLoS Negl Trop Dis* 2018, **12**:e0006566.

Orientia tsutsugamushi has the most highly repetitive bacterial genome published to date, hampering efforts to generate complete genome sequences of this organism. This report demonstrates the use of PacBio long-read sequencing to generate single-contig closed genomes of *Orientia tsutsugamushi*, and contributed complete genomes of six divergent strains bringing the total number of available *Orientia* genomes to eight.

145. Gillespie JJ, Driscoll TP, Verhoeve VI, Utsuki T, Husseneder C, Chouljenko VN, Azad AF, Macaluso KR: **Genomic diversification in strains of Rickettsia felis Isolated from different arthropods**. *Genome Biol Evol* 2015, **7**:35-56.