



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

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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.

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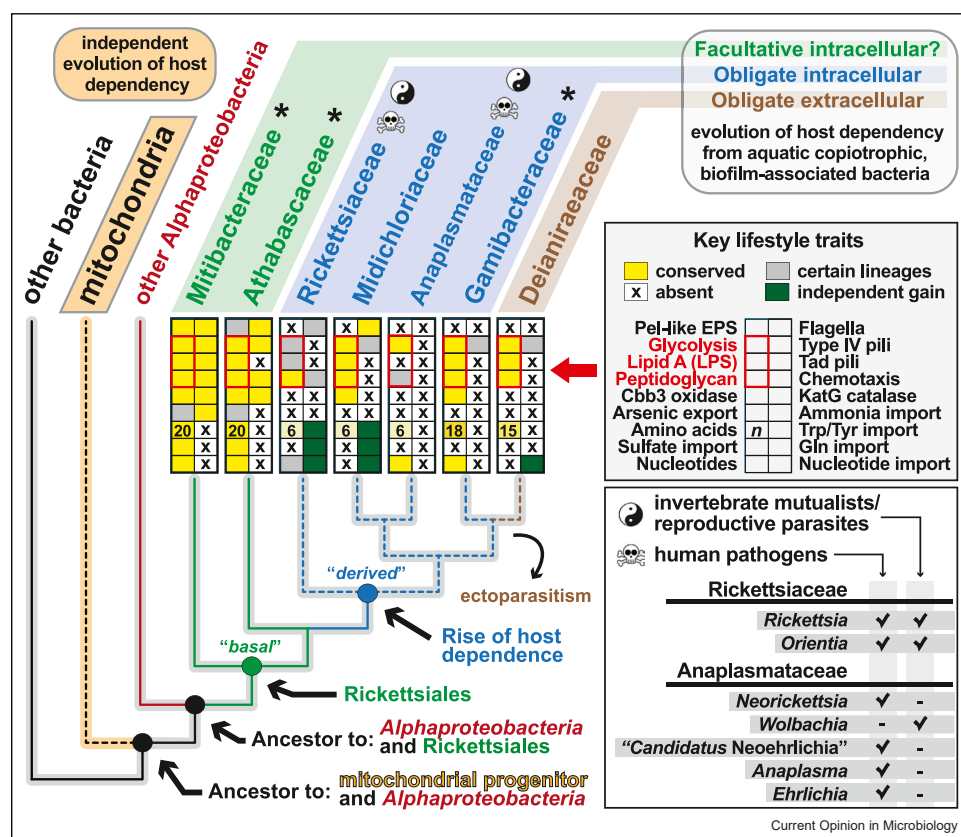
Rickettsiales and the emergence of host dependency

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 addition 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* Neorhlichia’, *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 Mitobacteraceae, 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

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 mucopeptide synthesis. Furthermore, along with three other divergent bacterial lineages (intracellular species in the *Gamma*proteobacteria, 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, however, 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 mucopeptide 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 (*psa*) of *Rickettsia conorii* diminished S-layer formation and rendered bacteria less pathogenic yet insensitive to bactericidal antibodies targeting O-antigen [95]. The *psa* 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.

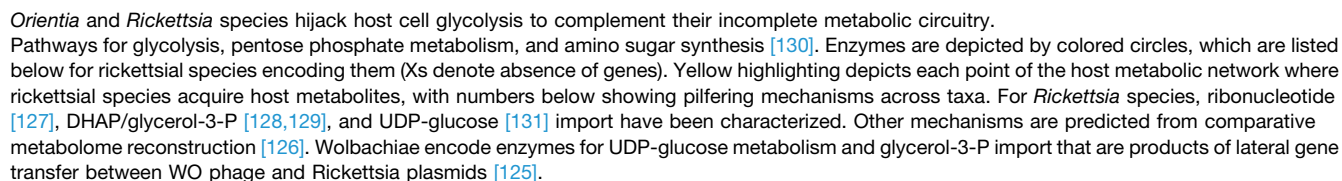
Thick as thieves: remarkable metabolic parasitism

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.

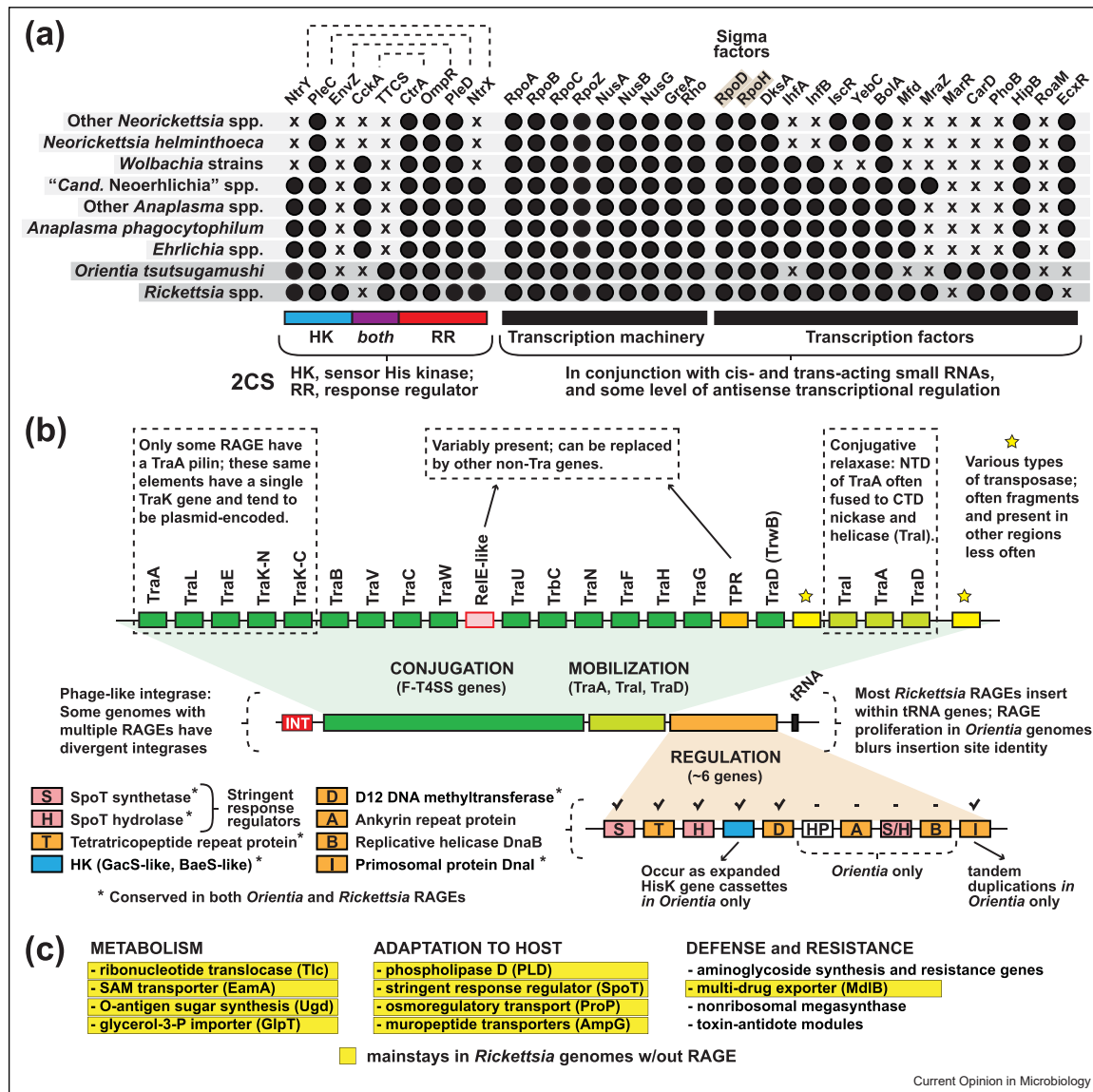
Remarkably, the revised phylogenetic framework for Rickettsiales indicates that *Orientia* species and all Anaplasmataceae species independently lost the capacity for LPS biosynthesis (Figures 1; 3). Furthermore, the collective rickettsial species synthesizing minimal PGN also converged on this cell 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.



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 ($\sigma 70$) and alternate heat shock RpoH ($\sigma 32$), 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.

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