MINIREVIEW

# The enigmatic biology of rickettsiae: recent advances, open questions and outlook

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**One sentence summary:** This review highlights the recent advances and insights gained into the unique biology of rickettsiae and key areas for future investigation.

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## ABSTRACT

Rickettsiae are obligate intracellular bacteria that can cause life-threatening illnesses and are among the oldest known vector-borne pathogens. Members of this genus are extraordinarily diverse and exhibit a broad host range. To establish intracellular infection, *Rickettsia* species undergo complex, multistep life cycles that are encoded by heavily streamlined genomes. As a result of reductive genome evolution, rickettsiae are exquisitely tailored to their host cell environment but cannot survive extracellularly. This host-cell dependence makes for a compelling system to uncover novel host–pathogen biology, but it has also hindered experimental progress. Consequently, the molecular details of rickettsial biology and pathogenesis remain poorly understood. With recent advances in molecular biology and genetics, the field is poised to start unraveling the molecular mechanisms of these host–pathogen interactions. Here, we review recent discoveries that have shed light on key aspects of rickettsial biology. These studies have revealed that rickettsiae subvert host cells using mechanisms that are distinct from other better-studied pathogens, underscoring the great potential of the *Rickettsia* genus for revealing novel biology. We also highlight several open questions as promising areas for future study and discuss the path toward solving the fundamental mysteries of this neglected and emerging human pathogen.

**Keywords:** *Rickettsia*; pathogenesis; microbial genetics; arthropod-borne pathogens; host–pathogen interactions; obligate intracellular bacteria

## INTRODUCTION

Bacteria dominate the biosphere and exhibit astounding diversity (Hug *et al.* 2016). Yet, we have merely scratched the surface of the bacterial world, and the vast majority of fascinating biology remains untapped. Much of what we know has come from studying model systems, like *Escherichia coli* and *Listeria monocytogenes*, for which there are powerful tools and large research communities. While these organisms still prove to be fruitful sources of discovery, we stand to make tremendous progress towards advancing our understanding of microbial life by expanding the range of bacteria we study to non-model systems (Alfred and Baldwin 2015; Blount 2015; Pizarro-Cerdá and Cossart 2019). With recent technological advances, especially in genetic manipulation, we are poised to delve into the molecular inner workings of diverse organisms and uncover novel biology (Alfred and Baldwin 2015; Matthews and Vosshall 2020).

In this review, we examine the unique biology of *Rickettsia*, a genus of obligate intracellular bacteria that includes several neglected and emerging human pathogens (Parola *et al*. 2013). Obligate intracellular bacteria are particularly remarkable and exciting for their extreme dependence on the host's intracellular environment. Over the course of evolutionary time, these bacteria evolved exquisitely tailored strategies to invade and hijack eukaryotic host cells to exploit them for their resources and for shelter from the humoral immune system. However, because they cannot survive extracellularly, obligate intracellular bacteria have historically been recalcitrant to laboratory manipulation, and progress towards understanding their biology has been stunted (Fields, Heinzen and Carabeo 2011; McClure *et al*. 2017). In the past decade, we have seen significant advancements in the study and genetic manipulation of rickettsiae (Burkhardt *et al*. 2011; McClure *et al*. 2017; Lamason, Kafai and Welch 2018; Kim *et al*. 2019; Narra *et al*. 2020). Here, we discuss these recent advances and how they have shed light on the unique infectious life cycles of rickettsiae. We also highlight several key unanswered questions that are likely to see progress over the next few years, thanks to advances in molecular biology, genetics and genomics.

## THE *RICKETTSIA* GENUS: BROAD HOST RANGE AND SPECIES-LEVEL DIVERSITY

All *Rickettsia* species are obligate intracellular endosymbionts of eukaryotic host cells (Weinert 2015). This genus of alphaproteobacteria belongs to the order Rickettsiales, which also includes other obligate intracellular pathogen genera like *Orientia*, *Ehrlichia* and *Anaplasma* (Palmer and Azad 2012). The *Rickettsia* genus has extraordinary diversity at the species level, with members found in freshwater, marine and terrestrial ecosystems (Weinert 2015). *Rickettsia* species also exhibit a fairly broad host range. While most are associated with arthropods (e.g. ticks, lice, mites and fleas), some species are associated with hosts including protozoa, algae, plants and vertebrates. Notably, all known human pathogens in the *Rickettsia* genus are vectored by arthropods (Barrett and Stanberry 2009; Weinert *et al*. 2009; Weinert 2015; Biggs *et al*. 2016; Blanton 2019).

Arthropod-associated *Rickettsia* species are mainly transmitted vertically to their offspring through the infected cytoplasm of egg cells (Barrett and Stanberry 2009; Socolovschi *et al*. 2009; Weinert *et al*. 2009; Weinert 2015). As a result, some species of *Rickettsia* have evolved strategies to increase their persistence within host lineages by manipulating reproductive behavior and physiology, mainly to increase the number, survival and fitness of egg-laying individuals in a population. These strategies include male-killing, induction of parthenogenesis, cytoplasmic incompatibility and feminization of male hosts (Perlman, Hunter and Zchori-Fein 2006; Weinert *et al*. 2009; Weinert 2015). Interestingly, rickettsia–arthropod symbioses may extend beyond parasitism to commensalism and mutualism, with some evidence even suggesting an obligate mutualistic relationship (Perotti *et al*. 2006; Zchori-Fein, Borad and Harari 2006; Weinert 2015). The evolutionary and molecular underpinnings of these symbioses are not well understood, nor are the incidence and relative frequencies of any of these strategies among *Rickettsia* species (Weinert 2015). Some *Rickettsia* species have also evolved strategies to increase their persistence during vertical transmission in arthropod hosts by transovarial interference (Burgdorfer and Anacker 1981; Macaluso *et al*. 2002; Wright *et al*. 2015; Gall *et al*. 2016; Tomassone *et al*. 2018). In this process, certain species of *Rickettsia* (members of spotted fever group and ancestral group, see below) preclude secondary infection with another rickettsial endosymbiont, though the molecular details are not understood. Greater environmental sampling and molecular characterization of rickettsia–arthropod interactions will be necessary to gain a complete understanding of how *Rickettsia* species persist within arthropod populations, including those that are vectors for human diseases.

Due to its extensive diversity, the *Rickettsia* genus is classified into several phylogenetic groups, including spotted fever group (SFG), typhus group (TG), ancestral group (AG) and transitional group (TRG) (Gillespie *et al*. 2007; Barrett and Stanberry 2009; Palmer and Azad 2012; Weinert 2015; Blanton 2019). These groups have distinct intracellular life cycles that are presumably driven by genetic differences, but this has not been demonstrated and much of the rickettsial genome is uncharacterized (McClure *et al*. 2017; Lamason, Kafai and Welch 2018; Narra *et al*. 2020). While SFG and TG rickettsiae were historically categorized using antigen-based methods, these classifications have since been corroborated by genome sequences (Barrett and Stanberry 2009; Sahni *et al*. 2013; Weinert 2015). More recent work characterizing novel *Rickettsia* species has revealed greater species-level diversity than previously thought, and some newer classification systems divide the genus into as many as twelve groups (Weinert 2015). As the field continues to sequence more genomes from diverse rickettsiae, as well as relatives from the Rickettsiales order, we will gain important insights into evolution and diversification within the *Rickettsia* genus. This review's primary focus will be SFG and TG *Rickettsia* species, which are the best-studied groups at a molecular and cellular level due to their relevance as human pathogens.

## *RICKETTSIA* CONTAINS GLOBAL, NEGLECTED AND EMERGING HUMAN PATHOGENS

The *Rickettsia* genus includes numerous important human pathogens, mainly in the SFG, TG and TRG clades (Barrett and Stanberry 2009; Parola *et al*. 2013; Weinert 2015; Biggs *et al*. 2016; Blanton 2019). Spotted fever rickettsioses are globally distributed and can have case-fatality rates as high as 55% without treatment, like in the case of Rocky Mountain spotted fever (Paddock and Alvarez-Hernández 2018), which the U.S. CDC considers the 'most deadly tickborne disease in the world' (CDC 2019a). Even with today's treatment options, Rocky Mountain spotted fever has case fatality rates of 5–10% (Biggs *et al*. 2016; Paddock and Alvarez-Hernández 2018). Several other SFG members cause life-threatening diseases, including *R. conorii* (Mediterranean spotted fever) and *R. japonica* (Japanese spotted fever; CDC 2019b; Barrett and Stanberry 2009; Parola *et al*. 2013; Blanton 2019). TG rickettsiae, which include *R. typhi* and *R. prowazekii*, exhibit mortality rates as high as 60% without treatment (Azad 2007; Barrett and Stanberry 2009; Angelakis, Bechah and Raoult 2016; Blanton 2019). The TRG member *R. felis* is a globally distributed flea-borne human pathogen that was first isolated in the U.S. and has more recently emerged as a common cause of non-fatal fever in Africa (Brown and Macaluso 2016). Improving our understanding of these neglected and emerging pathogens at the molecular and genetic levels will be crucial for controlling and treating these diseases.

The incidence of *Rickettsia*-associated diseases is currently on the rise. Around the world, there have been sporadic outbreaks of epidemic typhus and murine typhus, caused by *R. prowazekii* and *R. typhi*, respectively (Angelakis, Bechah and Raoult 2016; Blanton 2019; Caravedo Martinez, Ramírez-Hernández and Blanton 2021). Because typhus diseases spread via lice and fleas, they

are often brought on by improper sanitary conditions and over-crowding. Consequently, it disproportionately affects homeless populations, those living in correctional facilities and isolated rural communities. Typhus diseases also often follow wars, spurred on by the conditions faced by troops and other people affected by warfare, and have influenced the outcomes of several wars throughout history (Azad 2007; Barrett and Stanberry 2009; Angelakis, Bechah and Raoult 2016). SFG rickettsioses have increased by at least an order of magnitude in the U.S. over the past two decades (Blanton 2019; CDC 2020). This increase in SFG rickettsioses has been driven by a variety of factors, including climate change and human migration, both of which have led to more interactions between humans and pathogen-associated arthropod vectors (Parola et al. 2013; Blanton 2019; CDC 2020). Even still, the prevalence of rickettsioses is underestimated because they are often undiagnosed or misdiagnosed (Parola et al. 2013; Biggs et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019). As discussed below, rickettsioses present with similar symptoms as other common infectious diseases, especially in tropical and subtropical regions, and we currently lack fast point-of-care diagnostic methods (Parola et al. 2013; Paddock and Alvarez-Hernández 2018; Blanton 2019). Unfortunately, delays in treating rickettsioses lead to poorer outcomes and possible fatality (Biggs et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019), underscoring the need for improved methods for detection and diagnosis.

Rickettsial diseases commonly present with one or more symptoms, including fever, headache, myalgia, rash and eschars (dry necrotic scabs), though symptoms vary among different species (Barrett and Stanberry 2009; Parola et al. 2013; Angelakis, Bechah and Raoult 2016; Biggs et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019). Arthropod-borne rickettsiae are transmitted to vertebrate hosts during a tick or mite bite, or they are deposited via fecal matter from lice or fleas and are subsequently introduced into the host by abrasive scratching of the skin (Barrett and Stanberry 2009; Blanton 2019). Rickettsia species then invade cells of the vasculature where localized rickettsia replication results in host cell death, giving rise to eschars in certain rickettsioses (Chan, Riley and Martinez 2010). Later in infection, rickettsia proliferation leads to increased vascular permeability, which presents as a rash (Barrett and Stanberry 2009; Biggs et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019). While their primary niche inside vertebrate hosts is endothelial cells, rickettsiae can infect a wide array of cell types, including macrophages, neutrophils, hepatocytes and neurons, some of which may play a role in spread throughout the body (Barrett and Stanberry 2009; Angelakis, Bechah and Raoult 2016; Biggs et al. 2016; Curto et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019; Sekeyová et al. 2019). Several groups have recently made progress in developing new animal models for rickettsial infection (Burke et al. 2020b; Bechah et al. 2010; Banajee et al. 2015), which will be crucial for fully understanding pathogenesis and for developing new treatments.

One notable but poorly understood feature of epidemic typhus is a recrudescent infection, referred to as Brill–Zinsser disease (Brill 1910; Zinsser and Castaneda 1933; Barrett and Stanberry 2009; Angelakis, Bechah and Raoult 2016). In this disease, R. prowazekii enters a dormant state that can persist asymptomatically for more than 40 years after acute infection (Barrett and Stanberry 2009; Bechah et al. 2010; Faucher et al. 2012; Angelakis, Bechah and Raoult 2016). The reactivated pathogen causes a milder form of the disease relative to the initial infection, and the trigger of reactivation is unclear

(Barrett and Stanberry 2009; Bechah et al. 2010; Angelakis, Bechah and Raoult 2016). The mechanism of dormancy and identity of the host tissues in which these bacteria reside are also not well understood, though work using a recently developed mouse model identified adipose tissue as a potential reservoir (Bechah et al. 2010).

Given the status of some Rickettsia species as emerging and global health threats, as well as the classification of R. prowazekii as a select agent by the U.S. government (Azad 2007), effective therapeutics will be essential for preventing morbidity and mortality in all patients. Today, rickettsial diseases are primarily treated with tetracycline antibiotics, typically doxycycline. Chloramphenicol is the only alternative treatment option for patients with sensitivities to tetracyclines, but chloramphenicol has been associated with higher case fatality rates, and effective forms are often unavailable in hospitals in the U.S. (Barrett and Stanberry 2009; Biggs et al. 2016; Paddock and Alvarez-Hernández 2018; Blanton 2019). Many antibiotics, including $\beta$-lactams, macrolides, aminoglycosides and sulfonamides, are ineffective against rickettsiae (Biggs et al. 2016). There has been a worrying increase in antibiotic resistance among bacteria in general (Berendonk et al. 2015), and antibiotic resistance in rickettsiae is readily evolved in laboratory culture and has been found in natural isolates (Rachek et al. 1998; Troyer et al. 1998; Drancourt and Raoult 1999). Further, it is not clear how the widespread use of antibiotics and pesticides in agriculture (Berendonk et al. 2015; Antwis et al. 2017) might affect the evolution or distribution of Rickettsia species across the world. Taken together with the increased prevalence of rickettsial diseases, there is a clear need to improve our understanding of the fundamental biology and pathogenesis of rickettsiae, and to develop new strategies to diagnose, treat and prevent rickettsial infections.

## THE STATE OF GENETIC TOOLS IN RICKETTSIA

Most obligate intracellular bacteria are difficult to study because they cannot be cultured axenically and must be grown within eukaryotic cell culture systems. Perhaps most impactfully, the difficulties associated with culturing and manipulating these bacteria have stunted the development of genetic tools (McClure et al. 2017). For example, even though genetic transformation has been possible for 50 years in E. coli (Mandel and Higa 1970), the first transformation in Rickettsia was only performed in 1998 (Rachek et al. 1998). While these hurdles have impeded progress towards understanding the biology of obligate intracellular bacteria (Palmer and Azad 2012; McClure et al. 2017), we have seen significant advances in rickettsial genetics over the past two decades that have revealed unique molecular mechanisms underlying the infectious life cycles of rickettsiae.

In 2009, the first published example of targeted mutagenesis was performed in R. prowazekii, in which the pld (phospholipase D) gene was knocked out by allelic exchange using a linear DNA substrate (Driskell et al. 2009). This strategy has not been widely adopted though, likely due to the poor efficiency of transformation and allelic exchange in rickettsiae. The next attempts at targeted genetic manipulation came 6 years later, with two papers describing two different methods. In R. rickettsii, the ompA gene was knocked out using group II intron retrohoming (TargeTron; Noriea, Clark and Hackstadt 2015), a proprietary technology that is no longer sold or supported. In R. typhi and R. montanensis, peptide nucleic acids were used to silence the expression of the ompB and rickA genes (Pelc et al. 2015). The PNA-based knockdown yielded slightly reduced expression of

these abundant proteins when measured by western blot, with differences in expression only being detectable when comparing diluted input samples. To this date, these are the only reports of targeted genetic manipulation in *Rickettsia*, and the field still lacks the highly efficient and scalable tools for targeted genetic manipulation that exist in other bacteria.

As a complementary approach to targeted mutagenesis, methods for forward-genetic screening in rickettsiae have also seen significant progress. A *mariner*-based transposon system for random mutagenesis was developed for *R. prowazekii* in 2007 (Liu *et al*. 2007) and applied to *R. rickettsii* in 2010 (Kleba *et al*. 2010). In contrast to the targeted mutagenesis techniques described above, transposon mutagenesis allows for more efficient generation of a relatively large number of mutants from a single transformation. By improving the efficiency of the original *mariner*-based system from 2007, the first large-scale rickettsial mutant collection was published in 2018 using *R. parkeri* (Lamason, Kafai and Welch 2018). This screen isolated over 100 mutants with defects in mammalian cell infection, and the resulting collection has helped reveal the essential functions of several genes during the intracellular life cycle, including *ompB* (Engström *et al*. 2019), *rickA* (Reed *et al*. 2014; Harris *et al*. 2018), *sca2* (Burke *et al*. 2020b; Reed *et al*. 2014; Harris *et al*. 2018) and *sca4* (Lamason *et al*. 2016) (described below). These mutants have also enabled functional-genetic studies, including the first genetic complementation of a rickettsial mutant with a native promoter in 2016 (Lamason *et al*. 2016), which was accomplished using a shuttle vector that was developed for rickettsiae in 2011 (Burkhardt *et al*. 2011). A similar transposon screen using a Tn5 transposon-based system was recently conducted in *R. conorii* (Kim *et al*. 2019). A total of 53 mutant strains were isolated, including two transposon mutants that were used to demonstrate the role of O antigen in the production of Weil–Felix antibodies, which are associated with anti-rickettsial immunity and detected in serology-based diagnostics. Forward-genetic screening using transposon-based systems represents a promising avenue for identifying the roles of the many genes of unknown function in the rickettsial genome.

Other forward genetic methods, including signature-tagged mutagenesis and Tn-seq, have allowed researchers to determine gene essentiality in a wide array of bacteria in different environmental contexts (Hensel *et al*. 1995; Hutchison III 1999; Knuth *et al*. 2004; Goodman *et al*. 2009; van Opijnen, Bodi and Camilli 2009). These methods rely on negative selection of strains with deleterious transposon insertions, so they require high numbers of randomly generated transposon mutants. However, in rickettsiae, reaching the number of mutants needed to call gene essentiality by saturation is not currently feasible given the extremely low transformation efficiency (Clark *et al*. 2011; Palmer and Azad 2012). The development of more efficient methods for transformation and transposon mutagenesis will be necessary to propel *Rickettsia* into the realm of genome-wide screens. The introduction of more efficient and scalable methods for targeted gene knockouts or knockdowns will also be crucial for the rickettsial toolkit. The advent of tools like CRISPR-based genome editing, which has been employed in diverse bacterial species including those that are difficult to culture and manipulate (reviewed in Vigouroux and Bikard 2020), provides a promising avenue for future tool development in *Rickettsia* species. With more powerful tools, researchers will be able to dissect the genetic and molecular mechanisms underlying the unique rickettsial infectious life cycle.

# RICKETTSIAE EMPLOY COMPLEX AND UNIQUE INTRACELLULAR LIFE CYCLES TO ESTABLISH INFECTION

Rickettsiae are entirely dependent on eukaryotic host cells for survival and have evolved complex life cycles that allow them to establish intracellular niches (Fig. 1 and Table 1; Palmer and Azad 2012; Lamason and Welch 2017; Narra *et al*. 2020). The rickettsial life cycle begins with adherence to the host cell, followed by uptake into a phagocytic vacuole. After escaping the phagosome, rickettsiae reside freely within the host cytosol, and some species can achieve motility by hijacking host cell actin. After replicating within the host cytosol, rickettsiae can disseminate to neighboring cells, either through lysis of the host cell or by moving directly into neighboring cells by traversing cell–cell junctions (Palmer and Azad 2012; Lamason and Welch 2017; Narra *et al*. 2020). In this section, we review key insights into the molecular details of this infectious life cycle.

Intracellular bacterial pathogens invade eukaryotic host cells using either the zipper or trigger mechanisms (Cossart 2004). In the zipper mechanism, the invading bacterium binds to host cell receptors, which triggers signaling cascades that cause phagocytosis of the bacterium. In rickettsiae, bacterial surface proteins bind host cell receptors to promote adherence and zipper-mediated invasion (Chan, Riley and Martinez 2010; Palmer and Azad 2012). The host proteins Ku70 and $\alpha2\beta1$ integrin promote adherence and invasion by rickettsiae (Martinez *et al*. 2005; Reed, Serio and Welch 2011; Hillman, Baktash and Martinez 2013); however, an essential host cell receptor has not yet been identified. The *R. conorii* proteins OmpB (outer membrane protein B) and OmpA (outer membrane protein A) interact with Ku70 (Martinez *et al*. 2005) and $\alpha2\beta1$ integrin (Hillman, Baktash and Martinez 2013), respectively. Other rickettsial surface proteins have also been suggested to play a role in host cell adherence and invasion, including Sca2 (Cardwell and Martinez 2009), Adr1 (Balraj, Renesto and Raoult 2009) and Adr2 (Vellaiswamy *et al*. 2011). The binding of rickettsiae to host cell receptors triggers a signaling cascade beginning with the activation of host tyrosine kinases and phosphoinositide 3-kinase (Martinez and Cossart 2004; Reed, Serio and Welch 2011). These tyrosine kinases then activate Rho-family GTPases, including Cdc42 (Martinez and Cossart 2004; Reed, Serio and Welch 2011) and Rac1 (Reed, Serio and Welch 2011), which in turn activate WAVE proteins (Reed, Serio and Welch 2011). These signaling events ultimately promote the activation of Arp2/3, which is the primary driver of actin polymerization during the uptake of rickettsiae by the host cell (Martinez and Cossart 2004; Reed, Serio and Welch 2011).

In contrast to the zipper mechanism, bacteria that invade host cells by the trigger mechanism use their secretion machinery to inject bacterial effectors into the host cell to activate cytosolic activators of phagocytosis directly (Cossart 2004). Recent work has suggested that rickettsial invasion might also involve elements of a trigger mechanism, possibly blurring the line between these two invasion mechanisms. It has been speculated that RickA, which nucleates host actin during actin-based motility (see below), might also be involved in activating Arp2/3 during invasion (Reed, Serio and Welch 2011; Rennoll-Bankert *et al*. 2015), though this has not been demonstrated experimentally. The *R. typhi* protein RalF is predicted to be secreted into host cells during invasion in a trigger-like mechanism (Rennoll-Bankert *et al*. 2015, 2016). RalF recruits the host protein Arf6 to manipulate phosphoinositol metabolism at the host plasma membrane and promote engulfment of *R. typhi* (Rennoll-Bankert
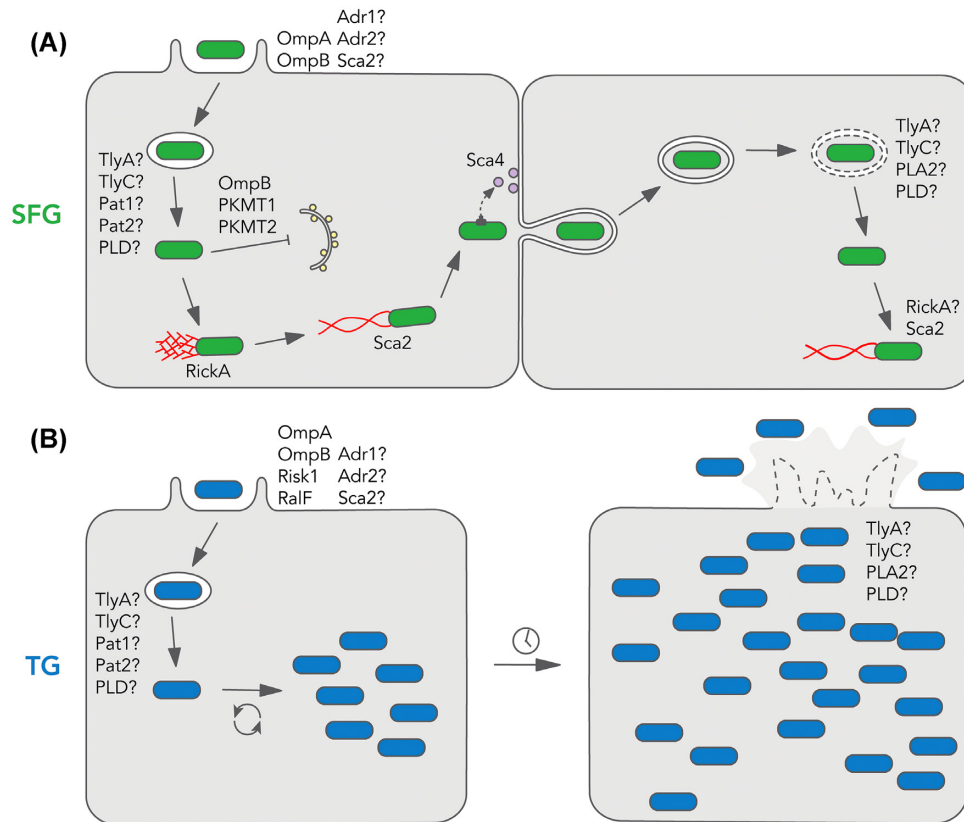
**Figure 1.** The intracellular life cycles of SFG and TG rickettsiae. **(A)** The SFG life cycle begins with host cell invasion, during which rickettsiae enter the host cell in a phagocytic vacuole. After escaping the phagosome, SFG rickettsiae initiate two phases of actin-based motility. SFG rickettsiae avoid host autophagy through lysine methylation of their surface proteins. Starting around 8 h after invasion, SFG rickettsiae begin replicating in the host cytosol. Subsequently, they undergo cell-to-cell spread, a process in which they directly traverse the cell–cell junction and enter the neighboring cell in a double-membrane vacuole. Upon escape, they can reinitiate the life cycle. **(B)** The TG life cycle begins similarly to SFG with invasion followed by rapid escape from the phagocytic vacuole. After replicating to a high density, the host cell lyses and TG rickettsiae can escape and spread to neighboring cells. (A and B) Rickettsial proteins that have been implicated in the life cycle are noted. Proteins that are speculated to be involved in a process are denoted with a question mark.

*et al*. 2015, 2016). Additionally, Risk1, a phosphatidylinositol 3-kinase effector from *R. typhi*, has been proposed to be secreted and act on the host cell membrane during invasion (Voss *et al*. 2020). However, without the ability to easily generate targeted knockouts in *Rickettsia*, we lack direct evidence for translocation of RalF or Risk1 into host cells during invasion. Furthermore, RalF has been lost or pseudogenized in other clades, including SFG (Rennoll-Bankert *et al*. 2015), suggesting that rickettsiae may have evolved diverse mechanisms for host cell invasion. Indeed, the prevailing model is that *Rickettsia* species have evolved multiple, redundant mechanisms to adhere to and invade host cells, which could contribute to their ability to infect numerous cell types in a range of host organisms (Reed, Serio and Welch 2011; Palmer and Azad 2012; Rennoll-Bankert *et al*. 2016). As more powerful genetic tools are developed for rickettsiae, we will be able to broaden our understanding of invasion by directly and comprehensively studying rickettsial adhesins and invasins.

Upon successful uptake into the host cell, rickettsiae quickly escape the phagocytic vacuole and enter the host cytosol. In SFG and TG rickettsiae, cytosolic bacteria are detectable as early as 3 min post-invasion, and the majority of internalized bacteria are in the cytosol by 20 min (Teysseire, Boudier and Raoult 1995; Palmer and Azad 2012). These vacuole escape kinetics are faster relative to other cytosolic pathogens, including *L. monocytogenes*, *Shigella flexneri* and *Francisella tularensis*, which escape into the

cytosol on the order of 15–60 min (Palmer and Azad 2012; Fredlund and Enninga 2014). While the vacuoles of these other intracellular bacteria associate with lysosomal markers (Fredlund and Enninga 2014), it is not known if rickettsia-containing vacuoles associate with lysosomal markers or if the faster escape kinetics allow them to avoid endosomal maturation. The rapid kinetics of vacuole escape suggest that rickettsiae may use mechanisms distinct from those employed by other cytosolic bacteria. The rickettsial genome encodes several enzymes that are suspected of breaking down the vacuolar membrane, including phospholipase D (PLD) (Whitworth *et al*. 2005; Driskell *et al*. 2009), the patatin-like phospholipase A2 enzymes Pat1 and Pat2 (Walker *et al*. 1983; Silverman *et al*. 1992; Blanc, Renesto and Raoult 2005; Rahman *et al*. 2010, 2013) and the hemolysins TlyA and TlyC (Whitworth *et al*. 2005). However, these genes have mostly been studied either indirectly (Walker *et al*. 1983; Silverman *et al*. 1992; Rahman *et al*. 2010, 2013) or by heterologous expression in bacteria like *Salmonella enterica* (Whitworth *et al*. 2005), and none have been ascribed specific roles in vacuole escape or infection dynamics. Directly probing the role of each gene has been difficult due to the lack of genetic tools, although a phospholipase D (*pld*) mutant was the first targeted genetic knockout created in *Rickettsia* (Driskell *et al*. 2009). Surprisingly, when *pld* was knocked out in *R. prowazekii*, no defects were observed in vacuole escape kinetics during *in vitro* infection

**Table 1.** Rickettsial proteins with known or predicted roles during infection. Summarized details of key rickettsial proteins discussed in this review. The presence of a protein in a specific rickettsial group is defined by a full or partial homolog found in the genome of at least one species. Asterisks (*) denote studies that directly characterized the protein in the native *Rickettsia* species by studying a mutant strain of rickettsia. SFG, spotted fever group; TG, typhus group; TRG, transitional group; AG, ancestral group.

| | Rickettsial Protein | Known host protein interactions | Function | Present | References |
|---|---|---|---|---|---|
| Secreted | TlyA | Unknown | Predicted hemolysin with possible role in vacuole escape and/or host cell lysis | SFG, TG, TRG, AG | *R. prowazekii* (Whitworth et al. 2005) |
| | TlyC | Unknown | Predicted hemolysin with possible role in vacuole escape and/or host cell lysis | SFG, TG, TRG, AG | *R. prowazekii* (Whitworth et al. 2005) |
| | Pat1 | Unknown | Phospholipase with possible role in vacuole escape and/or host cell lysis | SFG, TG, TRG, AG | *R. rickettsii* (Walker et al. 1983; Silverman et al. 1992), *R. typhi* (Rahman et al. 2010, 2013) |
| | Pat2 | Unknown | Phospholipase with possible role in vacuole escape and/or host cell lysis | SFG, TG, TRG, AG | *R. rickettsii* (Walker et al. 1983; Silverman et al. 1992), *R. typhi* (Rahman et al. 2010, 2013) |
| | PLD | Unknown | Predicted phospholipase with possible role in vacuole escape and/or host cell lysis | SFG, TG, TRG, AG | *R. prowazekii* (Whitworth et al. 2005, Driskell et al. 2009*) |
| | Sca4 | Vinculin | Disrupts interaction between vinculin and α-catenin promote cell-to-cell spread | SFG, TG, TRG, AG | *R. rickettsii* (Park et al. 2011), *R. parkeri* (Lamason et al. 2016*) |
| | Risk1 | Beclin-1 | Phosphatidylinositol 3-kinase activity promotes host cell colonization | SFG, TG, TRG, AG | *R. typhi* (Voss et al. 2020) |
| | RalF | Arf6 | Guanine nucleotide exchange factor of Arf6 that promotes invasion | TG, TRG, AG | *R. typhi* (Rennoll-Bankert et al. 2015, 2016) |
| | RARP-2 | Unknown | Cysteine protease that contributes to *trans*-Golgi fragmentation | SFG, TG, TRG, AG | *R. rickettsii* (Lehman et al. 2018; Aistleitner et al. 2020) |
| Surface or membrane | Tlc1 | Unknown | ATP/ADP symporter that imports ATP from host cells | SFG, TG, TRG, AG | *R. prowazekii* (Winkler 1976; Audia and Winkler 2006; Krause, Winkler and Wood 1985) |
| | OmpA | α2β1 integrin | Rickettsial surface protein involved in invasion | SFG, TRG, AG | *R. conorii* (Hillman, Baktash and Martinez 2013) |
| | OmpB | Ku70 | Rickettsial surface protein involved in invasion and autophagy avoidance | SFG, TG, TRG, AG | *R. conorii* (Martinez et al. 2005), *R. parkeri* (Engström et al. 2019*) |
| | PKMT1/2 | Unknown | Lysine methylation of rickettsial surface proteins to avoid autophagy | SFG, TG, TRG, AG | *R. parkeri* (Engström et al. 2020*), *R. prowazekii* (Abeykoon et al. 2012, 2014, 2016), *R. typhi* (Abeykoon et al. 2014, 2016) |
| | RickA | Arp2/3 | Mimics WASP to activate Arp2/3 to drive actin-based motility | SFG, TRG, AG | *R. conorii* (Gouin et al. 2004), *R. rickettsii* (Jeng et al. 2004), *R. parkeri* (Reed et al. 2014*) |
| | Sca2 | Actin, profilin | Formin-like activity that directly nucleates actin to promote motility | SFG, TG, TRG, AG | *R. rickettsii* (Kleba et al. 2010*), *R. parkeri* (Haglund et al. 2010, Reed et al. 2014*), *R. conorii* (Madasu et al. 2013) |

of macrophages. In contrast, this *pld* mutant strain was unable to induce fever or weight changes in a low inoculum guinea pig infection model, suggesting that phospholipase D does contribute to pathogenesis *in vivo* (Driskell *et al*. 2009). Given that rickettsiae encode multiple membranolytic enzymes, other genes might provide functional redundancy for *pld*. It is also possible that phospholipase D acts during another phase of the rickettsial infectious life cycle. For instance, it could contribute to host cell lysis or lipid metabolism, or it could play a role in different cell types that were not tested, like endothelial cells. It will be interesting to see how each of the rickettsial membranolytic enzymes promotes niche establishment and pathogenesis.

Rickettsiae avoid and suppress cellular immunity to promote successful infection. For example, *R. rickettsii* inhibits apoptosis in a manner dependent on NF-$\kappa$B (Clifton *et al*. 1998; Joshi *et al*. 2004). Pathogenic SFG rickettsiae also avoid detection by host autophagy machinery by shielding their surface proteins with lysine methylation (Abeykoon *et al*. 2012, 2014, 2016; Uchiyama, Kishi and Ogawa 2012; Engström *et al*. 2019). Indeed, transposon mutagenesis of the genes encoding two protein-lysine methyltransferases PKMT1 and PKMT2 and OmpB (Lamason, Kafai and Welch 2018) rendered these *R. parkeri* mutants vulnerable to autophagy-mediated clearance during infection (Engström *et al*. 2019, 2020). Intriguingly, *R. parkeri* was recently shown to exhibit more virus-like interactions with the innate immune response relative to facultative intracellular bacteria like *L. monocytogenes*, in that it is sensitive to IFN-I and exploits the inflammasome to avoid IFN-I (Burke *et al*. 2020a). The full repertoire of rickettsial factors that mediate interactions with host immunity remains to be elucidated, but future work focused on this is sure to yield valuable insights into how different cytosolic bacteria hijack their host cells.

Several *Rickettsia* species undergo actin-based motility, a process in which they polymerize host actin filaments at one pole of the bacterial cell surface to propel themselves throughout the host cytoplasm (Palmer and Azad 2012; Choe and Welch 2016; Lamason and Welch 2017). Unlike most cytosolic bacterial pathogens, some rickettsiae encode two distinct bacterial effector proteins for actin-based motility (Gouin *et al*. 2004; Jeng *et al*. 2004; Haglund *et al*. 2010; Kleba *et al*. 2010). The first, RickA, is found in nearly all SFG species (except *R. peacockii*) as well as members of AG and TRG (Jeng *et al*. 2004; Haglund *et al*. 2010; Choe and Welch 2016; Lamason and Welch 2017). RickA produces short, curved tails by mimicking WASP to activate Arp2/3 at the bacterial pole, which polymerizes branched actin networks capable of propelling the bacterium forward (Gouin *et al*. 2004; Jeng *et al*. 2004; Reed *et al*. 2014). This mechanism is similar to ActA in *L. monocytogenes* and BimA in *Burkholderia thailandensis* (Gouin *et al*. 2004; Jeng *et al*. 2004; Lamason and Welch 2017). The second rickettsial factor involved in actin-based motility is Sca2, which is found in SFG, AG and TRG species, as well as the TG member *R. typhi* (Haglund *et al*. 2010; Kleba *et al*. 2010). The best-characterized variant is the SFG Sca2, which polymerizes long, straight actin tails using a formin-like mechanism that is distinct from its eukaryotic counterpart (Haglund *et al*. 2010; Madasu *et al*. 2013; Reed *et al*. 2014). Unlike eukaryotic formins, which act as dimers and use formin homology domains, Sca2 exists as a monomer and uses N- and C-terminal repeat domains to nucleate and elongate actin filaments (Madasu *et al*. 2013). SFG Sca2-mediated motility is also dependent on several host factors, including fimbrin/T-plastin, profilin, capping protein and cofilin (Serio *et al*. 2010). Interestingly, members of TG and AG, like *R. typhi* and *R. bellii*, encode variants of Sca2 that lack the formin-like domains (Haglund *et al*. 2010; Madasu *et al*. 2013;

Choe and Welch 2016). These orthologs still contain WH2 motifs, which might facilitate a distinct form of actin-based motility, although this has not been demonstrated. It will be intriguing to see how the differences among Sca2 orthologs impact the intracellular life cycle and pathogenesis.

Notably, transposon insertions in *rickA* and *sca2* were isolated in *R. parkeri*, revealing two distinct phases of actin-based motility during its infectious life cycle (Reed *et al*. 2014; Lamason, Kafai and Welch 2018). Within the first 2 h of infection, RickA mediates early actin-based motility. Then, starting around 8 h post-infection, late motility is mediated by Sca2 (Reed *et al*. 2014). Members of SFG *Rickettsia* are unique among known cytosolic bacterial pathogens to undergo two phases of actin-based motility mediated by distinct effectors (Reed *et al*. 2014; Lamason and Welch 2017), and we are only beginning to learn how these two forms of actin-based motility shape the infectious life cycle of SFG rickettsiae and affect their pathogenesis. In mammalian cell culture, dissemination of SFG rickettsiae through the monolayer (i.e. cell-to-cell spread) is almost completely abolished in the *sca2::tn* mutant but only minorly impaired in the *rickA::tn* mutant (Reed *et al*. 2014; Harris *et al*. 2018). Given that the *sca2::tn* mutant is replication-competent (Reed *et al*. 2014; Harris *et al*. 2018), this suggests a model in which Sca2 is responsible for correctly positioning the bacteria within the host cell to initiate the process of cell-to-cell spread in mammalian tissues. *R. parkeri* Sca2 is required for efficient dissemination to host organs and lethality in an intradermal infection mouse model (Burke *et al*. 2020b), and *R. rickettsii* Sca2 has been linked to fever induction in a guinea pig infection model (Kleba *et al*. 2010). The primary function of RickA-mediated actin-based motility is less clear. For instance, we do not know whether it plays a small but direct role in cell-to-cell spread or if its modest phenotype in spread assays is an indirect effect stemming from other functions it has during the intracellular life cycle. RickA-mediated actin-based motility could potentially play a role in avoiding cellular immunity, similarly to ActA in *L. monocytogenes* (Yoshikawa *et al*. 2009; Cheng *et al*. 2018), though this has not been tested. Sca2 and RickA also mediate actin-based motility in tick cell culture (Harris *et al*. 2018), but they are not essential for cell-to-cell spread in this context. The *rickA::tn* mutant has a minor cell-to-cell spread defect in tick cells (Reed *et al*. 2014; Harris *et al*. 2018), but the *sca2::tn* mutant spreads similarly to wild-type *R. parkeri* in tick cells (Harris *et al*. 2018). In live ticks, both Sca2 and RickA promote early dissemination events, but the transposon mutants did not have an overall impact on dissemination at later timepoints of infection (Harris *et al*. 2018). Altogether, these data suggest that the roles of actin-based motility and the genetic determinants of cell-to-cell spread may vary across different host organisms. More detailed characterization of actin-based motility in diverse *Rickettsia* species and various host cell types will be needed to elucidate the various roles of actin-based motility during infection.

SFG rickettsiae undergo cell-to-cell spread, a process in which they directly move into neighboring cells by traversing cell–cell junctions, preserving access to the host cytoplasm and protection from the humoral immune system (Palmer and Azad 2012; Lamason and Welch 2017; Dowd, Mortuza and Ireton 2020). Cell-to-cell spread has also been observed in *L. monocytogenes* and *S. flexneri*, which use the force of actin-based motility to propel themselves into the cell–cell junction creating a protrusion into the neighboring cell (Kuehl *et al*. 2015; Lamason and Welch 2017; Dowd, Mortuza and Ireton 2020). This protrusion is then engulfed by the recipient cell, and the bacterium is taken up in a double-membrane vacuole. After vacuole escape, the
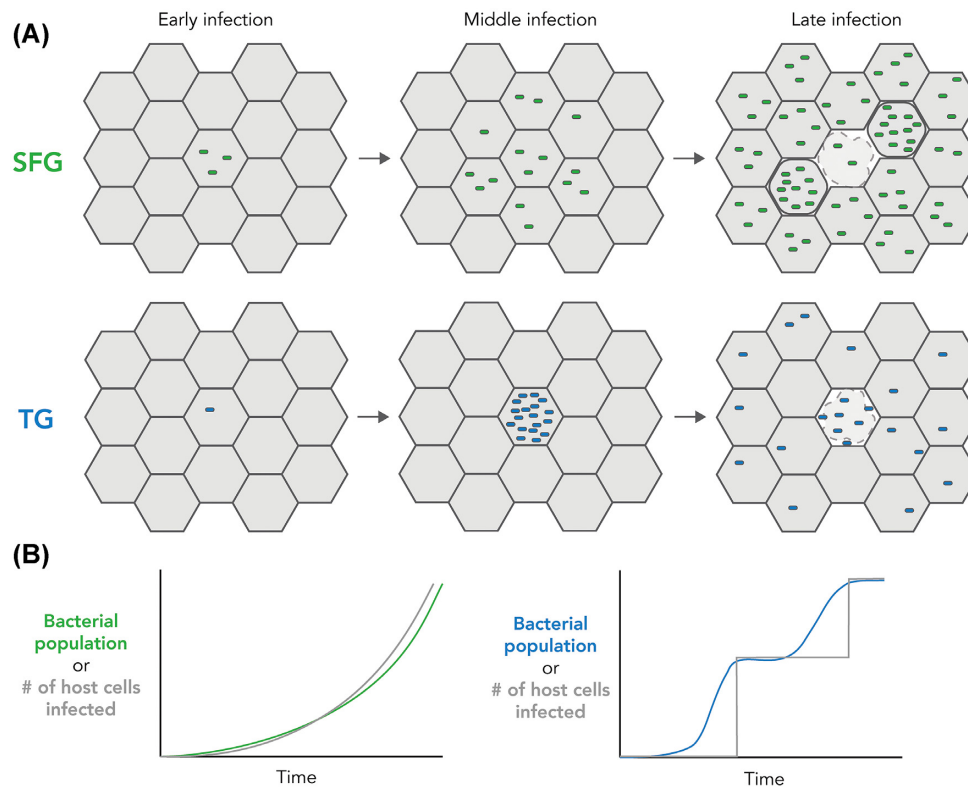
**Figure 2.** Growth dynamics of SFG and TG rickettsiae. (**A**, top) Shortly after entry into the host cell, SFG rickettsiae begin replicating. They maintain a constant growth rate and low bacterial densities within individual cells by initiating cell-to-cell spread early in infection. Late in infection, host cell lysis occurs releasing infectious bacteria into the extracellular space. (**A**, bottom) After a brief initial lag phase, TG rickettsiae replicate to high densities within individual infected cells. Once the infected cell becomes saturated with bacteria, the host cell lyses and releases large quantities of infectious bacteria that subsequently invade other host cells. (**B**) The dynamics of bacterial growth and host cell infection are represented graphically.

life cycle can be reinitiated. In stark contrast to this canonical mode of cell-to-cell spread, it was recently demonstrated that *R. parkeri* undergoes a dramatically different process of cell-to-cell spread (Lamason *et al.* 2016; Lamason and Welch 2017). Despite undergoing two distinct forms of actin-based motility (Reed *et al.* 2014), *R. parkeri* loses its actin tail before protrusion initiation (Lamason *et al.* 2016). Consequently, these protrusions are significantly shorter in length and lifetime compared with those of *L. monocytogenes* (Lamason *et al.* 2016). How SFG rickettsiae navigate to cell–cell junctions after losing actin tails and how they manipulate the host membrane in the absence of actin-based motility is not well understood. One clue has come from work examining the function of the *R. parkeri* secreted effector Sca4 (Lamason *et al.* 2016), which contains two vinculin-binding sites (Park *et al.* 2011; Lamason *et al.* 2016). This effector blocks the association between host cell adhesion proteins vinculin and α-catenin, which normally maintains intercellular tension at adherens junctions. Sca4-mediated inhibition of vinculin decreases intercellular tension and promotes protrusion engulfment, allowing for more efficient *R. parkeri* cell-to-cell spread. Notably, the *sca4::tn* mutant is not completely defective in spread, suggesting that SFG rickettsiae rely on a suite of to-be-identified bacterial proteins to alter cell-cell junctions and drive cell-to-cell spread (Lamason *et al.* 2016; Lamason, Kafai and Welch 2018). Further work will be needed to determine how conserved cell-to-cell spread is within the *Rickettsia* genus.

In contrast to SFG rickettsiae, TG rickettsiae have more limited actin-based motility and have not been observed to undergo direct cell-to-cell spread (Teysseire, Chiche-Portiche and Raoult

1992; Haglund *et al.* 2010; Palmer and Azad 2012; Choe and Welch 2016). As mentioned above, this is likely due to the loss or truncation of *sca2* in TG rickettsiae. Consequently, TG rickettsiae replicate in their host cells until reaching high densities (Wisseman and Waddell 1975; Palmer and Azad 2012), after which the host cells lyse and release large numbers of infectious bacteria that invade new host cells (Fig. 2). For this reason, the growth of TG rickettsiae within individual cells has been likened to the growth of bacteria in test tubes, with a brief lag phase followed by exponential growth (Palmer and Azad 2012). At high bacterial cell densities, TG rickettsiae display altered sizes and morphology (Wisseman and Waddell 1975; Silverman, Wisseman and Waddell 1980), although whether they reach a stationary phase of growth is not clear. In contrast, the growth of SFG rickettsiae has been compared to growth in a chemostat (Palmer and Azad 2012). Because SFG rickettsiae maintain low densities within individual host cells by initiating cell-to-cell spread early during their life cycle, they exhibit consistent replication rates throughout the course of infection (Fig. 2; Wisseman *et al.* 1976).

Somewhat counterintuitively, TG rickettsiae do not induce significant morphological changes to their host cell during infection until lysis (Silverman, Wisseman and Waddell 1980), while SFG rickettsiae cause substantial changes to their host cells (Walker *et al.* 1977; Silverman and Wisseman 1979; Walker and Cain 1980; Palmer and Azad 2012). Starting between 48–72 h post-infection with SFG rickettsiae, host cells display dilation of the nuclear envelope and the rough ER, dissociation of ribosomes from the ER, loss of Golgi apparatus morphology and swelling of mitochondria. Recent work showed that the secreted

effector RARP-2 contributes to the fragmentation of the *trans*-Golgi network during *R. rickettsii* infection (Lehman *et al.* 2018; Aistleitner *et al.* 2020); however, no other bacterial factors have been implicated in altering host organelle morphology. Further, it is unclear how structural changes to host organelles impact SFG rickettsial infection dynamics. It is also unknown what factors mediate host cell lysis in SFG or TG rickettsiae. More powerful tools will be essential for deciphering the genetic basis of how rickettsiae alter their host cell environment and the differences between TG and SFG rickettsiae.

In addition to the cytosolic niche, several SFG and AG *Rickettsia* species have been observed inside the nucleus of their host cells (Wolbach 1919; Pinkerton and Hass 1932; Philip *et al.* 1983; Ogata *et al.* 2006). Interestingly, intranuclear rickettsiae display high frequencies of actin tails with distinct dynamics relative to cytoplasmic actin tails (Heinzen *et al.* 1999; Ogata *et al.* 2006), which might be indicative of a specialized nuclear life cycle or a response to unique properties of the nucleoplasm. How these bacteria enter or escape the nucleus is not known. It is possible that rickettsiae become entrapped inside the nuclear envelope during host cell division or that they have evolved specialized mechanisms to actively invade the nucleus. However, it is not clear why other *Rickettsia* species, like TG members, are not observed in the nucleus. Further study of intranuclear rickettsiae will be important to determine their role in infection and pathogenesis. Moreover, the distinct behavior of intranuclear rickettsiae could provide a useful tool for studying the molecular constituents of the nucleoplasm.

## SECRETION SYSTEMS AND MANIPULATION OF THE HOST CELL THROUGH SECRETED EFFECTORS

Rickettsiae encode several secretion systems that foster manipulation of the host cell environment and progression through its life cycle by either secreting effector proteins into the host cell cytosol or attaching proteins to the bacterial cell surface. These include Type I, IV and V secretion systems, the Sec translocase, and the twin-arginine translocation (Tat) protein export pathway (Palmer and Azad 2012; Gillespie *et al.* 2014). However, the complete catalog of secreted proteins and their associations with the secretion machinery has not been elucidated.

Perhaps the most enigmatic of the rickettsial secretion machinery is the Type IV secretion system (T4SS; Fig. 3), which will be the main focus of this section (see (Gillespie *et al.* 2014) for a comprehensive review of rickettsial secretion systems). The rickettsial T4SS system (MPF-T class/P-T4SS) is related to the *vir* T4SS of the plant pathogen *Agrobacterium tumefaciens*, a distant relative within alphaproteobacteria, and the rickettsial T4SS genes have been renamed Rickettsiales *vir* homolog (*rvh*; Gillespie *et al.* 2014, 2016). T4SSs are typically comprised of 12 different subunits, although some bacteria also encode additional accessory genes, and some encode multiple complete T4SSs (Gillespie *et al.* 2014, 2016; Costa *et al.* 2015). The T4SS from the Rickettsiales order is unique because it has undergone several gene duplication events that have given rise to unprecedented complexity within a single T4SS (Gillespie *et al.* 2016). The *rvhB4*, *rvhB8*, *rvhB9* genes each have duplicate paralogs and the *rvhB6* gene has five paralogs. Each paralog is significantly divergent from one another, with as much as ~80% divergence between paralogs and some paralogs containing additional domains, like large N- and C-terminal extensions encoded
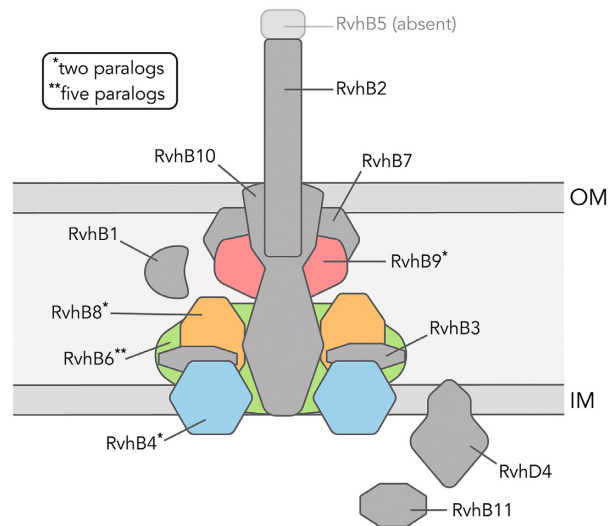


**Figure 3.** The rickettsial Type IV secretion system. The Type IV secretion system (T4SS) from the order Rickettsiales contains eleven distinct subunits termed Rickettsiales *vir* homologs (Rvh). Notably, the rickettsial T4SS has undergone extensive gene duplication. As a result, the RvhB4, RvhB6, RvhB8 and RvhB9 subunits have multiple paralogs (depicted in color). Also, the RvhB5 subunit is absent from the rickettsial T4SS. OM and IM indicate the rickettsial outer and inner membranes, respectively.

by several of the *rvhB6* genes. Both sets of *rvhB4*, *rvhB8*, *rvhB9* paralogs are expressed during a 24-hour window of *R. typhi* infection of HeLa cells (Gillespie *et al.* 2016). While expression of the *rvhB6* paralogs has not been examined in *Rickettsia*, all *rvhB6* paralogs are expressed during tick and human cell infection in *Ehrlichia chaffeensis*, a relative within the Rickettsiales order that encodes four *rvhB6* genes (Bao *et al.* 2009). How the rickettsial T4SS uses the expanded repertoire of subunits is not clear. It will be important to determine how these paralogous subunits are incorporated into T4SS complexes. For example, different paralogs may interact to form heterogeneous T4SS complexes containing multiple variants of each paralog. Alternatively, homogenous T4SS complexes might be assembled using a single paralog at a given time, which could enable rickettsiae to control effector secretion in a manner dependent on time, life cycle stage, or host cell type. It will be interesting to see how these paralogs impact substrate specificity and secretion efficiency and, ultimately, how they impact the infectious life cycle and pathogenesis.

As Type IV substrates are difficult to predict with bioinformatic approaches, we do not know the complete catalog of Type IV effectors in rickettsiae. Still, several recent studies have identified candidate Type IV effectors. In a bacterial two-hybrid experiment, the *R. typhi* protein RalF was shown to interact with RvhD4, a subunit of the T4SS involved in substrate recognition, suggesting that it is a Type IV effector (Rennoll-Bankert *et al.* 2015, 2016), similar to its homolog in *Legionella* (Folly-Klan *et al.* 2013). Another rickettsial protein, RARP-2, coimmunoprecipitates with RvhD4, suggesting it is also a T4SS substrate (Lehman *et al.* 2018; Aistleitner *et al.* 2020). Encouraged by the identification of RalF and RARP-2, a recent investigation sought to systematically identify Type IV effectors in *R. typhi* using coimmunoprecipitation with RvhD4 followed by mass spectrometry (Voss *et al.* 2020). This approach identified seven new putative Type IV effector proteins, including Pat2, a patatin-like phospholipase and Risk1 (Voss *et al.* 2020). However, this screen did not identify the full repertoire of Type IV effectors, as known Type IV effectors like RARP-2 were not detected. Therefore, more sensitive or

orthogonal approaches, including improved bioinformatic prediction software (Esna Ashari, Brayton and Broschat 2019), will be crucial in determining the full suite of effectors that interact with the rickettsial T4SS.

## A STREAMLINED AND MINIMALIST GENOME RELIANT ON HOST CELL METABOLISM

Rickettsiae are among a small number of bacterial pathogens that primarily reside in the cytosol of eukaryotic host cells, including *S. flexneri*, *L. monocytogenes*, *F. tularensis* and *B. pseudomallei*, with many other bacterial pathogens opting for a vacuolar-replicative niche (Ray *et al.* 2009). It is not clear why this uneven division between cytosolic and vacuolar bacterial pathogens exists, and to what extent the cytosol is permissive to bacterial growth. The eukaryotic cytosol is known to be strongly reducing (Hwang, Sinskey and Lodish 1992; Ray *et al.* 2009), but its precise nutritional content has not been fully defined and may vary across different cell types (Ray *et al.* 2009). Some modified strains of bacteria like *Bacillus subtilis* (Bielecki *et al.* 1990) and *E. coli* (Monack and Theriot 2001) can replicate efficiently in eukaryotic host cells, demonstrating that the cytosol can be growth permissive for bacteria that are not strictly evolved for that niche. Additionally, recent work has revealed an increasing relevance of the cytosolic niche for bacteria that were once thought to be strictly vacuolar, including *S. enterica* serovar Typhimurium, *Mycobacterium tuberculosis* and *Legionella pneumophila* (reviewed in (Fredlund and Enninga 2014)). Defining the metabolic interactions and exchange between rickettsiae and their host cells will help contribute to a deeper understanding of the eukaryotic cytosol, especially as a niche for bacterial pathogens at large. Such insights will also be crucial towards the development of an axenic medium for rickettsiae, which would transform the field by massively simplifying the process of culturing and genetic manipulation, similar to what has been done for the vacuolar obligate intracellular bacterium *Coxiella burnetii* (Omsland *et al.* 2008, 2009).

Over the course of evolutionary time, rickettsiae have undergone extensive reductive genome evolution, with an average genome size of around one million base pairs (Andersson *et al.* 1998; Blanc *et al.* 2007; Palmer and Azad 2012). The cytosolic lifestyle of rickettsiae allows them to scavenge nutrients from the host cell, relieving selective pressure to maintain key biosynthetic pathways in their genomes (Blanc *et al.* 2007; Fuchs *et al.* 2012; Palmer and Azad 2012; Driscoll *et al.* 2017). This defined niche has also allowed rickettsiae to lose genes needed for extracellular survival. Moreover, the intracellular environment may be less conducive to horizontal gene transfer, slowing the rate of gene gain in obligate intracellular bacteria (Newton and Bordenstein 2011; Weinert and Welch 2017). Genome reduction represents a common mode of evolution throughout all kingdoms of life (Wolf and Koonin 2013) and is associated with increased virulence in some bacterial pathogens (Moran 2002; Fournier *et al.* 2009; Weinert and Welch 2017; Diop, Raoult and Fournier 2018). For example, the loss of genes that negatively contribute to pathogenicity (e.g. antivirulence genes; Maurelli *et al.* 1998; McCormick *et al.* 1999) and immunogenic genes (e.g. flagella; Parkhill *et al.* 2001, 2003; Yang 2005) have been observed in several bacterial pathogens. Further, genome size can influence factors like cell size and replication rate, with smaller genomes leading to faster growth (Koskiniemi *et al.* 2012; Lee and Marx 2012). In *Rickettsia*, the loss of regulatory genes is associated with increased pathogenicity (Fournier *et al.* 2009), though this has

not yet been tested *in vivo*. Furthermore, no evidence was found for increased pathogenicity resulting from the gain of virulence genes in rickettsiae (Darby *et al.* 2007). Still, with a largely uncharacterized genome, more work is needed to fully understand the genetic determinants of pathogenicity in rickettsiae, as well as to provide insights into reductive genome evolution in bacterial pathogens.

Intracellular life has sculpted rickettsial genomes to become progressively more dependent on the host, while still maintaining a minimal set of genes required for infection and pathogenesis. The genomes of rickettsiae no longer encode numerous biosynthetic pathways in favor of scavenging nutrients from the host cell environment and have lost essential genes involved in glycolysis, gluconeogenesis, the pentose phosphate pathway, B vitamin synthesis and amino acid and nucleotide biosynthesis (Blanc *et al.* 2007; Palmer and Azad 2012; Driscoll *et al.* 2017). Reliance on the host cell environment for nucleotides is one potential explanation for the high AT-content of the genome (Dietel *et al.* 2019). Moreover, rickettsiae import energy-carrying molecules like ATP and $NAD^+$ from the host cell, despite being able to synthesize ATP on their own. Host cell-derived energy carriers may be essential for survival since the addition of ATP or $NAD^+$ partially restores the infectivity of extracellular rickettsiae (Bovarnick, Allen and Pagan 1953; Bovarnick and Schneider 1960). Although the $NAD^+$ transporter has not been identified, heterologous expression studies carried out in *E. coli* identified the nucleotide translocase Tlc1, an ATP/ADP symporter, as the mechanism of ATP import in rickettsiae (Winkler 1976; Krause, Winkler and Wood 1985; Audia and Winkler 2006). Additionally, the genomes of rickettsiae encode biosynthetic pathways that utilize isoprenoids but lack the genes needed for isoprenoid synthesis (Driscoll *et al.* 2017; Ahyong *et al.* 2019). Recent evidence has suggested that rickettsiae import isoprenoid precursors from the host cell (Ahyong *et al.* 2019). This study found that statins, FDA-approved drugs that inhibit the host cell isoprenoid synthesis, impaired *R. parkeri* infection *in vitro*, providing a promising route for future drug development. Continued characterization of the metabolic interactions between rickettsiae and their mammalian and arthropod hosts will reveal new avenues for therapeutic interventions, which will be crucial given the limited options available for treating rickettsial diseases.

## CONCLUSION AND OUTLOOK—CURRENT CHALLENGES AND THE PATH FORWARD

*Rickettsia* is a genus of diverse obligate intracellular bacteria with fascinating and unique biology but remains poorly understood at a molecular and genetic level. With several global, neglected and emerging human pathogens in the genus, it will be critical to gain a deeper understanding of their biology and virulence strategies. Since their discovery over 100 years ago, rickettsiae have proven challenging to study due to their obligate intracellular nature. One of the most significant roadblocks towards understanding the enigmatic biology of rickettsiae has been the lack of genetic tools. Still, this obstacle has started to diminish with recent developments in targeted knockout and transposon mutagenesis.

In this review, we highlighted several key exciting areas of unique biology in rickettsiae and the many fundamental questions that remain. We propose that future efforts in three fundamental and complementary approaches will pave the way towards unlocking these mysteries. First, more comprehensive

sampling and increased genome sequencing of *Rickettsia* species from diverse hosts in a variety of ecosystems will be critical for gaining a clearer picture of evolution and diversification within this genus. Second, further development of methods for profiling gene expression, metabolism and protein secretion will provide key insights into the molecular details of the intracellular life cycles of rickettsiae. Finally, new methods for genetic manipulation will be instrumental in determining the genetic basis of the intracellular life cycles and pathogenesis of rickettsiae. Altogether, the time is ripe for making fundamental advances in our understanding of rickettsiae, which will provide invaluable insights into the biology and evolution of obligate intracellular bacteria, host–pathogen interactions and eukaryotic cell biology.

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