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MINIREVIEWS

Phylogenomics Reveals a Diverse *Rickettsiales* Type IV Secretion System▽†‡

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With an obligate intracellular lifestyle, *Alphaproteobacteria* of the order *Rickettsiales* have inextricably coevolved with their various eukaryotic hosts, resulting in small, reductive genomes and strict dependency on host resources. Unsurprisingly, large portions of *Rickettsiales* genomes encode proteins involved in transport and secretion. One particular transporter that has garnered recent attention from researchers is the type IV secretion system (T4SS). Homologous to the well-studied archetypal *vir* T4SS of *Agrobacterium tumefaciens*, the *R*ickettsiales *v*ir *h*omolog (*rvh*) T4SS is characterized primarily by duplication of several of its genes and scattered genomic distribution of all components in several conserved islets. Phylogeny estimation suggests a single event of ancestral acquirement of the *rvh* T4SS, likely from a nonalphaproteobacterial origin. Bioinformatics analysis of over 30 *Rickettsiales* genome sequences illustrates a conserved core *rvh* scaffold (lacking only a *virB5* homolog), with lineage-specific diversification of several components (*rvhB1*, *rvhB2*, and *rvhB9b*), likely a result of modifications to cell envelope structure. This coevolution of the *rvh* T4SS and cell envelope morphology is probably driven by adaptations to various host cells, identifying the transporter as an important target for vaccine development. Despite the genetic intractability of *Rickettsiales*, recent advancements have been made in the characterization of several components of the *rvh* T4SS, as well as its putative regulators and substrates. While current data favor a role in effector translocation, functions in DNA uptake and release and/or conjugation cannot at present be ruled out, especially considering that a mechanism for plasmid transfer in *Rickettsia* spp. has yet to be proposed.

Type IV secretion systems (T4SSs) are macromolecular complexes that transport protein, DNA, and nucleoprotein across the bacterial cell envelope in both Gram-negative and Gram-positive species, as well as some wall-less bacteria and archaea (1, 32). Functioning in naked DNA uptake and release (60), conjugation (80), and the propagation of genomic islands (69), T4SSs are prominent factors in bacterial diversification and are responsible for the horizontal spread of antimicrobial resistance and virulence genes. T4SSs are also used by some species to deliver effector molecules (DNA and/or protein) into eukaryotic host cells (28), a process that facilitates infection and subsequent pathogenesis. It is assumed that all varieties of T4SSs form a channel that spans the cell envelope and culminates in a surface-exposed structure, such as a pilus (Fig. 1A). Despite this conserved architecture, genetic diversity in a multitude of features, including gene composition and organization, underlies the hundreds of T4SSs identified through genome sequencing. Recently, T4SSs have been classified into four groups: F, P, I, and GI (70). F-T4SSs and P-T4SSs (previously known as type IVA) are widespread systems represented by the archetypes encoded by the F plasmid of *Escherichia coli* (*tra* and *trb*) and the pTi plasmid of *Agrobacterium tumefaciens* (*vir*), respectively. I-T4SSs (previously known as type IVB) are typified by the *icm*/*dot* system of IncI plasmids, and examples in *Legionella* spp. and *Coxiella burnetii* are the best characterized. GI-T4SSs, distinct systems that function in transferring the genomic islands with which they are associated (70, 71), are also widespread and can be further classified into sublineages based on gene content and arrangement (73). The growing diversity of T4SSs will undoubtedly continue to challenge attempts at their classification and the unraveling of their evolutionary origins.

Alphaproteobacteria of the order *Rickettsiales* are diverse obligate intracellular species with a wide range of eukaryotic hosts (22, 23, 105, 125). Many species within the two well-characterized families, *Anaplasmataceae* and *Rickettsiaceae*, pose severe threats to livestock and human health. The agricultural and medical ramifications have resulted in the rapid accumulation of over 30 complete or nearly complete genome sequences from a diverse array of *Rickettsiales* taxa. Despite the common ancestry (127) and strictly intracellular lifestyles of *Rickettsiales*, the manner of genome reduction and reliance on host resources vary greatly across lineages (36, 63, 95).

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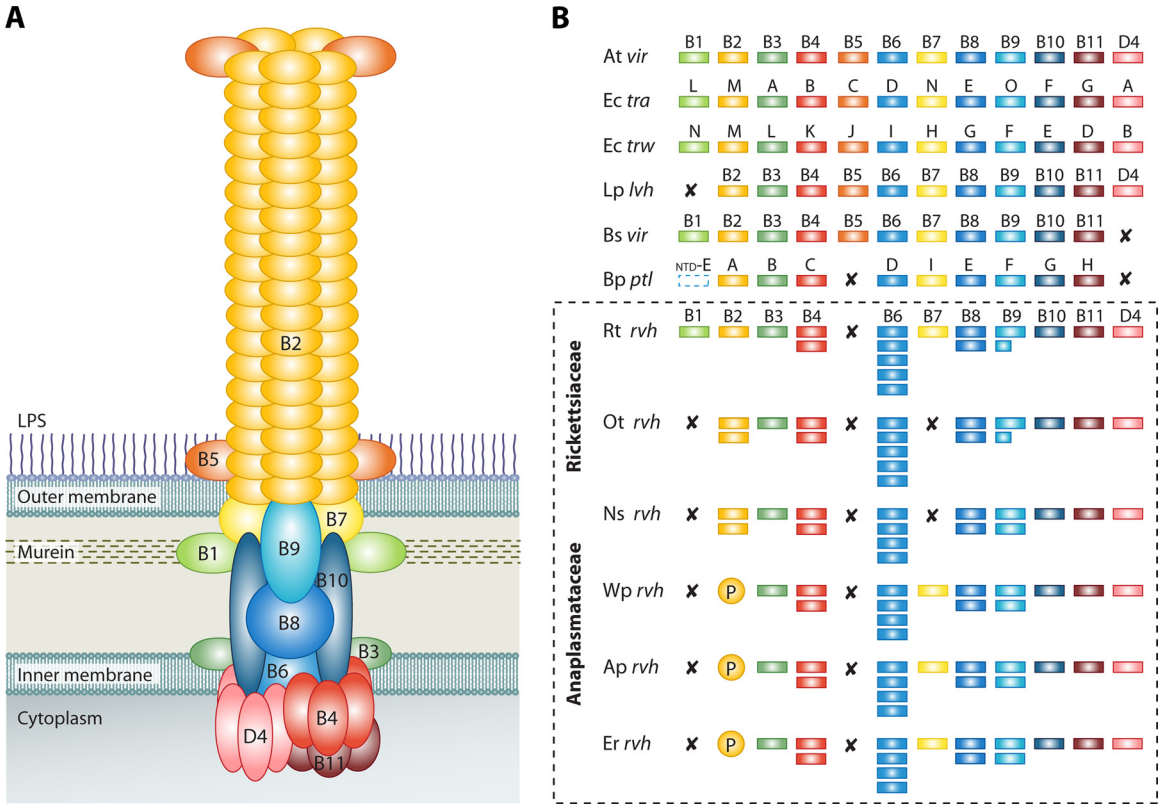

FIG. 1. P-T4SSs. (A) Model of the *vir* P-T4SS encoded on the pTi plasmid of *A. tumefaciens*. B1 to B11, VirB1 to VirB11; D4, VirD4. (B) Comparison of the *rvh* P-T4SSs from *Rickettsiales* with similar P-T4SSs from other bacteria. At *vir*, *A. tumefaciens* Ti plasmid P-T4SS; Ec *tra*, *E. coli* IncN plasmid pKM101 P-T4SS; Ec *trw*, *E. coli* plasmid R388 P-T4SS; Lp *lvh*, *L. pneumophila* P-T4SS; Bs *vir*, *Brucella suis* P-T4SS; and Bp *ptl*, *B. pertussis* P-T4SS. VirB1 of *B. pertussis* is depicted with the N-terminal glycohydrolase domain of PtlE (NTD-E) (107). The *rvh* examples are shown within the dashed-line box: Rt *rvh*, *R. typhi* P-T4SS; Ot *rvh*, *O. tsutsugamushi* P-T4SS; Ns *rvh*, *Neorickettsia sennetsu* P-T4SS; Wp *rvh*, *Wolbachia pipientis* P-T4SS; Ap *rvh*, *A. phagocytophilum* P-T4SS; and Er *rvh*, *Ehrlichia ruminantium* P-T4SS. X indicates that no gene for the component has been annotated and no subjects were detectable using tblastn; P represents the proliferation of *rvhB2* genes, putative VirB2-like encoding genes.

While few syntenic regions are found across *Rickettsiales* genomes (63), a conserved P-T4SS is a particularly definitive feature of these bacteria. Since the completion of sequencing of the first *Rickettsiales* genome, that of *Rickettsia prowazekii* (5), a reduced P-T4SS (lacking homologs of *virB1*, *virB2*, *virB5*, and *virB7*) has been uncovered in all subsequently sequenced genomes, with anomalous duplication of genes homologous to *virB4*, *virB6*, *virB8*, and *virB9* suggesting rich functionality and with genes split into multiple islets across the genomes. We recently performed a detailed informatics analysis of the P-T4SS of *Rickettsia* spp. and concluded that, relative to the canonical *vir* P-T4SS of *A. tumefaciens*, this transporter lacks only a homolog of *virB5*, the gene encoding the minor pilus subunit (55).

In this review, we expand our prior analysis of the *Rickettsia* T4SS, in which we named this transporter *rvh* (*R*ickettsiales *v*ir *h*omolog), to encompass T4SSs of all *Rickettsiales* (Fig. 1B). An assumption is made that the acquisition of a P-T4SS was pivotal in the transition from an extracellular to an obligate intracellular lifestyle. We address the nature of duplication of *rvh* components (*rvhB4*, *rvhB8*, and *rvhB9*) and proliferation of another component (*rvhB6*) and draw special attention to the components that tend to elude automated genome annotation (*rvhB1*, *rvhB2*, and *rvhB7*). The translocated proteins encoded by the latter genes define the most plastic attributes of the *rvh* P-T4SS and, coupled with the deletion of a *virB5* homolog, imply coevolution of the transporter and the bacterial cell envelope. Despite conservation of the *rvh* T4SS across the *Rickettsiales*, there is little information regarding *rvh* regulation and substrate transport. Learning more about the manner in which the various *rvh* T4SSs assemble and function in the bacterial outer membrane (OM) may present novel opportunities for vaccine development and drug targeting, and we discuss these possibilities in relation to *rvh* adaptations to host cell environments.

LATERAL ACQUISITION OF THE *rvh* P-T4SS

Previously, it was determined by phylogenetic analysis of P-T4SSs that lateral gene transfer (LGT) has spread these transporters across divergent bacterial lineages and that effector molecule translocation has evolved from the primitive function of conjugation (50). Consistent with this observation, it has been demonstrated previously that the *rvh* T4SS is related to P-T4SSs from certain *Gammaproteobacteria* (*Legionella* spp. and *Photobacterium profundum*) and *Epsilonproteobacteria*

(*Helicobacter pylori*, *Wolinella succinogenes*, and *Campylobacter jejuni*) (26, 93). Our phylogeny estimation for 47 P-T4SSs based on half of the *vir*-like components (*virB4*, *virB8* to *virB11*, and *virD4*) is in accord with this notion that the *rvh* P-T4SS was derived from a non-*Alphaproteobacteria* ancestor (Fig. 2). We recovered the *trw* P-T4SS, carried on plasmid pXcB of the *Gammaproteobacteria* species *Xanthomonas citri*, as the closest xenolog of *rvh*, as in a previous analysis based on 73 VirB4 and VirB4-like proteins (50). As demonstrated in other studies, the *lvh* P-T4SSs of *Legionella* spp., the *trb* P-T4SS of *P. profundum*, and the unnamed P-T4SS carried on plasmid pMLa of *Mesorhizobium loti* are the next closest xenologs of *rvh*, all having branched from the P-T4SSs of *H. pylori*, *C. jejuni*, and *W. succinogenes* that are involved in DNA uptake and release.

Our analysis, as well as the findings of prior studies (55, 106, 108), supports the vertical transmission of the *rvh* T4SS after the split of "*Candidatus* Pelagibacter" from the *Rickettsiales* ancestor. The most parsimonious recreation of an LGT event that equipped this ancestor with a P-T4SS involves a transporter containing 12 genes (*rvhB1* to *rvhB11* and *rvhD4*), with ancestral deletion, recombination, and duplication events creating a modified transporter comprising 18 genes (*rvhB1* to *rvhB3*, *rvhB4a* and *rvhB4b*, *rvhB6a* to *rvhB6e*, *rvhB7*, *rvhB8a* and *rvhB8b*, *rvhB9a* and *rvhB9b*, *rvhB10*, *rvhB11*, and *rvhD4*). The modern genomic distribution of this ancestral operon is fragmented across all sequenced *Rickettsiales* genomes (Fig. 3; see also Fig. S1 in the supplemental material) and, coupled with lineage-specific gene deletion, proliferation, and truncation events, defines a variable *rvh* P-T4SS.

NATURE OF *rvh* DUPLICATION

For *Rickettsia* spp., we reported previously that only one ortholog of each of the singly duplicated genes (*rvhB4a*, *rvhB8b*, and *rvhB9a*) adhered to the conserved properties of similar genes in other P-T4SSs (55). Both *rvhB4b* and *rvhB8a* encode proteins with similar defects in *Rickettsia* spp. and the other *Rickettsiales*, as observed in our prior informatics analysis (see Table S1 in the supplemental material). In contrast, the product of *rvhB9b*, which in *Rickettsia* spp. is missing the C-terminal domain, demonstrated previously via nuclear magnetic resonance (NMR) (16), cryo-electron microscopy (52), and crystallography (29) to bind VirB7 in other systems, is full-length in the *Anaplasmataceae* (see Table S1 in the supplemental material). We had hypothesized (55), given the mirrored arrangements of the *rvhB9a-rvhB8a* locus (antisense) and the *rvhB8b-rvhB9b* locus (sense), with *rvhB7* at the center of these loci (Fig. 3), that an intrachromosomal recombination event truncated *rvhB9b* in *Rickettsia* spp.; however, this pattern does hold throughout the *Rickettsiales* order. *rvhB9b* is also truncated in the genomes of *Orientia tsutsugamushi* strains, yet the *rvhB8b-rvhB9b* locus is well separated from the *rvhB9a-rvhB8a* locus in *O. tsutsugamushi* genomes, as it is in the *Anaplasmataceae* genomes. The exact manner in which *Rickettsiaceae rvhB9b* became truncated is unclear; either the entire C islet (*rvhB9a rvhB8a rvhB7 rvhB8b rvhB9b rvhB10 rvhB11 rvhD4*) exhibits the ancestral gene order and the *rvhB9a-rvhB8a-rvhB7* segment became separated outside *Rickettsia* spp. or a recombination event coupled the duplicate *rvhB9* and *rvhB8* genes in *Rickettsia* spp. to the remaining genes within islet C. Nonetheless, a major difference between the T4SSs of *Anaplasmataceae* and *Rickettsiaceae* is the presence of two full-length copies of *rvhB9* in the former and a truncated *rvhB9b* in the latter.

The proliferated *rvhB6* genes, encoding proteins RvhB6a to RvhB6d, are present in most sequenced *Rickettsiales* genomes and are arrayed contiguously within one predicted operon (Fig. 3). A fifth gene, *rvhB6e*, is found only in *Rickettsiaceae* genomes, and while arrayed with *rvhB6a* to *rvhB6d* in the *Rickettsia* genomes, the gene is well separated from the *rvhB6* operon in *O. tsutsugamushi* genomes. The proteins encoded by *rvhB6* are perhaps the most intriguing features of the *rvh* P-T4SS. While all the proteins contain a complete VirB6/TrbL (PF04610) domain that is comparable to full-length VirB6 and VirB6-like orthologs in other species harboring P-T4SSs, the *Rickettsiales* open reading frames (ORFs) encode additional flanking regions that lack similarity to any proteins in public databases (55). The nature of these "gangly arms" flanking the VirB6/TrbL domain, coupled with the unusual proliferation of the corresponding genes in the highly reduced *Rickettsiales* genomes, in which gene duplication is atypical, brings attention to these curious proteins. VirB6/TrbL proteins resemble ComEC channel proteins, which are involved in the uptake of environmental DNA (42). It is possible that RvhB6 proteins play a role in DNA import/export, with the various duplications forming multiple diverse channels that maximize the potential for LGT in environments (such as those in protozoa and macrophages) with high rates of congener contact. Given that VirB6 in *A. tumefaciens* has been demonstrated previously to facilitate substrate transfer (66) yet is not a component of the core P-T4SS channel (52), the multiple RvhB6 proteins may equip *Rickettsiales* with substrate selectivity in the various host environments encountered by these obligate intracellular bacteria. However, the strong selection keeping the *rvhB6* loci contiguous, presumably within tightly regulated operons (55), suggests that posttranscriptional regulation of *rvhB6* genes would be necessary for environment-specific utilization of the various duplicate proteins in the bacteria. This idea is supported by the *rvhB6e* gene of *O. tsutsugamushi* strain Ikeda, which is truncated and well separated from the remaining *rvhB6* genes in islet A (Fig. 3). However, all four *rvhB6* genes of *Ehrlichia chaffeensis* are coexpressed in tick and human cells, and various RvhB6-RvhB6 and RvhB6-RvhB9 interactions suggest more than one RvhB6 protein may assemble at the inner membrane (IM) channel region of the *rvh* T4SS (8). Nonetheless, given that two rickettsial RvhB6-encoding genes (*rvhB6d* in *R. bellii* strain OSU 85 389 and *rvhB6e* in *R. massiliae*) (see Table S2 in the supplemental material) have undergone pseudogenization, not all copies of these genes may be functional despite the near global conservation across the *Rickettsiales*.

THE ENIGMATIC RvhB2 PROTEINS

VirB2 and related proteins constitute the major subunit of the *Agrobacterium* T-pilus and related structures (77, 79). The signal sequences of these proteins are processed in the IM, with subsequent species-specific processing of the C and/or N termini (44, 45, 72, 78, 80, 92, 112). This role in T-pilus formation is supported by findings from various studies demon-

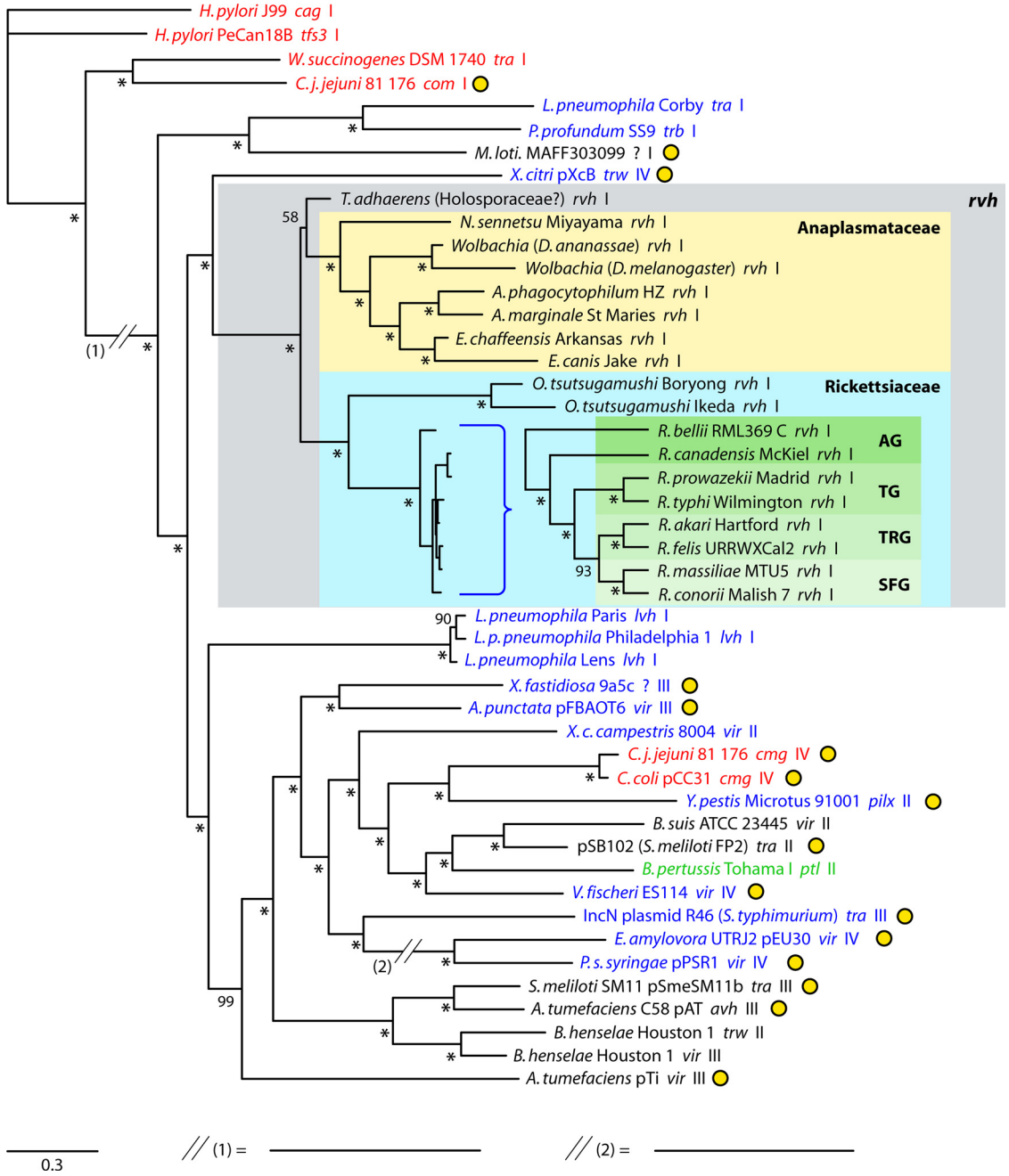

FIG. 2. Estimated phylogeny for 47 diverse P-T4SSs. Taxa are colored according to proteobacterial class: red, *Epsilonproteobacteria*; blue, *Gammaproteobacteria*; black, *Alphaproteobacteria*; and green, *Betaproteobacteria*. Each taxon name is appended with its P-T4SS nomenclature and a number (I, II, III, or IV) referring to recently proposed categories of P-T4SSs (93). Plasmid-encoded P-T4SSs are indicated by yellow circles. The 18 sampled *Rickettsiales* taxa are within a gray box. *Rickettsia* spp. are shown as a cladogram due to limited sequence divergence relative to that in the remaining taxa, with the following abbreviations: AG, ancestral group; TG, typhus group; TRG, transitional group; and SFG, spotted fever group (56, 57). The tree is from two independent Bayesian analyses of six P-T4SS proteins (VirB4, VirB8 to VirB11, and VirD4). The topology of the sampled tree with the greatest likelihood (Lnl = −201,623.248) is shown, with branch support assessed from probabilities of clade occurrence in the posterior distribution of 1,906 sampled trees. Asterisks indicate probabilities of 100%. For *Wolbachia* species, the species in which they are symbionts are listed in parentheses (*D. ananassae*, *Drosophila ananassae*; *D. melanogaster*, *Drosophila melanogaster*). *C. j. jejuni*, *C. jejuni* subsp. *jejuni*; *T. adhaerens*, "*Trichoplax adhaerens*"; *L. p. pneumophila*, *L. pneumophila* subsp. *pneumophila*; *X. fastidiosa*, *Xylella fastidiosa*; *A. punctata*, *Aeromonas punctata*; *X. c. campestris*, *Xanthomonas campestris* pv. *campestris*; *C. coli*, *Campylobacter coli*; *Y. pestis*, *Yersinia pestis*; *S. meliloti*, *Sinorhizobium meliloti*; *V. fischeri*, *Vibrio fischeri*; *S. typhimurium*, *Salmonella enterica* serovar Typhimurium; *E. amylovora*, *Erwinia amylovora*; *P. s. syringae*, *Pseudomonas syringae* subsp. *syringae*; *B. henselae*, *Bartonella henselae*.

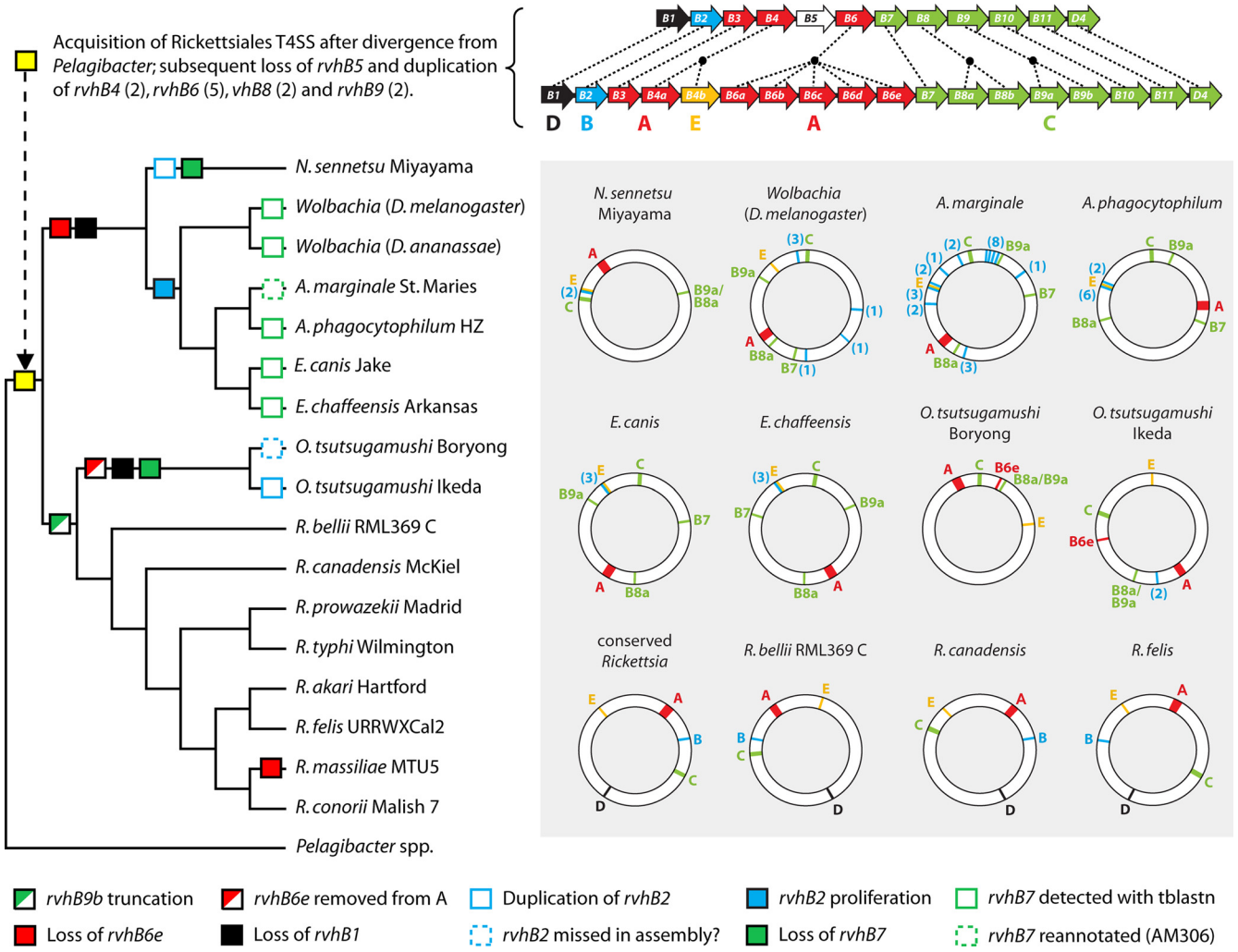

FIG. 3. Diversification in *rvh* T4SS architecture across the major lineages of *Rickettsiales*. The schematic at the top depicts the deletion and duplication events that possibly occurred prior to the split of the major *Rickettsiales* lineages (corresponding to the yellow box at the root of the cladogram). The color scheme for the *rvh* components is consistent throughout the figure and reflects the five islets (A to E) of the *Rickettsia rvh* P-T4SS described previously (55). The cladogram is simplified from data obtained by phylogeny estimation across 31 *rvh* P-T4SSs (see Fig. S1 in the supplemental material). Colored boxes in the cladogram depict deviations from the ancestral *rvh* P-T4SS and are explained at the bottom. Synteny maps (shaded inset) are shown for select taxa. Note that in *Rickettsiales* other than *Rickettsia* spp., *rvhB9a*, *rvhB8a*, and *rvhB7* are removed from islet C. Also, multiple *rvhB2* orthologs per genome are depicted, with corresponding numbers in parentheses.

strating the presence of VirB2 in complexes with VirB5 (the minor subunit of the T-pilus) and VirB7 (17, 67, 76, 86, 115, 132). In particular, the VirB2-VirB5 pilus complex is dependent on the periplasmic interaction between VirB4 and VirB8, which promotes the formation of extracellular pili (132). Unlike pTi-encoded VirB5 ($VirB5_{Ti}$), $VirB2_{Ti}$ is critical for substrate transfer through the P-T4SS scaffold (27, 64). Polymers (115) of these proteins probably span the entire periplasm (32), as made evident by the presence of at least two predicted transmembrane-spanning regions in nearly all available sequences (55). Recently, it was hypothesized that VirB2 polymers might "snake" along the VirB10 antenna domain, lining the outside of the entire P-T4SS channel from the IM to the OM (65) and binding VirB5 at the OM, with continual polymerization as a T-pilus in species that protract these extracellular appendages. However, it cannot be ruled out that VirB2 polymerizes within the chamber of the VirB7-VirB9-VirB10 core complex prior to binding VirB5 and extending extracellularly as the T-pilus (51), a hypothesis supported by the observation that the transmembrane helices within the TraF/VirB10 crystal appear to be caved in (in the absence of VirB2) (29).

Several prior reviews of T4SSs reported a lack of genes encoding either the major or the minor pilin subunit within *Rickettsiales* genomes. However, our recent bioinformatics analysis of 13 *Rickettsia* genomes identified a candidate VirB2-encoding ORF, *rvhB2*, predicted to be a single transcriptional unit well separated from other T4SS genes (55). In certain *Anaplasmataceae* genomes, a duplicated ORF previously named *orfX* (24, 94), associated with the major surface protein 2 (*msp2*) superfamily, has also recently been designated a putative *virB2* homolog (23, 99). Here, we draw attention to recently identified ORFs in the

O. tsutsugamushi and *Anaplasmataceae* genomes which suggest that VirB2 proteins are likely to be an essential component of the *rvh* P-T4SS (see references 35 and 114 and Fig. S2 in the supplemental material). Interestingly, unlike the genomes of *Rickettsia* spp., the remaining *Rickettsiales* genomes have *rvhB2* present at least twice and the genomes of *Wolbachia*, *Anaplasma*, and *Ehrlichia* species show proliferation of this gene (yielding three or more paralogs) (Fig. 3). Together with a closely related ORF named *orfY*, a total of 22 candidate *rvhB2* sequences in the *Anaplasma marginale* genome were identified (data not shown), exemplifying the operation of selection on the retention of numerous *rvhB2* paralogs in the derived *Anaplasmataceae*. Like that of other duplicate *rvh* genes in *Rickettsiales*, the expression of *rvhB2* paralogs may be specific to the host environment. Supporting this hypothesis, the *rvhB2* paralogs of *Anaplasma phagocytophilum* are differentially expressed in tick and mammalian cell cultures (99). Alternatively, based on its probable secretion to the OM and likely exposure to the host immune system, the major pilin component may have become co-opted into a diverse antigen family that increases the chance of host immune avoidance, a role other duplicate genes and functional pseudogenes in the *msp2* superfamily have (23). Supporting this possibility, the majority of *rvhB2* genes in *A. marginale* are arrayed with *msp2* and *msp3* genes and their associated functional pseudogenes (25). Regardless of the provisional role that RvhB2 plays in *rvh* assembly and function, experimental evidence suggests surface exposure of the protein. For example, in *A. marginale*, RvhB2 induces a T-cell response in cattle as part of a protective bacterial membrane vaccine (89, 119), and the two orthologs in *Neorickettsia risticii* are coexpressed by an operon and localized predominantly at the poles, where they form focal complexes (85).

RvhB7: THE NEEDLE IN THE HAYSTACK

T4SSs typically encode small lipoproteins under 100 amino acids that are secreted to the periplasm and are essential for substrate transfer. In *A. tumefaciens*, the small lipoprotein VirB7 primarily binds and stabilizes VirB9 in the OM (13, 47, 118) via an essential disulfide bond (4, 11, 62, 132). Structural studies of this interaction in the P-T4SS encoded by the plasmid pKM101 of *E. coli* illustrate that a disulfide bond is not universal (16, 52), suggesting that other protein interactions in VirB7-VirB9 heterodimer formation can suffice (9). Along with VirB10 and VirB9, VirB7 is a component of the core T4SS complex, and it is inserted into the OM with the C-terminal domains of VirB9 and VirB10 (52). Prior comprehensive studies of type IV secretion have noted that VirB7 and related proteins are not always encoded within T4SSs (26, 93). However, given their small size, it is likely that many ORFs encoding these lipoproteins are not annotated by automated gene prediction methods, especially if the genomic positions of these ORFs are not arrayed with those of other T4SS genes.

We recently identified a putative VirB7 homolog, RvhB7, encoded within all sequenced genomes of *Rickettsia* spp. (55). *rvhB7* is located upstream of the *rvhB8b-rvhB9b-rvhB10-rvhB11-rvhD4* cluster and is flanked by the *rvhB9a-rvhB8a* locus, which is carried on the opposite strand (Fig. 3). Bioinformatics analysis revealed that RvhB7 contains at least one Cys residue in addition to the conserved lipoprocessing Cys that is characteristic of all VirB7-like proteins, suggesting that RvhB7 is similar to $VirB7_{Ti}$ and other T4SS lipoproteins that may bind the C-terminal domain of VirB9 via a disulfide bridge. Other than these conserved features, RvhB7 proteins contain a candidate P(ILV)NK motif in the C-terminal region that is typical of most VirB7-like sequences (16). Additionally, a conserved sequence, (KI)KSP, directly flanking the second conserved Cys on the N-terminal side was found to be a feature shared only by the RvhB7 proteins of the *Rickettsia* spp. and the ComB7 proteins of *H. pylori* and *C. jejuni*, illustrating the possibility that *rvh* T4SSs descended from DNA competence systems (55).

Herein, we expand our analysis of putative rickettsial T4SS lipoproteins and illustrate that these molecules are likely to be part of the conserved *rvh* core as in other systems (Fig. 4). RvhB7 sequences of *Rickettsia* spp. were used to identify hypothetical proteins encoded in the genomes of *A. marginale* (hypothetical protein AM306) and the *Wolbachia* symbiont of *Culex quinquefasciatus* (hypothetical protein WPa_0823) that share the conserved characteristics of RvhB7 proteins. More refined tools revealed putative ORFs in most of the remaining sequenced *Anaplasmataceae* genomes that are currently not annotated as genes. Each of the protein sequences analyzed contains a predicted lipoprocessing site (Cys), a second conserved Cys, and several candidate P(ILV)NK motifs. The latter feature continues to become less conserved with the addition of more diverse VirB7-like sequences, and even simplifying the motif to PhN+, where h and + represent nonpolar and positively charged residues, respectively, still does not encompass the diversity in the C-terminal regions of these sequences or facilitate approaches to multiple-sequence alignment. The addition of these putative *Anaplasmataceae* sequences refines the conserved region shared by RvhB7 and the ComB7 proteins of *H. pylori* and *C. jejuni* to (K/R)SP, further supporting the evolution of *rvh* from competence systems. Additionally, we identified the (K/R)SP motif in the TrwH protein of *X. citri*, which is consistent with the phylogenetic position of the *trw* T4SS of *X. citri* as the closest xenolog of *rvh* (Fig. 2). Importantly, a recent study reports that the putative RvhB7 protein in *A. marginale* OM vaccine preparations is immunogenic for cattle vaccinees expressing several common major histocompatibility complex class II haplotypes (119). Altogether, these data strongly imply that RvhB7 is part of the conserved core *rvh* T4SS (RvhB7, RvhB9, and RvhB10) and is possibly surface exposed at the OM.

LOCALIZED MUREIN DEGRADATION IS SPECIFIC TO *RICKETTSIA*

Efficient T4SS transporter assembly across the cell envelope typically is associated with local disruption of peptidoglycan (PG) (Fig. 1). Descending from the free-living "*Candidatus* Pelagibacter," *Rickettsiales* have not been found to encode a complete pathway for the synthesis of PG that is independent of host resources (see Fig. S3 in the supplemental material). PG from *R. prowazekii* similar to PGs from other Gram-negative species has been purified and demonstrated to incorporate D-alanine (103), and it is likely that a murein layer is

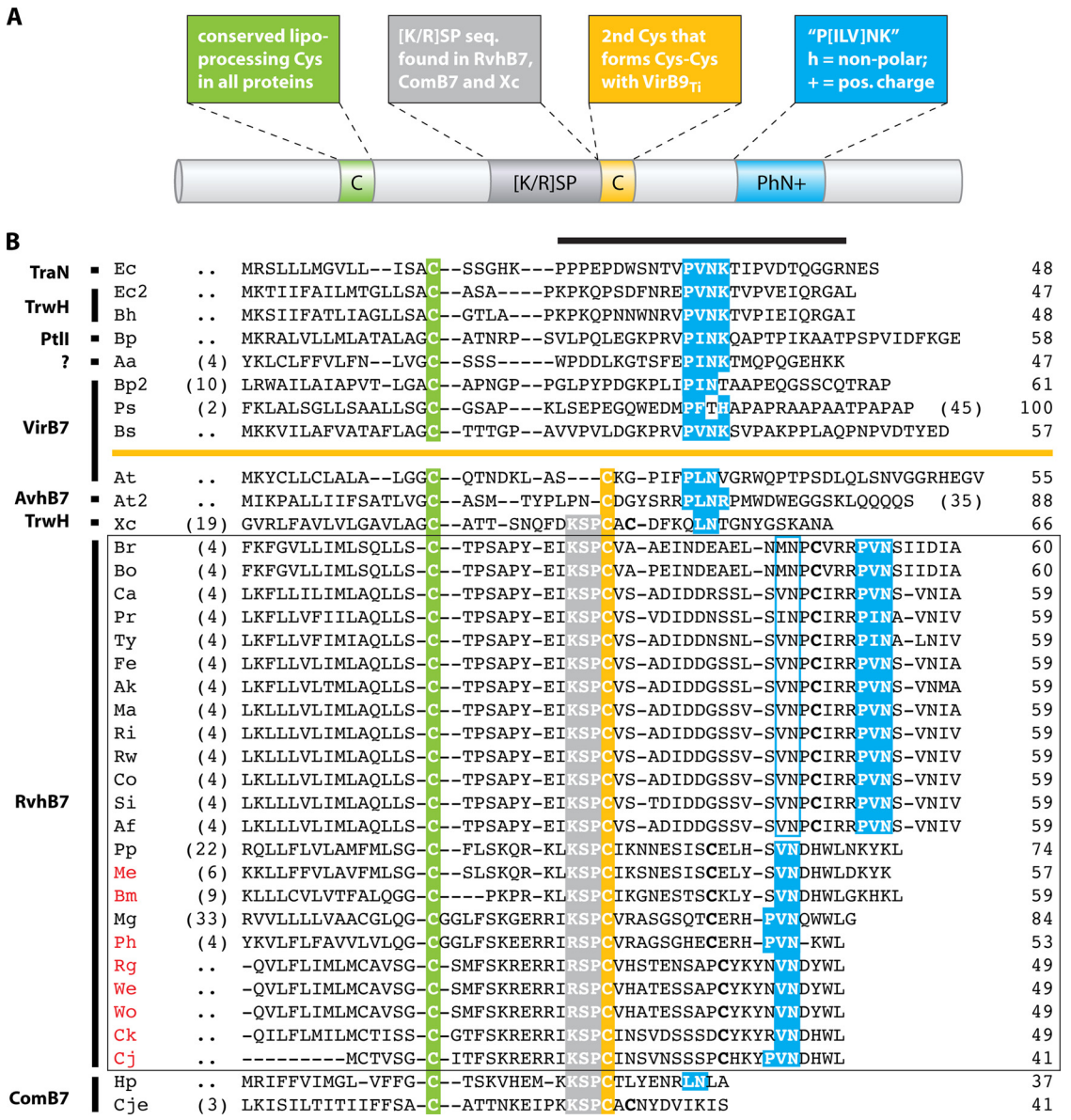

FIG. 4. P-T4SS lipoproteins. (A) Schematic depiction of typical characteristics of VirB7 and related proteins. The color scheme is used to identify distinct features in the alignment (see the text for explanation). (B) Manual alignment of 13 diverse VirB7 and VirB7-like lipoproteins with 23 RvhB7 lipoproteins (the latter are boxed). Coordinates for each sequence are shown to the right, with numbers in parentheses to the left referring to flanking residues not shown in the alignment. Protein annotation is shown to the left. The black horizontal bar over the sequences of the predicted processed lipoproteins identifies the region of the NMR structure for the interaction of the TraO C-terminal domain (VirB9-like protein) and TraN (VirB7), both encoded by plasmid pKM101 in *E. coli*. Sequences above the orange line depict lipoproteins without a predicted Cys-Cys interaction with VirB9 and VirB9-like proteins: Ec, *E. coli* TraN, encoded by plasmid pKM101 (accession no. NP_511194); Ec2, *E. coli* TrwH protein (accession no. FAA00034); Bh, *B. henselae* TrwH-like protein (accession no. AAM82208); Bp, *B. pertussis* TraI protein, encoded by plasmid pSB102 (accession no. NP_361043); Aa, *Aggregatibacter actinomycetemcomitans* lipoprotein (accession no. NP_067577); Bp2, *B. pertussis* putative bacterial secretion system protein (accession no. NP_882291); Ps, *P. syringae* subsp. *syringae* VirB7 (accession no. NP_940729); and Bs, *B. suis* VirB7 (accession no. AAN33275). Sequences below the orange line represent proteins predicted to interact with VirB9 and VirB9-like proteins via a Cys-Cys bond: At, *A. tumefaciens* VirB7 (accession no. NP_536291); At2, *A. tumefaciens* AvhB7 (accession no. NP_396098); Xc, *X. citri* VirB7 (accession no. NP_942612); Br, *R. bellii* strain RML 369-C RvhB7 (accession no. YP_538183); Bo, *R. bellii* strain OSU 85 389 RvhB7 (accession no. YP_001495873); Ca, *R. canadensis* strain McKiel RvhB7 (accession no. YP_001492545); Pr, *R. prowazekii* strain Madrid E RvhB7 (accession no. NP_220672); Ty, *R. typhi* strain Wilmington RvhB7 (accession no. YP_067241); Fe, *R. felis* strain URRWXCal2 RvhB7 (accession no. YP_246480); Ak, *R. akari* strain Hartford RvhB7 (accession no. YP_001493229); Ma, *R. massiliae* strain MTU5 RvhB7 (accession no. YP_001499186); Ri, *R. rickettsii* strain Sheila Smith RvhB7 (accession no. YP_001494506); Rw, *R. rickettsii* strain Iowa RvhB7 (accession no. YP_001649753); Co, *R. conorii* strain Malish 7 RvhB7 (accession no. NP_360023); Si, *R. sibirica* strain 246 RvhB7 (accession no. ZP_00142155); Af, *R. africae* strain ESF-5 RvhB7 (accession no. ZP_02336216); Pp, protein of the *Wolbachia* symbiont of *C. quinquefasciatus* (accession no. YP_001975581); Me, protein of the *Wolbachia* symbiont of *D. melanogaster*; Bm, protein of the *Wolbachia* symbiont of *Brugia malayi*; Mg, *A. marginale* protein (accession no. YP_153652); Ph, *A. phagocytophilum* protein; Rg, *E. ruminantium* strain Gardel protein; We, *E. ruminantium* strain Welgevonden (Erwe) protein; Wo, *E. ruminantium* strain Welgevonden (Erum) protein; Ck, *E. chaffeensis* strain Arkansas protein; Cj, *E. canis* strain Jake protein; Hp, *H. pylori* protein (accession no. CAA10654); and Cje, *C. jejuni* protein (accession no. NP_863349). RvhB7 taxon codes colored red indicate that sequences corresponding to putative unannotated ORFs were recovered from tblastn searches of the *Anaplasmataceae* database (sequence coordinates are listed in Table S2 in the supplemental material). Additional conserved Cys residues in the RvhB7 sequences are in bold. A second putative PhN+ motif in the *Rickettsia* RvhB7 sequences is shaded in blue.

synthesized in all *Rickettsia* spp., starting from host reserves of fructose-6-phosphate and/or glucosamine-6-phosphate. Prior studies have failed to detect PG in *O. tsutsugamushi* (3, 117), and a lack of enzymes able to convert fructose-6-phosphate to UDP-*N*-acetylglucosamine suggests the absence of PG in the cell envelope. Furthermore, *alr*, which encodes the enzyme that converts L-alanine to D-alanine, is deleted from *O. tsutsugamushi* genomes, as it is from all *Anaplasmataceae* genomes. Despite this finding, genes for most of the enzymes involved in amino sugar metabolism are present in the *O. tsutsugamushi* genomes (31, 98), as well as in *A. marginale* and *Wolbachia* genomes (49, 130). The same is true for genes encoding the enzymes responsible for the synthesis of lipid I and lipid II and the transport of anhydromuropeptides to the periplasm via the MurJ flippase (111). Genes encoding enzymes responsible for modification (i.e., transpeptidation and transglycosylation) of PG in the periplasm are highly conserved in *Rickettsia* spp. but found sporadically in the remaining *Rickettsiales* genomes. Outside the *Rickettsiaceae*, only *A. marginale* contains *slt*, the gene encoding the soluble lytic transglycosylase (LT) responsible for the excision of PG subunits from the murein layer. *Rickettsia* spp. encode the most complete pathway for recycling PG, with atypical proliferation of the IM AmpG permeases involved in PG subunit import to the cytoplasm (discussed below). Thus, from a genomics perspective, *Rickettsia* spp. and *A. marginale* are the only *Rickettsiales* that likely incorporate PG into their cell envelopes. The lack of rigid cell envelopes in *Anaplasmataceae* aside from *A. marginale* supports this viewpoint (33, 84, 109).

It is common for type II, III, and IV secretion systems, as well as DNA competence systems and bacteriophages, to encode LTs that hydrolyze PG (14, 40, 74, 81, 96). These specialized LTs (75) facilitate the local disruption of PG, allowing for efficient transporter assembly across the entire cell envelope or for host cell penetration in the case of bacteriophages. While typically much smaller than Slt and related PG autolysins, specialized LTs contain similar lysozyme-like folds and belong to an ancient glycohydrolase superfamily that includes plant chitinases, bacterial chitosanases, goose and hen g-type lysozymes, and phage T4 lysozymes (110). One of the best-characterized specialized LTs is $VirB1_{Ti}$, which aside from its N-terminal lysozyme domain contains a processed C-terminal region that is secreted extracellularly and possibly involved in T-pilus formation (10, 133). While $VirB1_{Ti}$ is critical for T-pilus biogenesis, $VirB1_{Ti}$ mutants weaken but do not entirely abolish substrate transfer (15, 18, 53, 77), suggesting that Slt and/or other PG autolysins suffice in degrading PG for transporter assembly. VirB1-like proteins are also nonessential in other systems (14, 38, 39, 129); however, in the *cag* P-T4SS of *H. pylori*, the specialized LT is critical for both CagA translocation/phosphorylation and interleukin-8 induction in host cells (48).

We recently identified a *virB1* homolog (*rvhB1*) that is conserved in all sequenced genomes of *Rickettsia* (55), supporting the likelihood that PG is a component of *Rickettsia* species cell envelopes. While *rvhB1* is located independently of the other *rvh* genes in these genomes (Fig. 3), there is strong *in silico* evidence supporting RvhB1 as a specialized LT (55). Informatics data suggest that no RvhB1 homologs are encoded in the remaining *Rickettsiales* genomes (data not shown). Thus, if PG is synthesized in *A. marginale* and/or *Wolbachia* spp., the manner in which the T4SS scaffold assembles and spans the periplasm is likely to be different from that in *Rickettsia* spp.

Rvh REGULATION, SUBSTRATES, AND PUTATIVE FUNCTION

The *vir* P-T4SS of *A. tumefaciens* and the *icm/dot* I-T4SS of *Legionella pneumophila* are regulated by two-component systems (54, 128, 134), suggesting tight correlation of T4SS gene expression and function. Other single genes have been demonstrated previously to control the expression of the *vir* loci of *Brucella* spp. (37, 41, 116). Regarding several intracellular species, it has been demonstrated that host conditions upregulate T4SS operons (21, 30, 113). Clustering of T4SS genes into one or a few operons undoubtedly enhances their coordinated regulation (32, 82). However, the conserved *rvh* T4SS is scattered in small islets throughout the genome in all lineages (Fig. 3), and in *Rickettsia* genomes, the 18 genes can be grouped into five islets (55). Counterbalancing this architectural anomaly, a single T4SS transcriptional regulator, *ecxR* (ECH_0795), has been identified in *E. chaffeensis* and induces coordinate expression of several of the *rvh* islets (30). Informatics data indicate that *ecxR* homologs are present in all genomes of the *Anaplasmataceae* but are not present in the *Rickettsiaceae* (see Table S3 in the supplemental material). Previously, we identified two conserved genes in *Rickettsia* spp. that are carried within predicted *rvh* operons. A RelA/SpoT-encoding gene adjacent to *rvhD4* is of interest given that a stringent response protein similar to RelA/SpoT, Rsh, regulates the *vir* loci of *Brucella* spp. (41). A second gene encoding a conserved hypothetical protein is immediately downstream of the *rvhB1* gene in all sequenced genomes, possibly having a role associated with type IV secretion. Undoubtedly, there will be further characterized *rvh* regulators given the need to coordinately express the scattered *rvh* islets.

Despite the *rvh* P-T4SS's being one of the features most conserved across *Rickettsiales* genomes, the function of the *rvh* P-T4SS is poorly characterized (55). The AnkA protein of *A. phagocytophilum*, which penetrates neutrophil nuclei and is essential for host infection (83, 104), has been described as the first *rvh* T4SS substrate and is able to be secreted via the *vir* P-T4SS of *A. tumefaciens* (83). However, no other protein or DNA molecules have been identified as substrates of the *rvh* T4SS, and the exact manner in which substrates are presented to and secreted by the transporter are unknown. For *Rickettsia* spp., two genes have garnered attention for possibly encoding *rvh* substrates. *ralF*, a gene encoding a Sec7 domain-containing protein, is known in prokaryotes only from *Rickettsia* spp. and *Legionella* spp. (34), and in *L. pneumophila* the protein is an I-T4SS effector that functions as a guanine nucleotide exchange factor in the recruitment of the ADP-ribosylation factor to occupied phagosomes (97). While the precise role of RalF in *Legionella* pathogenesis is unknown, an analogous function associated with phagosomal modification in *Rickettsia* spp. is unlikely given the immediate lysis of the phagosome upon host cell invasion. Furthermore, pseudogenization has eliminated nearly the entire *ralF* ORF in all sequenced genomes of spotted fever group rickettsiae. The second putative *rvh* effector is RickA, a protein considered to activate host

Arp2/Arp3 complexes, resulting in actin nucleation (58, 68), which permits intercellular spread of some rickettsiae (61). Based on informatics data, which predict neither membrane nor periplasmic association of RickA (data not shown), and the fact that the protein is localized to the bacterial surface (59), it was hypothesized that RickA is secreted via the T4SS (58). Like *ralF*, the RickA gene is not present in all sequenced genomes of *Rickettsia* spp.; hence, if either or both of these candidates are true T4SS effectors, the *rvh* T4SS would secrete different substrates in various species/strains of *Rickettsia*, possibly contributing to lineage-specific pathogenicity.

It was speculated previously that the *rvh* T4SS must function in virulence factor secretion versus plasmid transfer because not all sequenced rickettsia genomes contain plasmids (102). Interestingly, the same investigation not only uncovered the first case of a plasmid system in a rickettsia (*R. felis*) but also provided electron microscopy images of pilus-like structures that were assumed to be associated with conjugation (102). As there is no complete set of genes in the *R. felis* genome encoding type II or type IV pili (data not shown) and the absence of a *virB5* homolog would presumably prevent the formation of a T-pilus (as discussed above), the exact genetic architecture underlying these extracellular appendages in *R. felis* remains unknown. Similar pilus-like structures were observed in *R. bellii* strain RML 369-C (101) and *R. massiliae* (19), which both carry a nearly full set of genes related to the *tra-trb* operon of the F plasmid of *E. coli*, despite only the latter's harboring a plasmid system. Other rickettsiae harboring plasmids (e.g., *R. africae*, "*R. monacensis*," and *R. peacockii*) do not carry full *tra-trb*-like operons, and no pilus-like structures have been reported. Thus, the significance of a *tra-trb* operon in relation to extracellular appendages and their possible role in the conjugation of rickettsial plasmids remains quite nebulous. What is clear is that a large mobile genetic element, which carries an F-T4SS highly similar to the F plasmid *tra-trb* operon, is detectable as products of pseudogenization across the sequenced *Rickettsiales* genomes (data not shown) and is highly proliferated in the genomes of *O. tsutsugamushi* strains (31, 98). Similar conjugation genes have also been detected in various rickettsiae associated with arthropods with no known association with vertebrates (124). Recent sequencing of the rickettsial endosymbiont of *Ixodes scapularis* (REIS) has revealed the largest number of conjugation genes within a *Rickettsia* genome, suggesting that ancient conjugative plasmids have propagated mobile elements across the *Rickettsiales*. Strong reductive evolution has eliminated the majority of these elements in most genomes, in particular a complete F-T4SS likely to be essential for conjugative transfer of plasmids. The ability of the *rvh* P-T4SS to function in conjugation cannot be ruled out, but the absence of any identified plasmids or pilus-like structures in the *Anaplasmataceae* argues strongly against a role in conjugation. However, a function in naked DNA uptake and release from host environments, particularly given the close phylogenetic relatedness of *rvh* to T4SSs that are involved in DNA competence (Fig. 2), cannot be overlooked (55). *Rickettsiaceae* may benefit from scavenging nucleotides upon DNA uptake since genes involved in *de novo* synthesis of nucleotides have been deleted relative to the genomes of *Anaplasmataceae*, which due to their vacuole-enclosed lifestyle do not have access to cytoplasmic nucleotides and have thus retained genes involved in purine and pyrimidine biosynthesis (23).

RICKETTSIALES: DIVERSE SCAFFOLDS CORRELATED WITH CELL ENVELOPE ARCHITECTURE?

Hundreds of sequenced bacterial genomes provide a foundation for understanding the diversity of T4SSs that define the lifestyles of many pathogens, as well as contribute to the continuum of speciation through the transfer of components of the bacterial mobile gene pool. While informatics tools and laboratory studies are useful for unveiling T4SS architecture and function, evolution has done the crucial experiments, and many T4SSs can be seen as the end products of millions of years of bacterial coevolution with various host and vector cells. This information, combined with a wealth of experimental data that define the archetypal *vir* P-T4SS of *A. tumefaciens* (Fig. 5A), allows for the observation of structural and functional diversification across a wide range of bacterial species. For example, in relation to the *vir* P-T4SS, the *ptl* P-T4SS of *Bordetella pertussis* has undergone three innovations that likely correlate with the secretion of its sole substrate, the pertussis holotoxin (PT) (Fig. 5B). First, a *virB1* homolog is absent in the *ptl* system, yet given the presence of PG in *B. pertussis*, a mechanism for local degradation of PG is required. This task is accomplished by the fusion of a glycohydrolase domain to the N-terminal region of PtlE (a VirB8 homolog) that has demonstrated peptidoglycanase activity in both *B. pertussis* and *E. coli* (107). Thus, because VirB8 proteins are bitopic in the IM, this N-terminal glycohydrolase domain is located in the periplasm, allowing for simultaneous degradation of PG and T4SS channel assembly. Second, a homolog of the type IV coupling protein (T4CP), VirD4, is deleted in the *ptl* system. This is explained by the *sec*-dependent secretion of PT subunits to the periplasm (88, 100), with holotoxin assembly likely driving the formation of the core Ptl scaffold around PT prior to secretion (121). Thus, a T4CP would not be needed for entry of substrates from the cytoplasm to the periplasm, with the energetics generated from the remaining two IM ATPases, VirB4 (PtlC) and VirB11 (PtlH), enough to drive translocation of PT out of the bacterial cell. Lastly, the lack of a *virB5* homolog in the *ptl* system correlates with the lack of an observed T4SS pilus in *B. pertussis* (126), as host cell contact is not required for secretion of PT.

Similar comparisons between the T4SSs of members of the *Rickettsiales* and the *vir* system of *A. tumefaciens* can be made for inferring structural and functional diversification of the *rvh* transporter (Fig. 5C and D). Like the *B. pertussis* genome, all *Rickettsiales* genomes lack a *virB5* homolog, suggesting that *rvh*-mediated secretion occurs in the absence of a T-pilus-like structure. As recent studies identify VirB5 as an adhesin involved in host cell recognition (2, 7, 131), a minor pilin homolog (and a T-pilus) would be unnecessary in *Rickettsiales*, as substrates would be directly secreted to and/or imported from the host environment (55). Thus, RvhB2 polymerization across the periplasm should terminate near the OM in all *Rickettsiales* species. As *Rickettsia* spp. are the only *Rickettsiales* lineage to synthesize lipopolysaccharide (LPS) (data not shown), RvhB2 may be highly surface exposed in the remaining *Rickettsiales*. This suggestion is supported by an RvhB2-induced T-cell re-

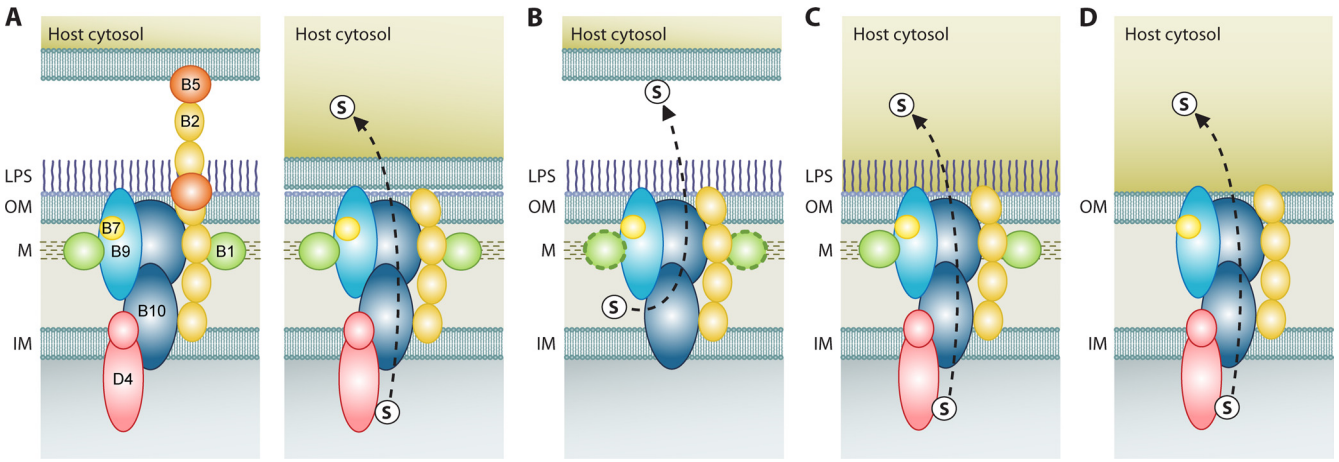

FIG. 5. P-T4SS structural and functional diversification. The dashed-line arrows illustrate the mode of secretion, with substrates depicted by an encircled "s." The color scheme of P-T4SS components VirB1 (green), VirB2 (light orange), VirB5 (dark orange), VirB7 (yellow), VirB9 (blue), VirB10 (dark blue), and VirD4 (pink) across four secretion systems implies homology. Distinct N and C termini of VirB10 and VirD4 are depicted. M, murein layer. (A) Model of transport for the *A. tumefaciens vir* P-T4SS. The two-step process of substrate attachment (left) and substrate transfer upon sloughing off of the T-pilus (right) is shown (51). (B) Model of transport for the *B. pertussis ptl* P-T4SS. VirB1 is distinguished to depict the N-terminal glycohydrolase domain of PtlE, a VirB8 homolog (107). (C) Model of transport for the *Rickettsia rvh* P-T4SS. (D) General model of transport for the *O. tsutsugamushi rvh* P-T4SS and for P-T4SSs in species of *Anaplasmataceae* that are not predicted to completely synthesize PG and LPS.

sponse in cattle immunized with an *A. marginale* OM fraction (89). The lack of LPS may also expose a portion of the outer cap of the core T4SS, as antibodies specific for RvhB9 have been detected in dogs infected with *Ehrlichia canis* (46) and cattle infected with *A. marginale* (6, 122). Furthermore, both RvhB9 and RvhB10 elicit robust antibody and T-cell responses from cattle immunized with a protective *A. marginale* OM fraction (89–91). The loss of LPS synthesis in the *Anaplasmataceae* may have lead to greater exposure of the T4SS at the OM and may account for RvhB2 duplication and proliferation as a consequence of host cell immune system avoidance. In contrast to *Rickettsiaceae* genomes, all sequenced *Anaplasmataceae* genomes have full-length RvhB9 gene homologs, which may add to antigenic complexity in the OM portion of the T4SS. While the cell envelope composition of *O. tsutsugamushi* differs greatly from that of *Rickettsia* spp. and does not contain LPS (117), prediction of surface exposure of the T4SS is difficult since only one genome (that of strain Ikeda) contains the VirB2 paralog genes (Fig. 3). Nonetheless, as VirB9 is predicted to be surface exposed in other systems (16, 64), it is probable that some regions of the *rvh* transporter are surface exposed at the OM, thus making it worthy for exploration as a vaccine target.

It has been hypothesized previously that species of *Rickettsiales* that invade vertebrate immune cells would benefit from a lack of PG synthesis (63). This proposal is consistent with evidence from other systems that host receptors of PG, nucleotide-binding oligomerization domain 1 (Nod1) and Nod2 proteins, detect by-products of PG degradation via inefficient anhydromuropeptide recycling (20, 120). As all members of the *Anaplasmataceae* replicate within intracellular vacuoles (43), discarded PG fragments may still be undetected by host cells in species that potentially synthesize PG. For free-living *Rickettsiaceae*, either PG synthesis does not occur (as in the case of *O. tsutsugamushi*) or a strategy may exist for "hiding" PG fragment release from immune cells. As discussed above, *Rickettsia* spp. contain many genes involved in the degradation and recycling of PG (see Fig. S3 in the supplemental material). Exceptionally, all sequenced genomes of *Rickettsia* spp. contain three to four copies of *ampG*, which encodes a permease involved in the import of PG monomers from the periplasm to the cytoplasm. As such genes are typically present only once in bacterial genomes, *ampG* proliferation in *Rickettsia* spp. may hint at increased necessity to recycle PG subunits rather then shed them freely into the host cytoplasm. The presence of a *virB1* homolog in only the genomes of *Rickettsia* spp. suggests that PG fragments are likely to be released during the assembly of the *rvh* T4SS and that this process of cell envelope rearrangement may make various components of the T4SS scaffold vulnerable to the host immune response. Furthermore, the processing of the C-terminal region of $VirB1_{Ti}$ (VirB1*) (10) and its subsequent extracellular secretion and role in T-pilus formation (87, 133) are likely not specific to the *A. tumefaciens vir* T4SS. Processed VirB1 products in the cell lysate from *Brucella abortus* were identified previously (39), and informatics data suggest that a conserved Ala is the likely cleavage site in many VirB1 homologs, with all predicted VirB1* sequences containing tracts of repeated residues, particularly Pro-rich tracts (55). Across 13 *Rickettsia* spp., *rvhB1* has the highest average number of codons evolving under positive selection among the 18 *rvh* genes (55), suggesting possible coevolution with host cell components. Nonetheless, RvhB1, as well as a putative RvhB1* form, may pose novel vaccine targets specific for species of *Rickettsia*.

CONCLUSIONS

Despite the genetic intractability of *Rickettsiales* as obligate intracellular bacteria, advances in understanding the mechanisms involved in the pathogenicity of these species are being made (12, 36, 123). This work outlines major achievements in the identification and characterization of components of the

rvh T4SS, as well as its putative regulators and substrates. Past and present bioinformatics approaches have greatly facilitated our understanding of the genetic architecture of the *rvh* scaffold, and experimental evidence has identified several promising vaccine targets. Our synopsis here suggests a single event of inheritance of the *rvh* T4SS in the *Rickettsiales* progenitor, with lineage-specific diversification of *rvh* components likely a result of modifications to cell envelope structure. This coevolution of the *rvh* T4SS and cell envelope structure is likely driven by adaptations to various host cells and thus identifies the transporter as an important target for vaccine development. While current data favor a role in effector translocation, functions in DNA uptake and release and/or conjugation cannot at present be ruled out, especially considering that no mechanism for plasmid transfer in *Rickettsia* spp. has yet been proposed. Furthermore, the genomes of several attenuated strains of *Rickettsia* (*R. prowazekii* Madrid E and *R. rickettsii* Iowa) and species with no known pathogenicity in arthropod or vertebrate cells (REIS and *R. peacockii* Rustic) carry most or all of the *rvh* genes. Taking this finding into consideration, we expect major advances in the near future regarding knowledge about the *rvh* T4SS and its potential involvement in host disease.

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ADDENDUM IN PROOF

Studies by Y. Rikihisa and M. Lin (Curr. Opin. Microbiol. **13**:59–66, 2010) and H. Niu, V. Kozjak-Pavlovic, T. Rudel, and Y. Rikihisa (PLoS Pathog. **19**:e1000774, 2010) were published during the production of this paper. They add substantial information regarding *rvh* substrates of the *Anaplasmataceae*.

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Editor: H. L. Andrews-Polymenis

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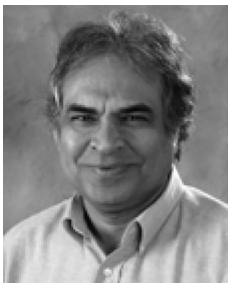
Kelly P. Williams (Virginia Bioinformatics Institute) has broad interest in bacterial molecular biology and evolution, with special interests in RNAs, genomic islands, and phylogeny. His Ph.D. work (at the University of California, San Diego) was on the biochemistry of bacteriophage transcription. Postdoctoral research during appointments at the Salk Institute, the Consiglio Nazionale delle Ricerche (Rome, Italy), and the Whitehead Institute employed *in vitro* selection to explore diverse RNA functions. His laboratory at Indiana University focused on the mechanism and evolution of bacterial tmRNA.

Marco A. Quevedo Diaz was born in Lima, Peru. He gained his M.S. degree in microbiology from the Faculty of Natural Sciences, Comenius University, in Bratislava, Slovakia, studying *pdr* genes in *Saccharomyces cerevisiae*. From 1998 to 2003, he was enrolled in the Ph.D. program in the Department of Rickettsiology at the Institute of Virology, Slovak Academy of Science, studying *Coxiella burnetii* phase variation and genetic transformation. Since that time, he has been fascinated with the biology of obligate intracellular bacteria, particularly rickettsiae and their interaction with eukaryotic hosts. Currently, he is at the Department of Microbiology and Immunology, School of Medicine, University of Maryland.

Wendy C. Brown was born and raised in upstate New York. She obtained her B.A. degree in microbiology from Smith College and M.P.H. and Ph.D. degrees from Yale University, studying infectious disease epidemiology and T-cell immunology. She transitioned to research on tick-borne pathogens at the International Laboratory for Research on Animal Diseases in Nairobi, Kenya, where she studied vaccine development for theileriosis, and then went to Texas A&M University (as an associate professor), where she studied T-cell responses to *B. bovis*, and finally to Washington State University, where she has also worked on T-cell immunity to the rickettsial pathogen *A. marginale.* There, she is a Regents Professor in the Department of Veterinary Microbiology and Pathology and the School for Global Animal Health. Of particular interest is targeting outer membrane protein complexes, specifically the bacterial type IV secretion system, for vaccine development. She resides with her family in the Palouse.



Abdu F. Azad is a professor of microbiology and immunology at the University of Maryland School of Medicine, Baltimore. He obtained his Ph.D. from the Johns Hopkins University School of Public Health, investigating the biology of a liver nematode, *Capillaria hepatica*, in Norway rats. His diverse scientific interests started with the epidemiology of human intestinal parasites, flea taxonomy (he described five new species), and the ecology and natural history of mammal-borne pathogens in Asia and Africa. His current research is focused primarily on the biology of arthropod-borne rickettsial pathogens, particularly understanding how these bacterial agents with reduced genomes cause infection and disease. Additionally, he has continued his long-term interest in investigating the genetic and molecular bases of preerythrocytic stages of malaria parasites in protective immunity. Aside from his research, he considers training as his major professional endeavor and is very proud of the scientific accomplishments of his former students and postdoctoral fellows.

Bruno W. Sobral was born in Brazil. His undergraduate education was in agricultural engineering and his Ph.D. was in genetics at Iowa State University, with postdoctoral work in molecular evolution. After starting his own research group at the California Institute of Biological Research, La Jolla, in 1991, he went on to be the vice president of scientific programs at the National Center for Genome Resources in Santa Fe, NM. In 2000 he started the Virginia Bioinformatics Institute (VBI) at Virginia Tech as the founding executive and scientific director and as a professor in the Department of Plant Pathology, Physiology, and Weed Science. His Cyberinfrastructure Section at VBI has focused on bioinformatics, computational biology, and informatics-based approaches to infectious disease research. He is particularly interested in and focused on transdisciplinary approaches to research and development and currently is a professor and the director of the Cyberinfrastructure Section.