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NOTES

A Typhus Group-Specific Protease Defies Reductive Evolution in Rickettsiae▽†‡

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Phylogenomics reveals extreme gene loss in typhus group (TG) rickettsiae relative to the levels for other rickettsial lineages. We report here a curious protease-encoding gene (*ppcE*) that is conserved only in TG rickettsiae. As a possible determinant of host pathogenicity, *ppcE* warrants consideration in the development of therapeutics against epidemic and murine typhus.

Alphaproteobacteria of the genus *Rickettsia* are obligate intracellular residents of a wide range of eukaryotes (6, 27, 33). The biology and diversity of *Rickettsia* species are poorly understood, with much knowledge stemming from decades of research on the species that cause mild to severe human disease. Understandably, the first dozen sequenced rickettsial genomes comprised definitive or tentative human pathogens. However, recently sequenced genomes of the attenuated strain Iowa of *Rickettsia rickettsii*, along with two groups with no known host pathogenicity, *Rickettsia peacockii* and *r*ickettsial *e*ndosymbiont of *Ixodes scapularis* (REIS), now provide a clearer framework for the elucidation of lineage specific virulence determinants.

Like other obligate intracellular bacteria, *Rickettsia* species have small genomes that reflect the reductive evolutionary processes of a lifestyle dependent on many eukaryotic host resources (3). Previously, we identified genes that define traits such as arthropod host species and disease phenotypes (13, 16). The smallest rickettsial genomes, belonging to the typhus group (TG) rickettsiae (*Rickettsia prowazekii* and *Rickettsia typhi*), lacked 53 genes present in all other sequenced rickettsial genomes. Additionally, TG genomes harbored very few pseudogenes, repetitive sequences, and genes typical of the bacterial mobile gene pool, relative to what was found for other species of *Rickettsia*. Thus, we concluded that some defining virulence factors associated with epidemic and murine typhus may comprise gene loss and strict reliance on host resources, a hypothesis previously put forward explaining the evolution of pathogenicity in all virulent rickettsiae (10).

Our prior analyses identified only three TG rickettsia-specific genes; we report here another TG-specific gene that encodes a putative prolyl oligopeptidase (POP) protein (Fig. 1A). POPs belong to the S9 serine proteases and are characterized by the presence of an N-terminal seven-bladed β propeller and a C-terminal α/β hydrolase fold domain (Fig. 1B). The unusual β propeller is considered a gating filter for substrate entry into the catalytic triad of the peptidase domain (12). The detection of a conserved TG-specific POP gene, named the postproline cleaving enzyme (*ppcE*) gene, was previously hampered by the clustering of highly fragmented *ppcE* remnants into this ortholog group (16). Thus, *ppcE* has undergone substantial pseudogenization in all non-TG *Rickettsia* species (Fig. 1C) and is undetectable even as a pseudogene in all *Rickettsiales* genomes basal to the three derived rickettsial groups (see Fig. S1 in the supplemental material). Furthermore, it is completely absent from the REIS genome and split in the *Rickettsia felis* genome. Despite a stop codon separating the catalytic triad from the remaining open reading frame, *R. felis ppcE* is highly conserved with TG rickettsial *ppcE*; this prompted us to confirm the split gene with DNA sequencing (see Fig. S2 in the supplemental material). We also failed to detect a suppressor mutant tRNA in the *R. felis* genome that could incorporate Glu for UAA. Thus, while TG rickettsiae and *R. felis* are the only species in our analysis with definitive insect hosts, *ppcE* is apparently not essential for life inside insects.

The POP family contains at least four subfamilies: the type prolyl oligopeptidase, dipeptidyl peptidase, oligopeptidase B, and acylaminoacyl peptidase (28, 29). PpcE is a member of the type prolyl oligopeptidase subfamily. *Rickettsia* species also harbor a ubiquitous POP-encoding gene, typically annotated as *ptrB* or the protease II gene, that is split in the genomes of both the RML369 C strain of *Rickettsia bellii* and *R. peacockii*. Fur-

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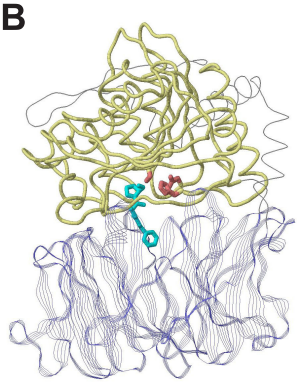
ID	Protein	Description	Reference
RT0615/ RP624	RelA	GTP pyrophosphokinase	(16)
RT0328/ RP338	COG3274	acyltransferase, putative membrane-association	(16)
RT0156/ RP164	HP	hypothetical protein	(16)
RT0165/ RP174	PpcE	post-proline cleaving enzyme (POP: prolyl oligopeptidase family)	This study



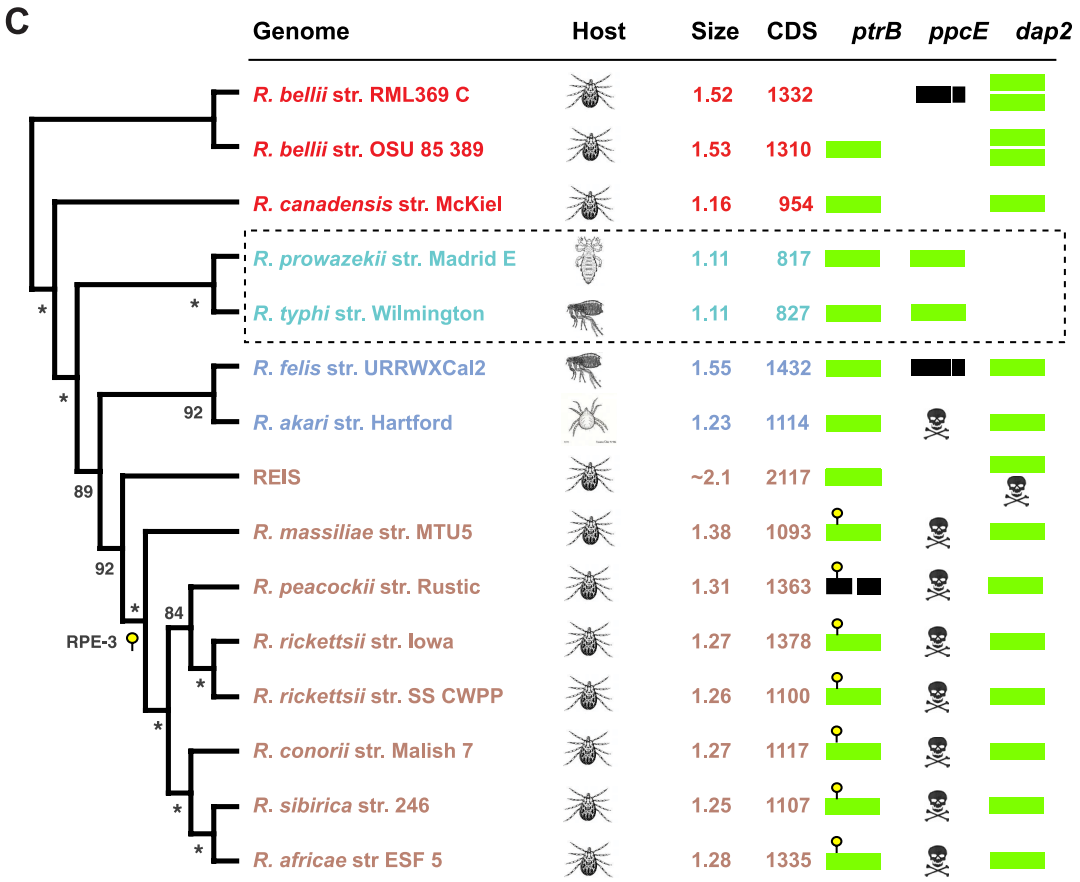


FIG. 1. Prolyl oligopeptidase (POP) genes of rickettsiae. (A) TG-specific genes identified previously (16) or in this study. Annotation is from PATRIC (32). (B) Tertiary structure of *Myxococcus xanthus* prolyl endopeptidase (31), illustrating the major features of POP proteins: blue, β-propeller domain; yellow, α/β hydrolase fold domain; red, catalytic triad (Ser-Asp-His); cyan, substrate. (C) Distribution of POP genes across 15 rickettsial genomes. Phylogeny was estimated from 18 genes encoding the *rvh* type IV secretion system as previously described (14). Branch support is from 1 million bootstrap replications; asterisks indicate 100% bootstrap support. The rickettsial classification scheme (15, 16) was as follows: red, ancestral group; turquoise, TG; blue, transitional group; and brown, SFG. Ticks are listed as the principal arthropod vector for all species except *R. prowazekii* (louse), *R. typhi* (flea), *R. felis* (flea), and *R. akari* (mite). Genome statistics were compiled from the PATRIC database (32). *ptrB*, oligopeptidase B gene (EC 3.4.21.83); *ppcE*, postproline cleaving enzyme gene (EC 3.4.21.26); *dap2*, dipeptidyl aminopeptidase 2 gene (EC 3.4.19.1). Full-length POP genes are colored light green, with truncated and split open reading frames colored black. Skulls and crossbones symbols depict gene fragments (three or more) detected with a TBLASTN search against the NCBI *Rickettsiales* database (taxid:766). Rickettsial palindromic element 3 (RPE-3) is depicted with a yellow ball-and-stick at its approximated insertion point in all derived SFG rickettsiae.

thermore, all spotted fever group (SFG) rickettsiae derived from REIS contain an insertion sequence (*Rickettsia* palindromic element 3) in the 5′ region of the gene. This nearly ubiquitous POP gene, herein named *ptrB*, is best described as a member of the oligopeptidase B subfamily. *ptrB* is also present in the sequenced genomes of *Orientia* and *Pelagibacter* species (see Fig. S1 in the supplemental material), suggesting that the gene has been lost from all *Anaplasmataceae* genomes and possesses typical characteristics of many other genes within the *Rickettsiales* that are undergoing pseudogenization.

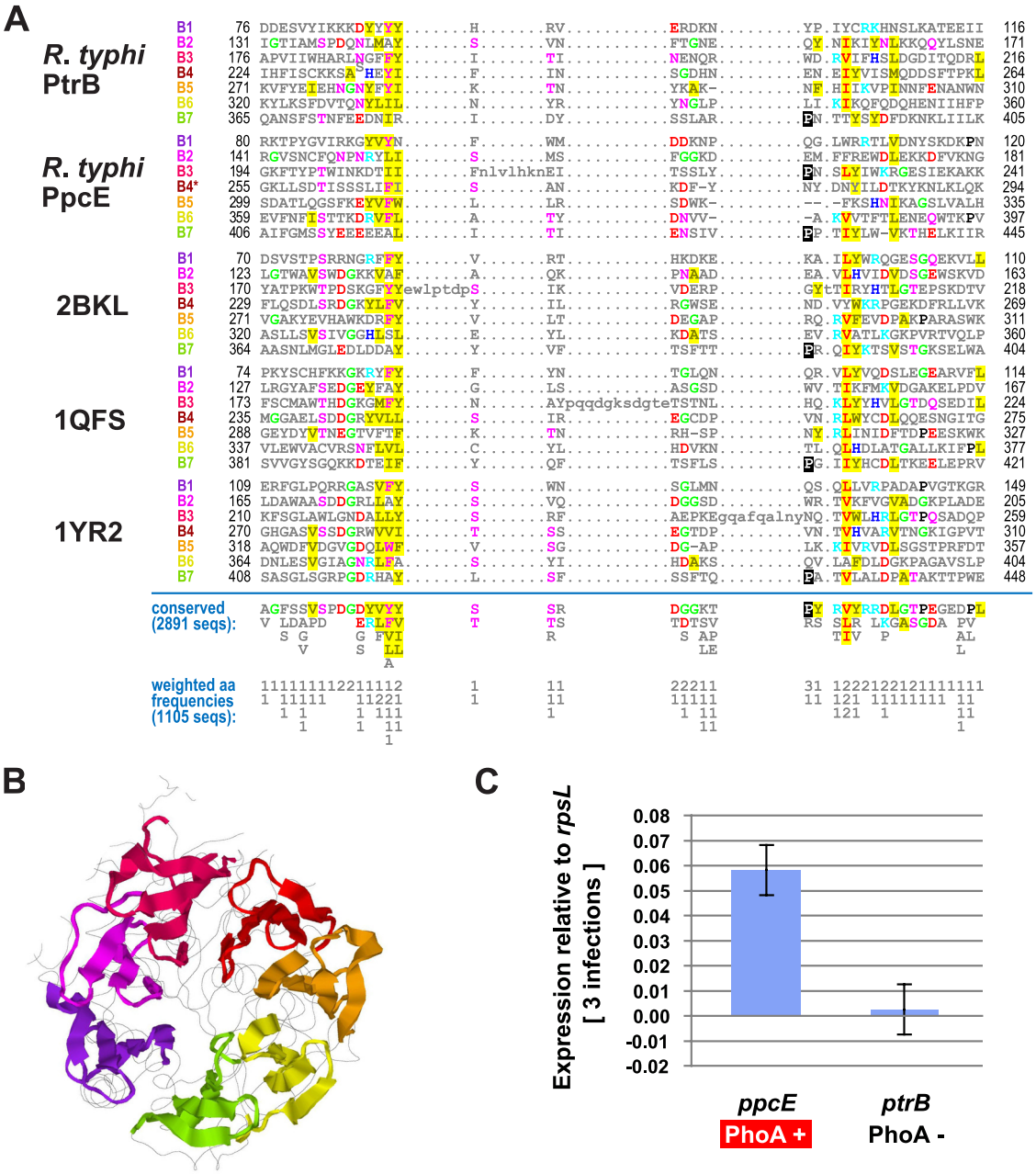


FIG. 2. Predicted sequence locations of β-propeller blades within *R. typhi* PpcE and PtrB. Using as queries those sequence regions corresponding to the β-propeller domains of bacterial prolyl endopeptidases of known structure, PSI-BLAST (1) iterative searches of the NCBI NR database were preformed with a very conservative E value cutoff of 10^{-40}. Those database sequence regions homologous to the queries were pooled, and both closely related sequences (those sharing ≥80% identity) and sequences less than 315 amino acids in length were removed. The remaining 380 sequence regions were used as input to a Bayesian Markov chain Monte Carlo sampling procedure for detecting subtly conserved internal repeats (22–24). This resulted in the detection and alignment of 2,891 putative β-propeller blades, including those shown in the alignment in panel A, which corresponds to regions within the *R. typhi* PpcE and PtrB sequences and to β-propeller blades of known structure within prolyl endopeptidases from *Myxococcus xanthus* (31) (as indicated in panel B), *Sus scrofa* (11), and *Novosphingobium capsulatum* (31) (Protein Data Bank accession numbers 2BKL, 1QFS, and 1YR2, respectively). The seven propeller blades are labeled in the N- to C-terminal direction as B1 to B7 in panel A and are colored purple, magenta, scarlet, red, orange, yellow, and green, respectively, in panel B. Note that the Bayesian sampler failed to detect the putative blade B4 within *R. typhi* PpcE (as indicated by an asterisk); instead, an optimal alignment of this region against a profile of the detected repeats was obtained independent of the sampler. The most commonly occurring residues at each position (within all 2,891 blades) are shown directly below the alignment, and directly below these, the corresponding (weighted) residue frequencies are given in integer tenths; for example, "2" indicates that the corresponding pattern residue occurs in 20% to 30% of the aligned sequences at that position. The most commonly occurring residue types are highlighted in the alignment, with chemically similar residues colored similarly, as previously described (21). The β-propeller domains in panel B are oriented perpendicularly to the structure shown in Fig. 1B. (C) Gene expression of *R. typhi* PpcE and PtrB in HeLa cells was as previously described (2). On the basis of Student's *t* test, the *P* value (for RT0165 versus RT0272) was 0.022503 ($P < 0.05$). The ability of the proximal N-terminal sequence of PpcE to translocate *E. coli* alkaline phosphatase (PhoA) to the periplasm is illustrated.

Comparison of *R. typhi* PtrB and PpcE with five diverse POP sequences illustrates the limited conservation in the β-propeller domain relative to the α/β hydrolase fold domain (see Fig. S3 in the supplemental material). Structural analysis of β-propeller domains from a large number of divergent POPs suggests that both PtrB and PpcE contain at least seven blades (Fig. 2A). Despite minimal sequence similarity, refined alignment with reference to several crystal structures (e.g., Fig. 2B) illustrates a hypervariable β-propeller domain. This is not surprising, as catalytic divergences have been demonstrated for POPs, ranging from hydrolysis of small peptides to cleavage of larger polypeptides, including triple-helical collagen fibers, fibronectin, and histones (7, 8, 17, 19, 20, 26, 30). Of note, a third POP-encoding rickettsial gene, named here *dap2*, was uncovered in our structural analysis despite having a β-propeller domain that is undetectable in BLASTP searches using PpcE or PtrB as a query (data not shown). Interestingly, *dap2* is present at least once in all rickettsial genomes except TG rickettsiae (Fig. 1C) and, like *ppcE*, is undetectable in other *Rickettsiales* genomes (see Fig. S1 in the supplemental material). The converse distribution of these two highly divergent genes, relative to what was found for *ptrB*, further defines TG rickettsiae and accentuates another novel correlate to murine and epidemic typhus.

In addition to the strong bioinformatics-based evidence that PpcE represents a unique and functional protein specific to the TG rickettsiae, we found that *ppcE* is transcribed by *R. typhi* during infections in mammalian cells (Fig. 2C). HeLa cells were infected with *R. typhi* strain Wilmington with a multiplicity of infection of approximately 10; at 4 days postinfection, the rickettsiae were isolated for RNA extraction, and gene expression was analyzed using two-step, real-time quantitative reverse transcription-PCR, as previously described (2). In this model system, *ppcE* was significantly more highly expressed than *ptrB*, indicating that PpcE is likely important for the *R. typhi* intracellular lifestyle. Both of the TG PpcE homologs are predicted to encode an N-terminal signal peptide (18), suggesting possible extracytoplasmic secretion. We analyzed the function of the *R. typhi* PpcE putative signal peptide in an *Escherichia coli*-based system by fusing the predicted PpcE signal peptide to the *E. coli* alkaline phosphatase (PhoA) moiety lacking its native signal peptide (2). In *E. coli*, PhoA is a Sec-dependent protein that is enzymatically active only after its translocation into the bacterial periplasm, and its phosphatase activity can be visualized by the addition of a colorimetric substrate to the culture medium. We found that the putative PpcE signal peptide was able to direct the translocation of PhoA into the periplasmic space of *E. coli*, indicating that its cognate protein (PpcE) is likely extracytoplasmic. Taken together, these data indicate that PpcE has a biologically relevant role within the TG rickettsiae, with its probable extracytoplasmic localization suggesting it could be involved in host cell interactions. In support of this, it was recently found that *R. prowazekii ppcE* (RP174) is expressed in L929 cells and significantly upregulated following heat shock (4).

Recently sequenced rickettsial genomes have revealed the prevalence of plasmids and a high number of laterally acquired genes (32), exemplifying the streamlined nature of TG rickettsial genomes. A TG-specific *ppcE* gene warrants attention, as POPs have been implicated in virulence in other systems (5, 20, 25) and are present in the secretome of *Bacillus anthracis* (9). Interestingly, PpcE of *Trypanosoma cruzi* is secreted extracellularly by trypomastigotes and involved in nonphagocytic mammalian cell invasion (17, 30). TG rickettsial *ppcE* presents a new target for elucidating the mechanisms of pathogenicity underlying murine and epidemic typhus.

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