

S4 Fig. Characteristics of the extended C-terminal domain of *Rickettsia* RalF proteins.

All alignments were performed using MUSCLE (default parameters) [1]. (A) Sequence alignment of the variable sequence with Pro-rich region (VPR) and T4SS signal sequence (T4S) across select *Rickettsia* species. *Rickettsia* species are organized based on recent estimated phylogeny [2], with classification scheme as follows: red, ancestral group (AG); blue, transitional group (TRG); aquamarine, typhus group (TG); and brown, spotted fever group (SFG). *R. helvetica* and *R. tamurae* (black) are unclassified following recent recommendations [3]. Red circles denote full-length RalF_R proteins, with NCBI GenBank accession numbers for these proteins provided in S2 Fig. Accession numbers for all other proteins as follows: *Rickettsia canadensis* str. McKiel, YP_001492434; *R. tamurae*, WP_032139614; *R. helvetica*, WP_010423297; *R. akari* str. Hartford, YP_001493241; REIS (*Rickettsia* endosymbiont of *Ixodes scapularis*), EER21763; *Rickettsia* sp. ISO7, KDO03638; *R. monacensis* str. IrR/Munich, CDI29630; “*Candidatus Rickettsia amblyommii*” str. GAT-30V, YP_005365253; *R. aeschlimannii*, WP_032074125; *R. rhipicephali* str. 3-7-female6-CWPP, YP_005390352; *R. montanensis* str. OSU 85-930, YP_005392197; “*Candidatus Rickettsia gravesii*”, WP_017443103; *R. heilongjiangensis* str. 054, YP_004764228; *R. japonica*, WP_024704516; *R. honei*, WP_032850143 and WP_016916690; *R. philipii* str. 364D, YP_005300602; *R. rickettsii* str. Brazil, YP_005292780 and YP_005292779; *R. slovaca* str. D-CWPP, YP_005426261, YP_005426262 and YP_005426263; *R. conorii* str. Malish 7, NP_360145 and NP_360146; *R. parkeri* str. Portsmouth, YP_005392764; *R. sibirica*, WP_026054964. Multiple accession numbers for several species denote chimeras comprised of multiple fragments corresponding to the full-length VPR sequences; green highlighting demarcates the boundaries of the individual ORFs within these chimeras, with green dots depicting missing sequence. For several proteins, misaligned terminal residues were stitched back to their respective ends (shown in red), though their homology to these positions is doubtful. Above the alignment, the blue bar depicts the VPR, and the orange bar depicts the T4S. Gray shading illustrates the large deletion within the VPR of RalF proteins from TG rickettsiae. Purple shading depicts the Pro-rich region with the VPR, with Pro residues colored purple. (B) Alignment of the T4S across full-length RalF_R proteins. Conserved residues are highlighted yellow, with positively charged residues (Arg, His, Lys) colored blue. One conserved positively charged residue (-12 from the C-terminus) is highlighted blue. (C) The T4S does not affect RalF_{Rt} localization. HeLa cells expressing YFP tagged RalF_{RtCTDΔT4S} (green, described in Fig. 2B) were stained with Alexa Fluor 594 Phalloidin probe to label actin (red). DAPI (blue) is shown in the merged image. Cytoplasmic (C) and membrane (M) localization was confirmed via membrane fractionation of

HEK293T cells Lipofectamine 2000 transfected with the indicated plasmids followed by immunoblotting. Immunoblot: 1, primary Ab, rabbit anti-GFP (Life Technologies); 2, rabbit anti-Calnexin (Abcam); 3, mouse anti-GAPDH (Abcam). (D) Alignment of the C-terminal sequences across select RalF_L proteins. NCBI GenBank accession numbers for all proteins are provided in **S2 Fig**. For *L. pneumophila* subsp. *pneumophila* str. Philadelphia 1, the Leu residue at position -3 (relative to the C-terminus) is within a black box. This L372 residue has been shown to be critical for RalF translocation via the *dot/icm* T4SS [4]. Unlike the C-terminal sequences of RalF_R proteins (panel B), the C-terminal sequences of RalF_L proteins are not conserved, with no other RalF_L sequences harboring a Leu or other hydrophobic residue at position -3, and only some sequences harboring a hydrophobic residue(s) in the terminal five aa positions (colored orange). (E) The VPR sequences across select *Rickettsia* species contain a predicted coiled-coil motif. Conserved residues are highlighted yellow, with residues within predicted coiled-coil motifs colored green. A consensus coiled-coil motif is depicted at the top of the alignment. Red arrows demarcate the region of the VPR that is absent from TG rickettsiae RalF proteins. Coiled-coil motifs were predicted using the COILS server [5]. (F) Schema depicting the prototypical RalF_R protein. See text for further details. Alignments provided for the S7D (**S2 Fig**), SCD (**S3 Fig**) and extended C-terminal domain (VPR and T4S) (panel A) provide the delineations of these discrete domains. (G) Breakdown of the amino acid composition within four discrete regions (S7D, SCD, VPR, T4S) for six full-length RalF_R proteins. Within each region, amino acid frequencies were calculated and grouped into four major categories: non-polar/aliphatic (Ala, Gly, Ile, Leu, Met, Val), polar (Asn, Cys, Gln, Pro, Ser, Thr), aromatic (Phe, Trp, Tyr), positively charged (Arg, His, Lys), and negatively charged (Asp, Glu). While non-polar, proline was considered polar since it is typically solvent-exposed, despite having an aliphatic side chain.

References

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D

L. shakespeare ----EQI--PRESQPEIGNINTPRO-----
L. moravica STWKSEI--GEEREAIKQDSSQRTOMQLK-----
L. p. p. Philadelphia 1 ----EGV--PKDPDAEMQKEKGRQMKF-----
L. p. Corby ----GAM--FRTPNAEQQETS KSA TKLNTDYDINEDTETIKDESKSSYK
L. p. Lens ----GSI--FRTPNAEQRETS KSA TRVTNTDYDINEDTVTRKDESRSSYK
L. p. Paris ----GSM--FRKPNAEQQETS KSA TRTLNTDYDINEDTVTRKDESRSSYK
L. p. Leg01/11 ----GSM--FRKPNAEQQETS KSA TRTLNTDYDINEDTVTRKDESRSSYK
L. longbeachae NSW150 ----EKLRFQRPCSEDNKEELMKSNGLK-----
L. sainthelensi ----EKLRFQRPSEKENTQEELMKANELKNHN-----
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E



● <i>R. bellii</i> RML369-C	HNKIK---ETTAKVQQADPEIIPDTKSIPLASETNFQDLKEAETIGIQPAK-----TQPVE
● <i>R. bellii</i> OSU 85-389	HNKIK---ETTAKVQQADPEIIPDTKSIPLASETNFQDLKEAETIGIQPAK-----TQPVE
● <i>R. australis</i> Cutlack	HKNIK---EITAIVQQTNQOIQPE---KPLASKESFEDLTQAEIGIKPVEILIKSSSQPVE
● <i>R. akari</i> Hartford	HKNIK---EITEIVQQTNQOIQPE---KPLASKEIFEDLTQAEIGIKPVEILIKSSYQOVK
● <i>R. felis</i> URRWXCal2	HKNIK---EITEIVQQTNQOIQPE---KPLASKESFEDLTQAEIGIKPVEILIKSSSQPVE
<i>R. monacensis</i> IrR/Munich	HKNIQ---EITKDLRKHDQPIPDTSKKPLASRENFEDLIHSDLG-KPVR-----SKPL-
<i>R. aeschlimannii</i>	HKNIQ---EITKDLRKHDQPTPDTSKKPLASRENFEDLIHSDLG-KPVR-----NKPL-
<i>R. rhipicephali</i> 3-7-female6-CWPP	HKNIQ---EITKDLRKHDQPIPDTSKKPLASRENFEDLIHSDLG-KPVR-----NKPL-
<i>R. montanensis</i> OSU 85-930	HKNIQ---EITKDLRKHDQPIPDTSKKPLASRENFEDLIHSDLG-KPVR-----NKPL-
Candidatus <i>R. gravesii</i>	HKNIQ---EITKDLRKHDQPIPDTSKKPLASRENFEDLIHSDLG-KPVR-----NKPL-
<i>R. heilongjiangensis</i> 054	NKNIQ---EITKGLRKHDQPIPDGKPLASRENFDDLHSDLG-QPVR-----NKPL-
<i>R. japonica</i>	NKNIQ---EITKGLRKHDQPIPDTSKKLLASRENFDDLHSDLG-QPVR-----NEPL-

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● <i>R. bellii</i> RML369-C	KPKFNTPAEELAWRNAQKKQAIIDTPVTKNEEPKAQIDNHKKRLEELKQKQEDLENKKKEL
● <i>R. bellii</i> OSU 85-389	KPKFNTLVEELAWRNAQKKQAIIDTPVTKNEEPKAQIDNHKKRLEELKQKQEDLENKKKEL
● <i>R. australis</i> Cutlack	KPKFNSFIEELAWKSVQRKQNIIDTPISQNKPKIQTDNHAKILEELKQKQKEDLKKKGL
● <i>R. akari</i> Hartford	KPKFNSFIEELAWKSAQRKQNVDTPIQNKPKIQTDNHAKVLEELKQKQKEDLKKKGL
● <i>R. felis</i> URRWXCal2	KPKFNSFIEELAWKNAQRKQNIIDTPISQNKPKIQTDNHAKILEELKQKQKEDLKKKGL
<i>R. monacensis</i> IrR/Munich	----SFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
<i>R. aeschlimannii</i>	----AFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
<i>R. rhipicephali</i> 3-7-female6-CWPP	----AFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
<i>R. montanensis</i> OSU 85-930	----SFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
Candidatus <i>R. gravesii</i>	----SFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
<i>R. heilongjiangensis</i> 054	----SFDEELK-KKVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL
<i>R. japonica</i>	----SFDEELK-KVQSRQVKSSEQQTKITEEKIDNHAKRIEELKQKQKEDLDRKGL

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● <i>R. bellii</i> RML369-C	EEKKANSNITKEEKWRIVAELEGVTKHLESIKKEHNTINTAEREKQF-NKSSQSSALKG
● <i>R. bellii</i> OSU 85-389	EEKKANSNITKEEKWRIVAELEGVTKHLESIKKEHNTINTAEREKQF-NKSSQSSALKG
● <i>R. australis</i> Cutlack	EEKKANSNITREERWKIVAELESINNQFKAIEKYVNTINSAADREKKFDNKNNPASPTKG
● <i>R. akari</i> Hartford	EEKKANSNITREERWKIVAELESINNQFKAIEKEYNINSASDREKKFDNKNNPSSPTKG
● <i>R. felis</i> URRWXCal2	EEKKANSNITREERWKIVAELESINNQFKAIEKERSNINSASDREKKFDNKNNPSSPTKG
<i>R. monacensis</i> IrR/Munich	EEKKANSNITREERWKIVAELEANNQFKAIEKEHNTINSASDREKKFDNKNKHLTSTKR
<i>R. aeschlimannii</i>	EEKKANSNITREERWKIAAELEANNQFKAIEKEHNTINSASDREKKFDNKNKHLTSTKR
<i>R. rhipicephali</i> 3-7-female6-CWPP	EEKKANSNITREERWKIAAELEANNQFKAIEKEHNTINSASDREKKFDNKNKHLTSTKR
<i>R. montanensis</i> OSU 85-930	EEKKANSNITREARWKIAAELEANNQFKAIEKEHNTINSASDREKKFDNKNKHLTSTKR
Candidatus <i>R. gravesii</i>	EEKKANSNITRDERWKIAAELEANNQFKAIEKEHNTINSASDREKKFDNKNKHLTSTKR
<i>R. heilongjiangensis</i> 054	EEKKANSNITREERWKIAAELEANNQFKAIEKEHNTINSASDREKQFDNKNKYLSTTKR
<i>R. japonica</i>	EEKKANSNITREERWKIAAELEANNQFKAIEKEHNTINSASDREKQFDNKNKYLSTTKR

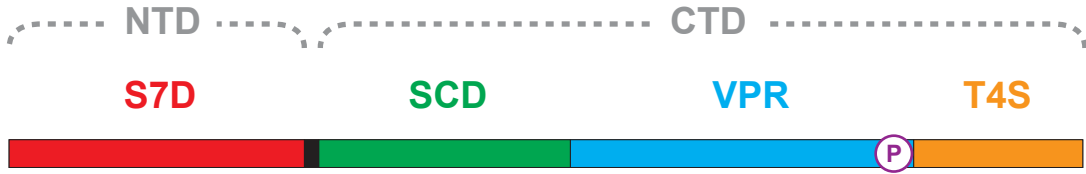
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● <i>R. bellii</i> RML369-C	EPTAADKQQQAAMELIRLRKEQRLQKEAASKAIIA
● <i>R. bellii</i> OSU 85-389	EPTAADKQQQAAMELIR-LRKQRLQEEAASKAIIA
● <i>R. australis</i> Cutlack	EPTAADLQQQALAAIKRMKEEKIKQQQA-EAVNQ
● <i>R. akari</i> Hartford	APTAAADLQQQALAAIKRMKEEKIKQQQA-EAANQ
● <i>R. felis</i> URRWXCal2	EPTAADLQQQALAAIKRMKEEKIKQQQA-EATNQ
<i>R. monacensis</i> IrR/Munich	EPTAADLQQQALAVIKRMKEEKIKQQQA-EAVNQ
<i>R. aeschlimannii</i>	EPTAADLQQQALAAIKRMKEEKIKQQQA-EVVNQ
<i>R. rhipicephali</i> 3-7-female6-CWPP	EPTAADLQQQALAAIKRVKEEKIKQQQA-EAVNQ
<i>R. montanensis</i> OSU 85-930	EPVAADLQQQALAAIKRMKEEKIKQQQA-EAVNQ
Candidatus <i>R. gravesii</i>	EPTAADLQQQALAAIKRMKEEKIKQQQA-EAVNQ
<i>R. heilongjiangensis</i> 054	EPTAADLQQQALAAIKRMKEEKIKQQQA-EAVNQ
<i>R. japonica</i>	EPTAADLQQQALAAIKRMKEEKIKQQQA-EAVNQ

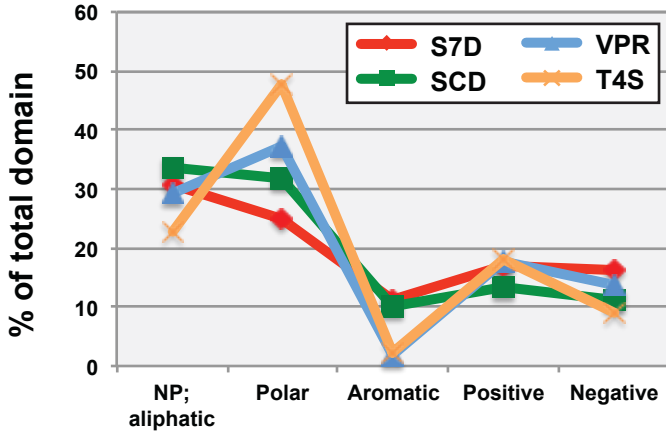
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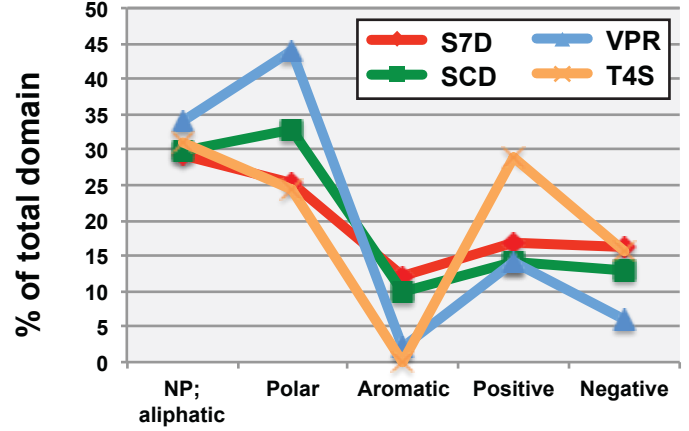


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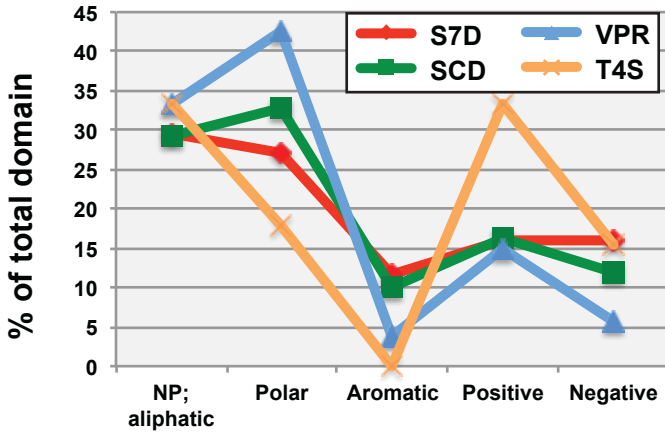
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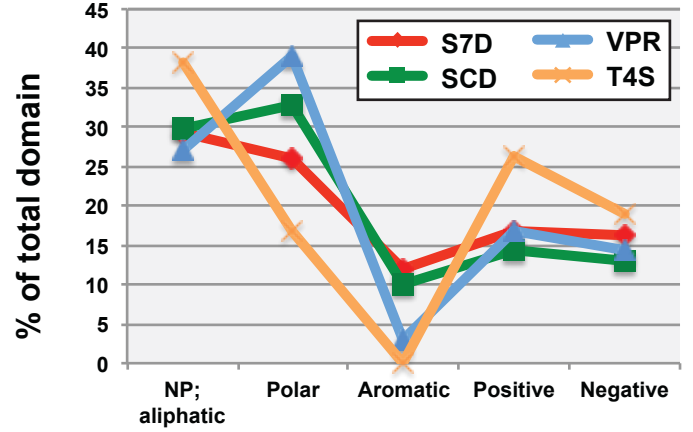
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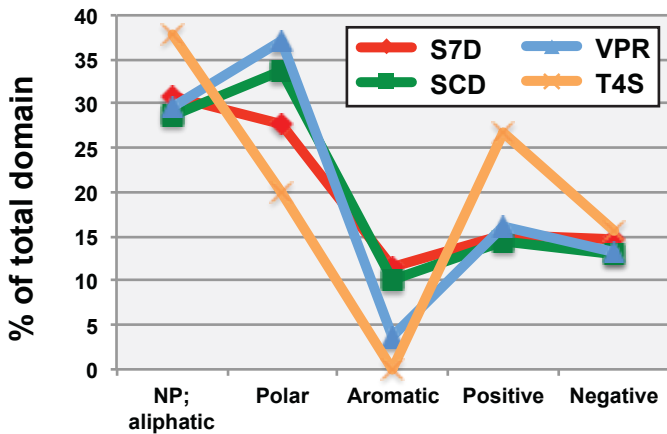
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R. felis str. URRWXCa12



R. akari str. Hartford



R. australis str. Cutlack

