

1 **FIGURE S1. Characteristics of the Rickettsiales *vir* homolog (*rvh*) type IV**
2 **secretion system (T4SS). (A)** Schema depicting genome-based Rickettsiales
3 phylogeny estimation, after Schön *et al.* (28). Host dependency evolved after the
4 divergence of basal families Mitibacteraceae and Athabascaceae (orange). The *rvh*
5 T4SS was present in the Rickettsiales ancestor (yellow). **(B)** Description of the general
6 *rvh* T4SS characteristics, summarized from prior studies (38, 42, 45, 89). **(C)** *rvh* genes
7 are arrayed in clustered islets or single transcriptional units throughout *Rickettsia*
8 genomes (e.g., *R. typhi* str. Wilmington). Asterisks denote genes for RvhB9-II, RvhB8-II
9 and RvhB4-II. Similar *rvh* gene arrangements are found in other Rickettsiales genomes
10 (38). **(D)** Proposed model for conserved *rvh* gene duplication (*rvhB9*, *rvhB8* and *rvhB4*)
11 based on divergent characteristics of RvhB9-II, RvhB8-II and RvhB4-II (44, 45).

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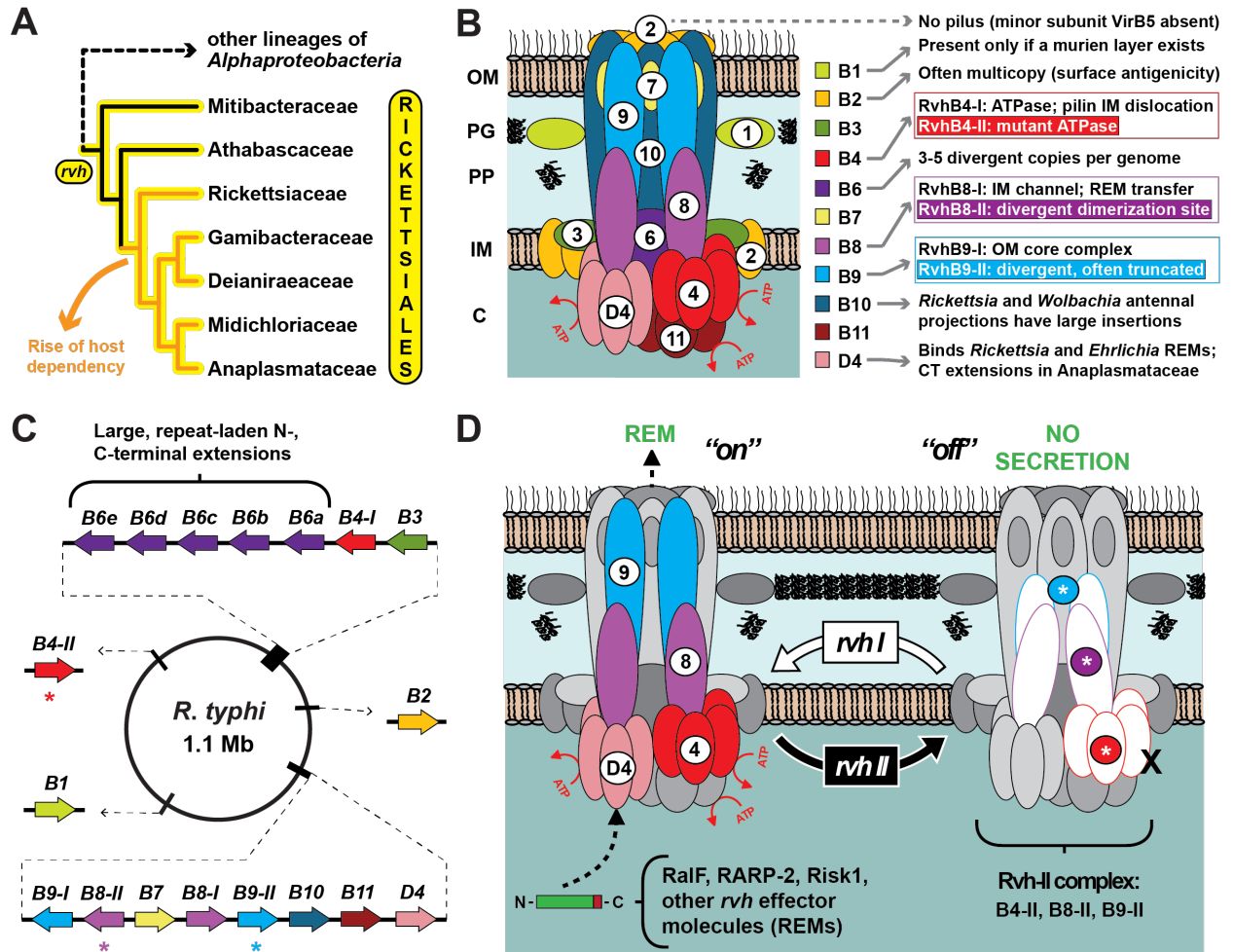


Fig. S1

15 **FIGURE S2. RvhB4-based phylogeny estimation.** Phylogeny estimated from
16 concatenated alignments of RvhB4-I and RvhB4-II proteins from 153 rickettsial genome
17 assemblies. *Agrobacterium tumefaciens* str. F4 VirB4 was used as an outgroup. See
18 **Table S1** for sequence information. RvhB4-I and RvhB4-II proteins were separately
19 aligned using MUSCLE (default parameters) with both alignments concatenated (1974
20 positions). TRIMAL (184) was used to create a second dataset with less conserved
21 positions masked (1613 positions). For both unmasked and masked alignments, a
22 maximum likelihood-based phylogeny was estimated with PhyML (185), using the Smart
23 Model Selection (186) tool to determine the best substitution matrix and model for rates
24 across aa sites (LG (G+I+F) for both alignments). Branch support was assessed with
25 1,000 pseudo-replications. **(A)** Statistics for the phylogeny estimated on the unmasked
26 alignment, which is shown as a phylogram in panels **C** and **D**, and as a cladogram in
27 **FIG. 1** and **FIG. 2**. **(B)** Statistics for the phylogeny estimated on the masked alignment.
28 This tree is congruent in topology and branch support to the tree generated on the
29 unmasked alignment. **(C)** Non-Rickettsiaceae lineages (taxa not previously described at
30 the family level are in black text). **(D)** Rickettsiaceae lineages. “*Candidatus* Sneabacter
31 namystus” (arrow), which lacks *rvh* genes but carries a type VI secretion system (see
32 **FIG. S3**) is shown on the phylogram per prior phylogeny estimation (187, 188). Black
33 boxes provide short names for 29 MAGs from Davison *et al.* (73) (NOTE: the green
34 colored clade comprises genus *Tisiphia* though “*Rickettsia*” reflects NCBI taxonomy as
35 of Feb. 26th, 2023). Asterisks, multiple genome assemblies for a species. STG, Scrub
36 Typhus Group; BG, Bellii Group; TRG, Transitional Group; TG, Typhus Group; TIG,
37 Tamurae-Ixodes Group; SFG, Spotted Fever Group.

A PhyML statistics (unmasked):

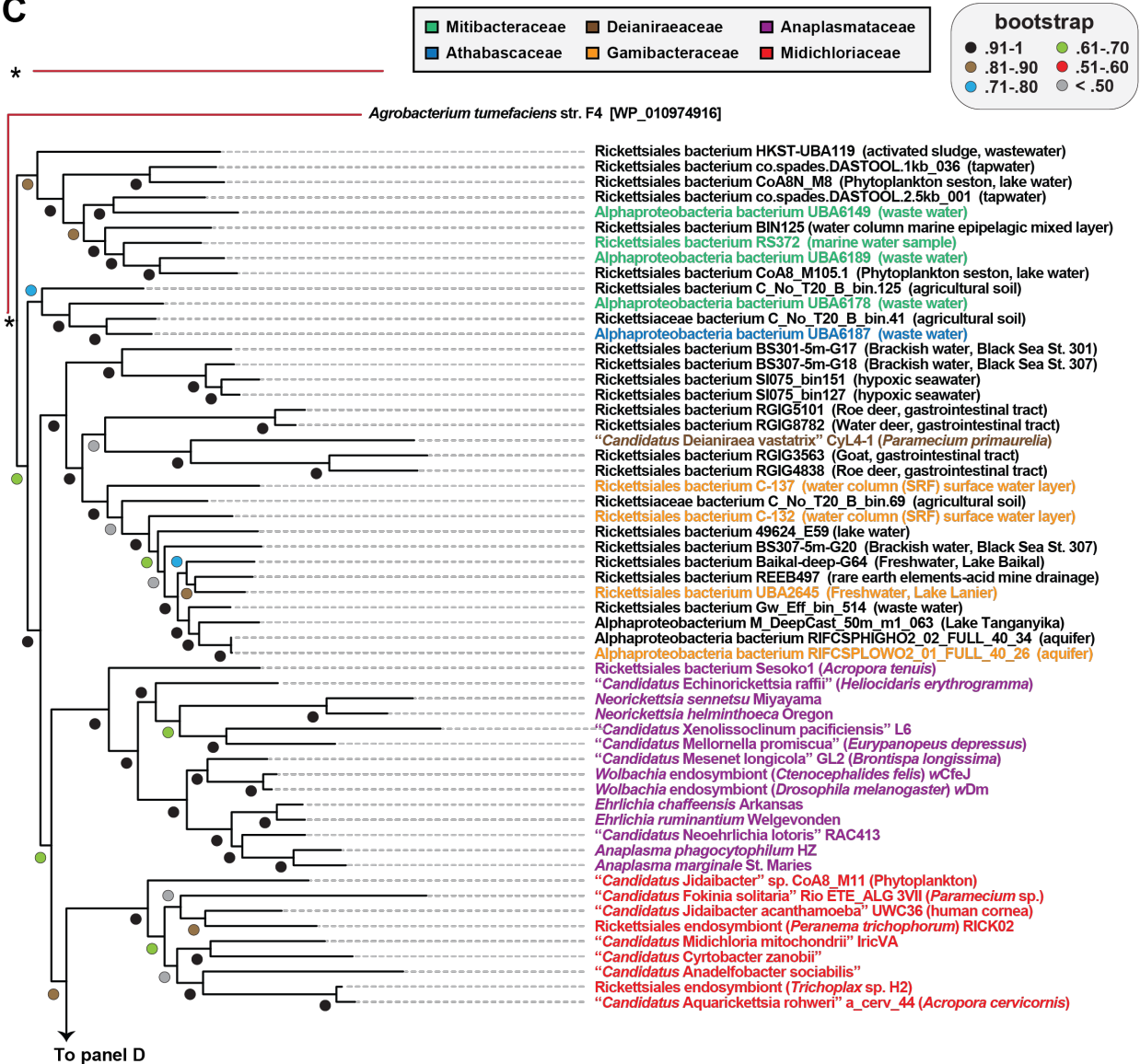
. Model of aa substitution: LG (+G+I+F)
 . Number of taxa: 153
 . Log-likelihood: -177643.39527
 . Unconstrained log-likelihood: -14907.80653
 . Composite log-likelihood: -866892.01804
 . Tree size: 52.48531
 . Discrete gamma model: Yes
 - Number of classes: 4
 - Gamma shape parameter: 0.883
 - Relative rate in class 1: 0.11306 [freq=0.250000]
 - Relative rate in class 2: 0.43852 [freq=0.250000]
 - Relative rate in class 3: 0.97754 [freq=0.250000]
 - Relative rate in class 4: 2.47088 [freq=0.250000]
 . Proportion of invariant: 0.039

B PhyML statistics (masked):

. Model of aa substitution: LG (+G+I+F)
 . Number of taxa: 153
 . Log-likelihood: -170882.36583
 . Unconstrained log-likelihood: -11869.35617
 . Composite log-likelihood: -708273.33174
 . Tree size: 48.13634
 . Discrete gamma model: Yes
 - Number of classes: 4
 - Gamma shape parameter: 0.891
 - Relative rate in class 1: 0.11473 [freq=0.250000]
 - Relative rate in class 2: 0.44135 [freq=0.250000]
 - Relative rate in class 3: 0.97930 [freq=0.250000]
 - Relative rate in class 4: 2.46463 [freq=0.250000]
 . Proportion of invariant: 0.045

Fig. S2

C



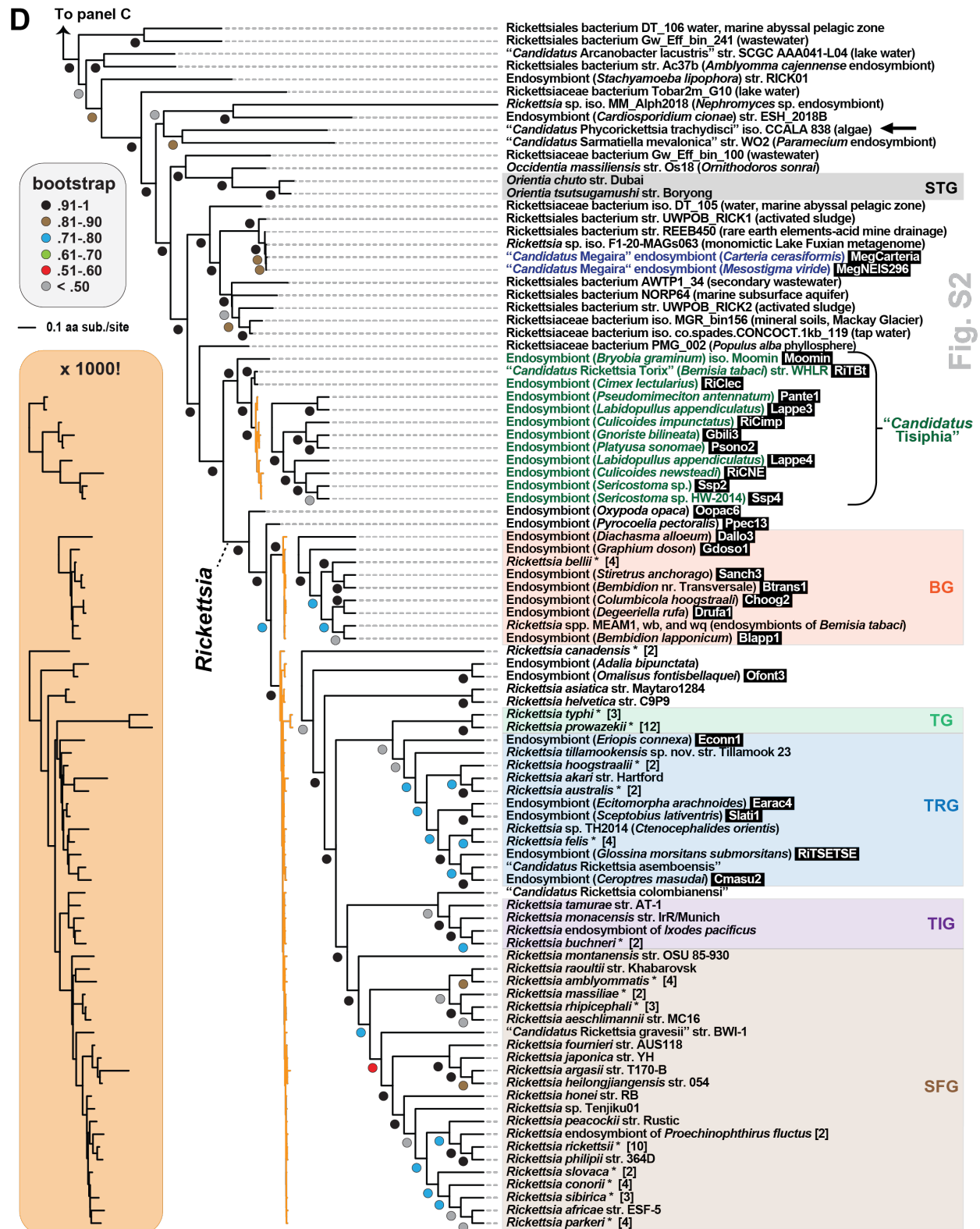


FIGURE S3. Non-orthologous replacement of secretion machines in Rickettsiales.

(A) Predicted proteins encoded on the “*Candidatus Sneabacter namystus*” unnamed plasmid, which mostly encodes type VI secretion system (T6SS) components. Protein colors depict their position in panel **B**. Plasmid map created with Proksee (<https://proksee.ca/>). **(B)** Model of a typical T6SS. The assignment of QEK39893 as a TssD protein is putative (see panel **D**). **(C)** HaloBlast analysis of 12 “*Candidatus S. namystus*” T6SS proteins. BlastP searches were performed against specified NCBI taxon databases (see **TABLE S3** for retrieved sequences). Highlighting: Sm score ($= b * I * Q$, where b is the bitscore of the match, I is the percent identity, and Q is the percent length of the query that aligned) (26, 81, 204). **(D)** Alignment of the putative TssD protein of “*Candidatus S. namystus*” with annotated TssD proteins from *Gemmata palustris* (Planctomycetia, WP_210656154) and *Denitrobaculum tricleocarpae* (Rhodospirillales, WP_142896164). Alignment done with MUSCLE (189) using default parameters. Amino acid coloring is described in the **FIG. 3** legend. **(E)** Detection of a second complete T6SS in the MAG Rickettsiales endosymbiont NP11 (NCBI BioSample SAMN07620291) (205). Another MAG, Rickettsiales endosymbiont EAC13 (SAMN07620031) (205), contained fewer T6SS genes. Neither MAG harbors *rvh* genes (data not shown), indicating that other Rickettsiales species may utilize a T6SS in place of a T4SS.

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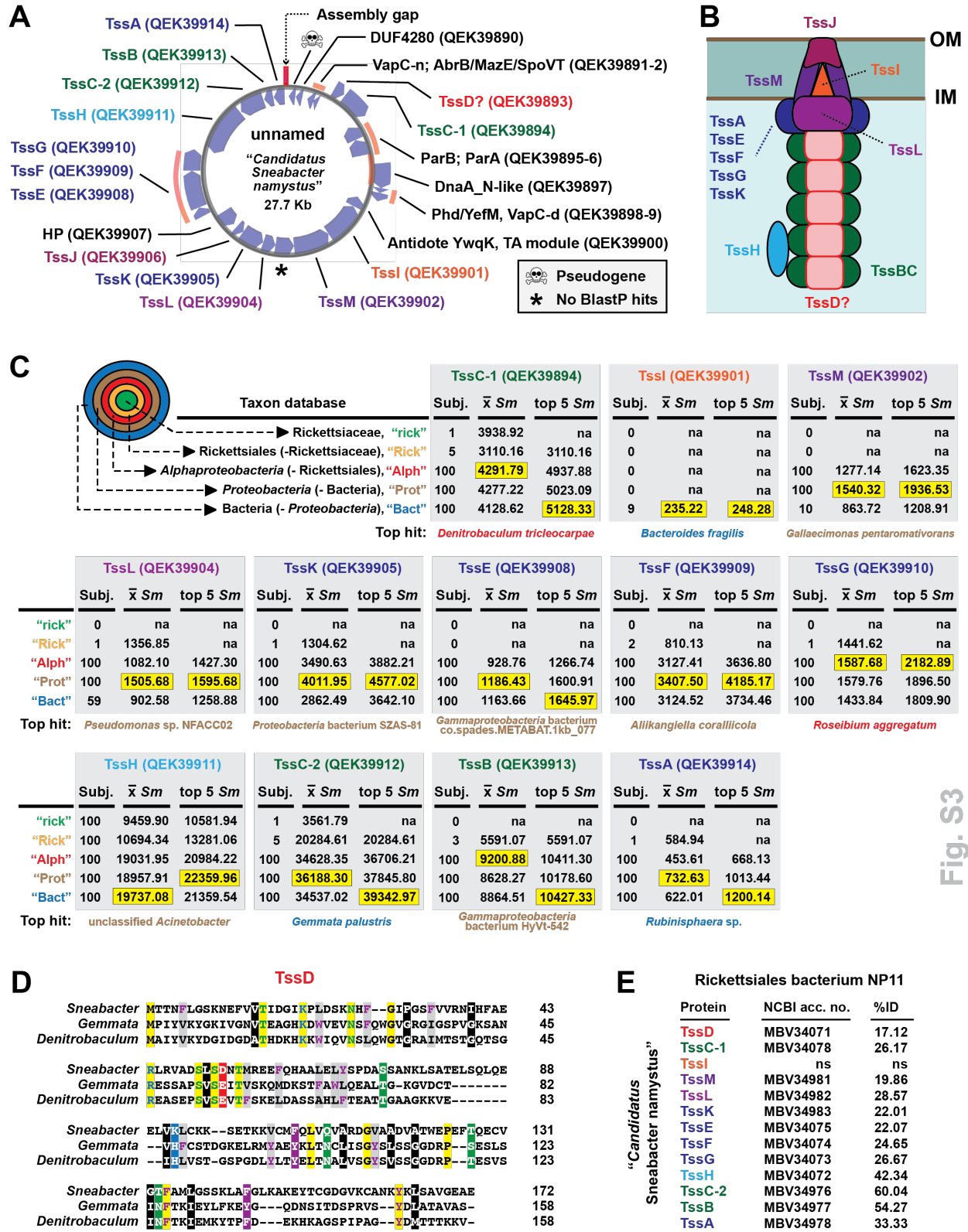


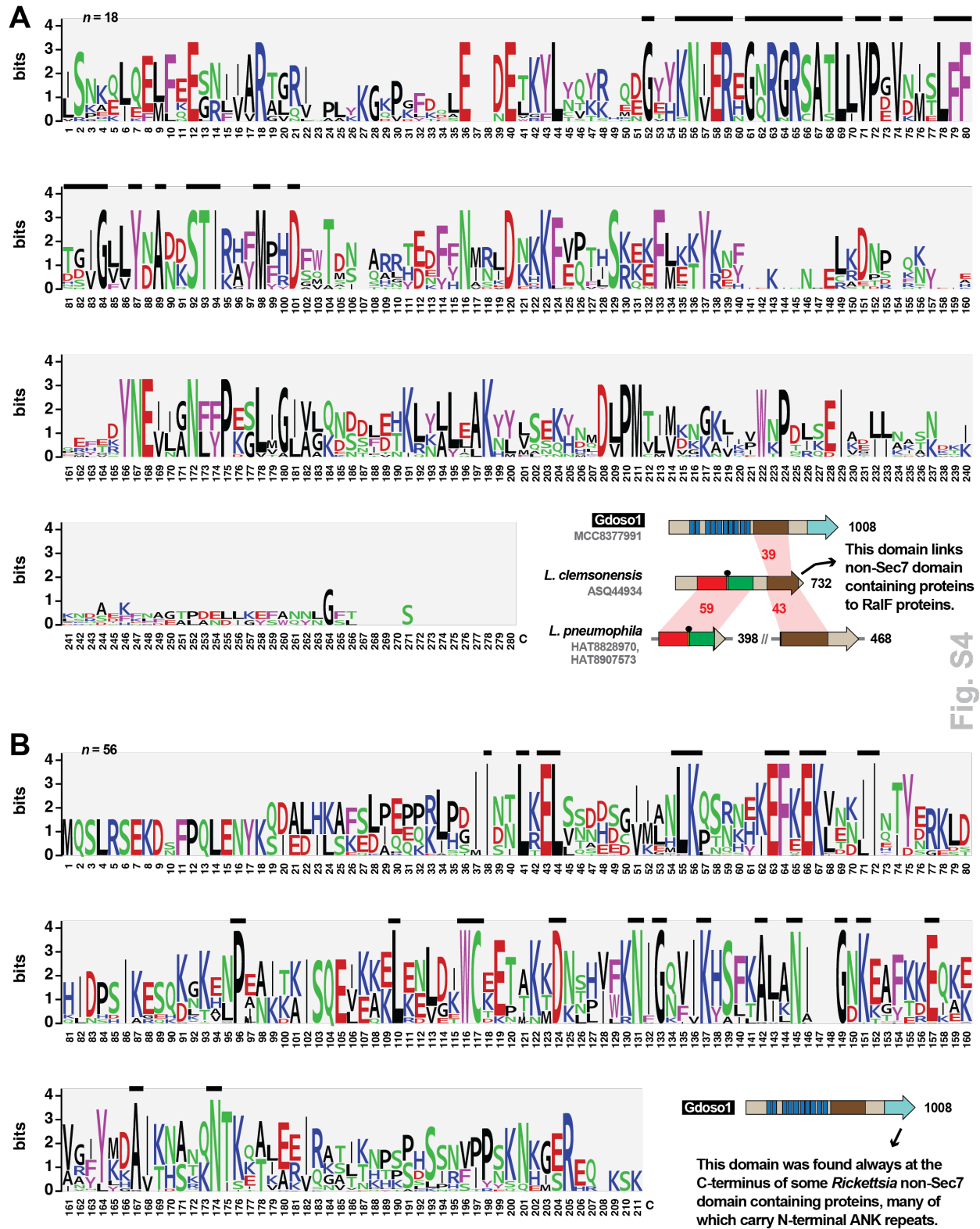
Fig. S3

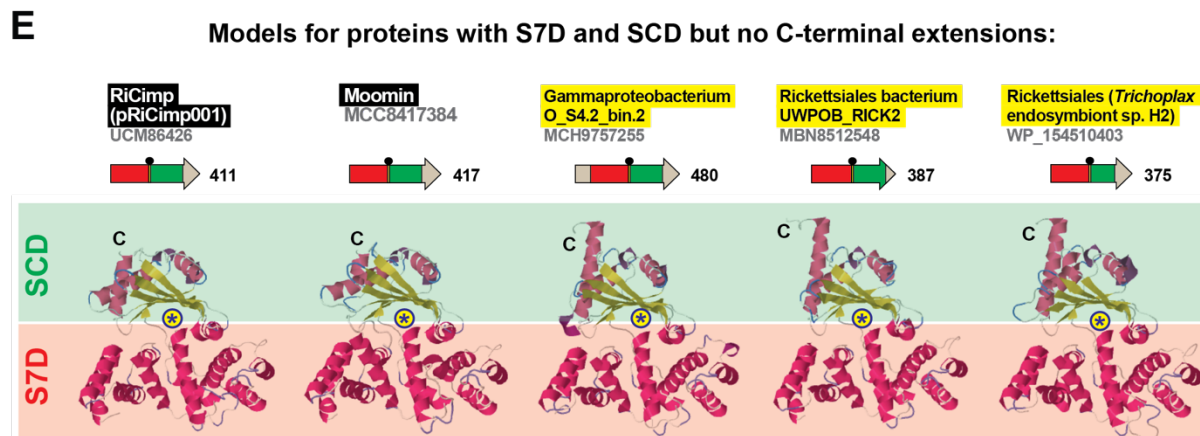
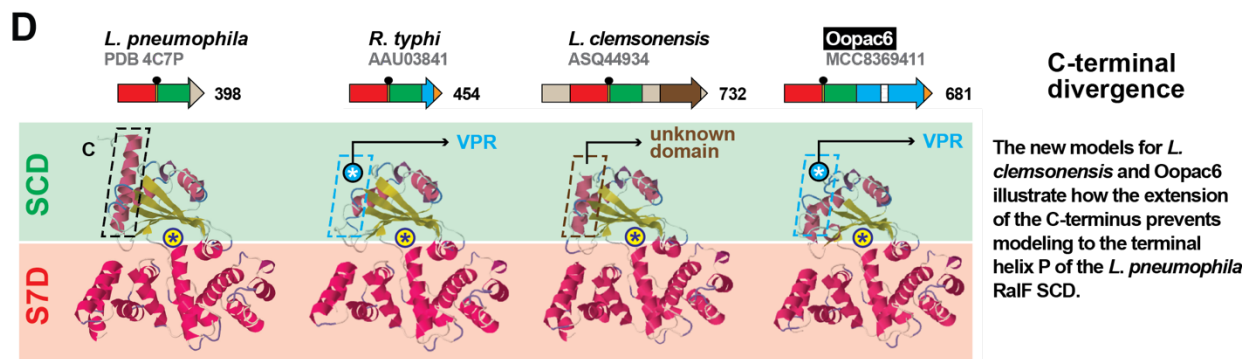
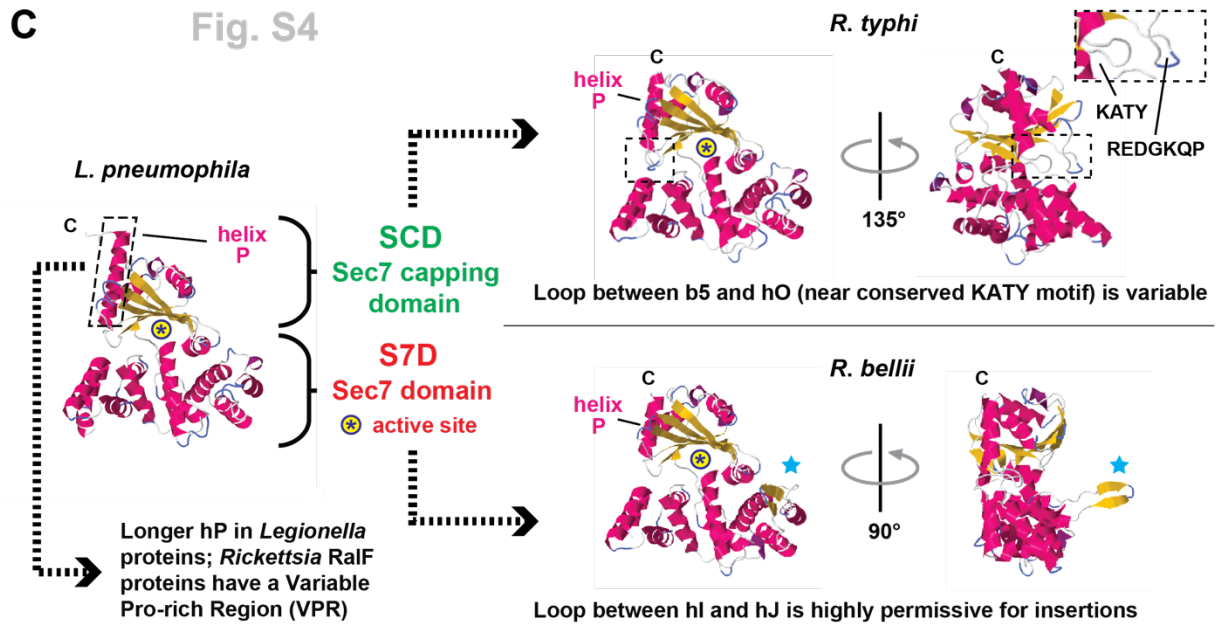
62 **FIGURE S4. Phylogenomics and bioinformatics analyses of RalF proteins.** Amino
63 acid coloring is described in the **FIG. 3** legend. Black boxes provide short names for
64 MAGs from Davison *et al.* (73). These and additional newly discovered RalF-like
65 proteins (highlighted yellow) substantially expand the prior RalF diversity. **(A, B)**
66 Sequence logos (190) illustrating conservation within two novel domains present in
67 some *Legionella* and *Rickettsia* proteins. Sequence information is provided in **Table S2**.
68 Sequences were aligned with MUSCLE (189) using default parameters. These two
69 domains are carried in a single protein (MCC8377991) from *Rickettsia* endosymbiont of
70 *Graphium doson* (Gdoso1). **(A)** Sequence conservation ($n = 18$) within the Gdoso1
71 MCC8377991 central domain. **(B)** Sequence conservation ($n = 56$) within the Gdoso1
72 MCC8377991 C-terminal domain. **(C)** Structural characteristics of the RalF S7D-SCD
73 architecture. **Left:** *L. pneumophila* RalF structure (PDB 4C7P) (86). The delineation of
74 the Sec7 domain (S7D, red) and Sec7-capping domain (SCD, green) is shown, with an
75 approximation of the active site Glu (asterisk). **Right:** regions of variability within the
76 predicted structures of *R. typhi* str. Wilmington (RT0362) and *R. bellii* str. RML369-C
77 (RBE_0868) RalF proteins. Modeling done with Phyre2 (195). *Rickettsia* full length RalF
78 proteins contain an extended C-terminal domain relative to *L. pneumophila* RalF (56).
79 **(D-F)** Gallery of predicted structures for diverse RalF and RalF-like proteins. All protein
80 domains are described in the gray inset. **(G)** Sequence comparison of diverse RalF and
81 RalF-like proteins (S7D and SCD only). An initial alignment with MUSCLE (default
82 parameters) was manually adjusted with reference to the Phyre2 structure models to *L.*
83 *pneumophila* RalF (PDB 4C7P). The secondary structure of *L. pneumophila* RalF is
84 superimposed over the alignment. Conserved residues are highlighted yellow. S7D: two

85 highly conserved regions (motif 1 and motif 2) that together form the Sec7 active site
86 are boxed in red (206); the active site Glu of Motif 1, which is essential for Arf1
87 recruitment to the LCV (85) and Arf6 recruitment to the plasma membrane during
88 *Rickettsia* invasion (148), is noted with an asterisk; divergent residues within motif 2 of
89 *R. bellii* RalF are colored black. SCD: aromatic clusters comprising the membrane
90 sensor region are enclosed in purple boxes; following previous mutagenesis analysis of
91 the SCD (87), # denotes residues in *L. pneumophila* RalF permuted to the
92 corresponding residues in *R. prowazekii* RalF (and vice versa) and ^ denotes residues
93 in *L. pneumophila* RalF permuted to the corresponding residues in *R. prowazekii* RalF
94 but not reciprocated. The conserved KATY motif, which contacts the S7D remote from
95 the active site and is thought to function as a hinge for the conformational change that
96 activates RalF (87), is colored black.

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F Models for proteins with no (or degraded) S7D; N- or C-terminal extensions may be present

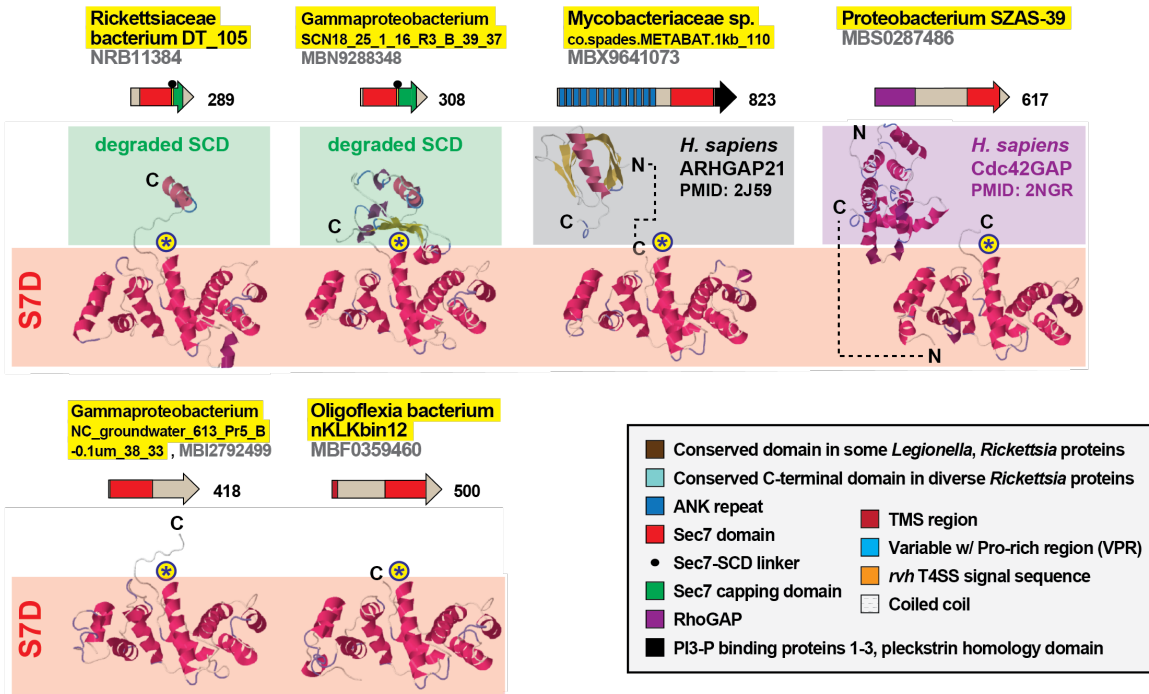
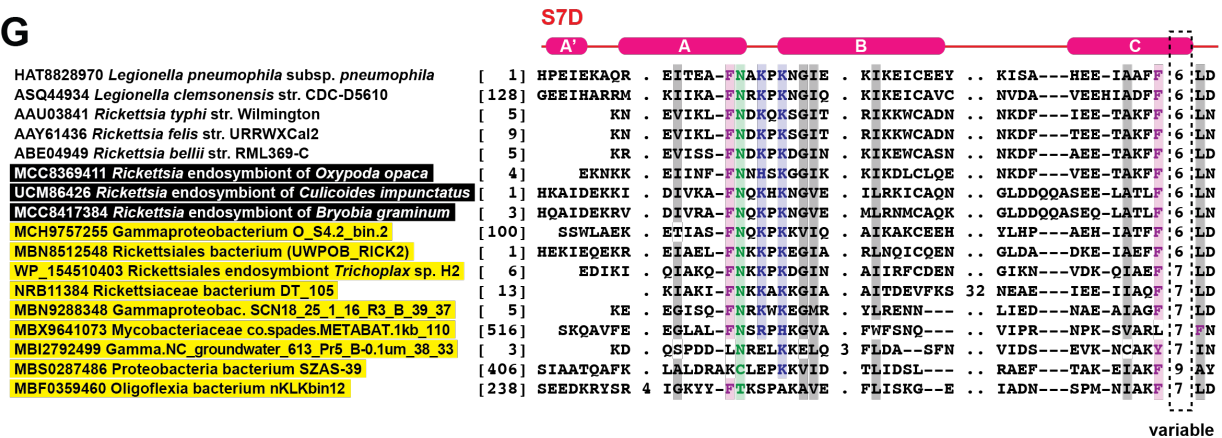


Fig. S4

G



G (cont'd.)

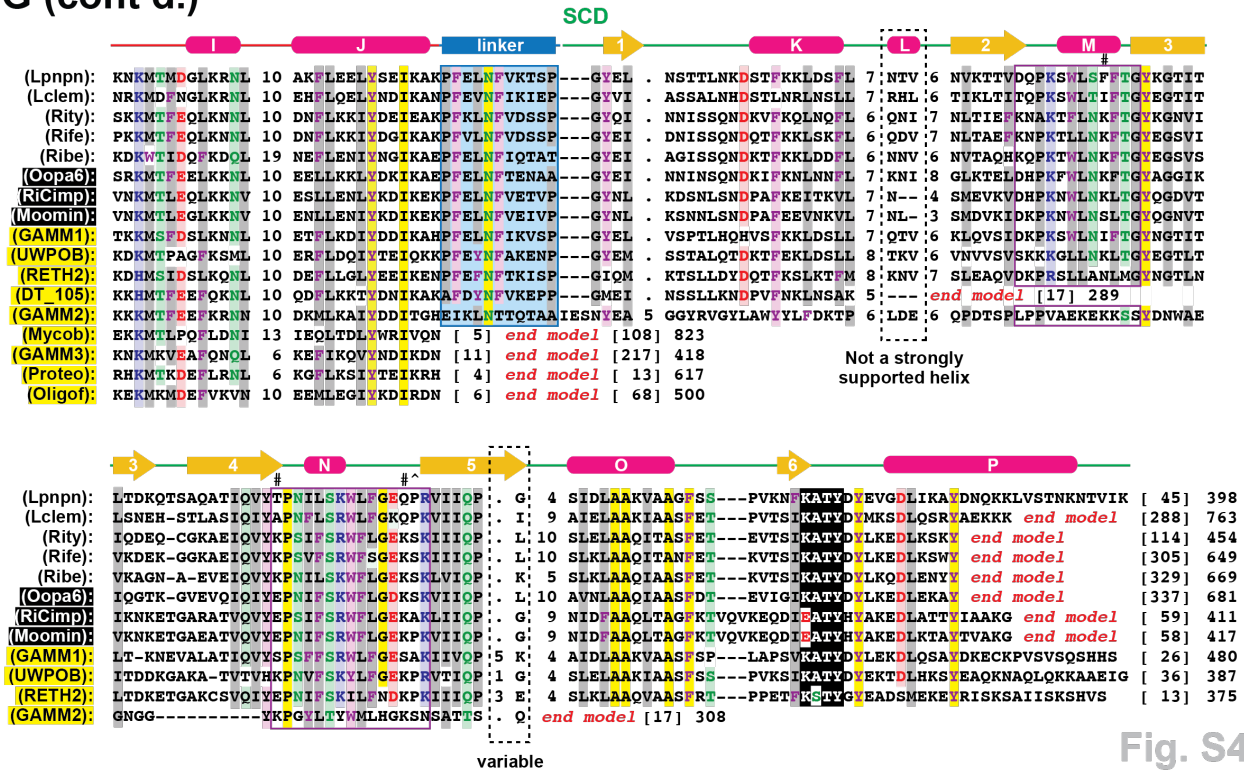


Fig. S4

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109 **FIGURE S5. The N-terminal domain of *Rickettsia Sca4* proteins is recurrent and**
110 **widespread in other rickettsial proteins.** Amino acid coloring is described in the **FIG.**
111 **3** legend. Black boxes provide short names for MAGs from Davison *et al.* (73). **(A)**
112 Alignment of the Schuenke Walker Antigen (SWA) domains of select *Rickettsia Sca4*
113 proteins with analogous domains in SWA-Risk2 chimeras (SWA-PIK) and SWA modular
114 proteins (SWAMPs). Domains were retrieved from BlastP searches against the NCBI nr
115 protein database using the *R. typhi* SWA domain as a query. Sequences were aligned
116 with MUSCLE (189) using default parameters. Structural assignment above alignment
117 corresponds to the modeling of *Rickettsia Sca4* N-terminal domains to the *R. rickettsii*
118 SWA domain (PDB ID: 4LQ8) (112). **(B)** Illustration of the SWA domain of *R. typhi Sca4*
119 and the two Vinculin Binding Sites (VBS); VBS-C of *R. rickettsii* interacts with the head
120 domain of human vinculin (76). **(C)** Gallery of diverse architectures for select SWAMPs
121 and predicted structures of SWA domains. Phyre2 (195) was used to model all SWA
122 structures to the *R. rickettsii* SWA domain, as well as the PIK domain of Rickettsiales
123 bacterium str. UWPOB_RICK1 to a portion of the LepB protein of *L. pneumophila* (PDB
124 ID: 4JW1) (201). Domains on schemas were predicted with SMART (191). **(D)** Sequence
125 logos (190) depict a consensus from an alignment of all 385 SWA domains retrieved
126 from a BlastP searches against the NCBI nr protein database using the *R. typhi* SWA
127 domain as a query (sequence information in **Table S2**).
128

A

SWA-VBP <i>R. typhi</i> Wilmington WP_011190939	} "Sca4"	[52]	SQSTPSPISVLSNSLPHGQDKS	PITKAVRETHIQPKDNLIEQI	ITREYVNTNPPELAAQIAK	EE
SWA-VBP <i>R. peacockii</i> WP_012736358		[57]	AQSTPSPMSALSGNISPSDQTS	PITKAVRETHIQPKDNLIEQI	KDLAALTDRLAEQRRKEIEE	
SWAMP plasmid pRRP, <i>R. peacockii</i> Rustic WP_228368942		[8]PITSAHKKHIMQKORE	QRLNPLAVRNPELDEY	TS	
SWA-PIK Pante1 MCC8371988		[506]KVLSVTLAFRQSHIAKQOT	TKNIPLEANIKTDAGAVANLIV		
SWAMP Pante1 MCC8372349		[27]	...LVVLTNSVSSSFLDTEEL	PITKAVRETHIQPKDNLIEQI	ITRGYVQNHHDVTVT	NY
SWAMP Pante1 MCC8371757		[27]	...LVVLTNSVSSSFFIEEV	PITKAVRETHIQPKDNLIEQI	ITREYVQNHDLTVT	NY
SWAMP Pante1 MCC8372295		[45]PITKAVRETHIQPKDNLIEQI	ITREYVQNHDLTVT	NY	
SWAMP Pante1 MCC8371625		[165]PITRAHRTQHLAKORE	LIVTAVKNAIVKQEEL	N	
SWA-PIK Rickettsiaceae bacterium MGR_bin156 MBA2628750		[471]PITQAHREDEVLAKQOL	YLAQEAIAKTIIPVGERNHFL	DQ	
SWA-PIK Rickettsiales bacterium UWPOB_RICK2 MBN8512064		[687]PISIQVIRDEEVLTKQOL	YVQQAETAKHIPVSRNGFL	DL	
SWA-PIK MegNEIS296 UCM93966		[453]PITTEAIRKSHIAEOK	LITQOTAHLSDPAVK	DM	
SWA-PIK MegNEIS296 UCM94396		[466]PVA-LVRESHLNKQIQ	ITKEAVLHLPDIT	GL	
SWA-PIK MegNEIS296 UCM94616		[989]PVA-LVRESHLNKQIQ	ITKEAVLHLPDIT	GL	
SWA-PIK Rickettsiales bacterium UWPOB_RICK1 MBN8523153		[1014]PVS-LVRESHLNKQIQ	ITKEAVLHLPDIT	SL	
SWAMP <i>Orientia tsutsugamushi</i> Boryong WP_011944657		[383]PVS-LVRESHLNKQIQ	ITKEAVLHLPDIT		

<i>R. typhi</i> :	DDKKRAFLSNQDN--YALNKAPE--DPETRKN----	EEVIVGKRNILST	SAASGYEGGQFVQVQENQVSAS	DL	SVVVNDA	EEETCALN	SHIKTNS
<i>R. peacockii</i> :	KDKTLSTFGNPAN--REFDKALE--NPELKKK----	ESIEIACQNVNHT	SAASGYEGGQFVQVQENHVSAS	DL	RAVVVND	QDLCALN	SHVKTNP
pRRP:	DDERCTKELMHLNNEQRKFNDALE--SEKVKAA----	EQIIVGKRNHTS	AAENVEGGQFRMDVSGESELN	TS	QAVVND	SSHCALN	SHKQHTTP
Pante1 1:	NSPEPKPEVNNNE--LIQKTA--SSITRNTVTQAKD--RQSQGKIHQ----	ANHRPLT	SEQASID--NITDD	SR	AVK	CEEFR	TAHIKTS
Pante1 2:	DEQFAEVLKNNIN--RAIDQAF--NTQVQTE----	EKTEIVGKRVVHNN	ADQFETMNVQDGRQG	NV	SOIVND	QDELA	SHKHPKIN
Pante1 3:	DDQFAEVLKNNIN--RAIDQAF--NSKVQTE----	EKTEIVGKRVVHNN	ADQFETMNVQDGRQG	NV	SOIVND	QDELA	SHKHPKIN
Pante1 4:	DDQFAEVLKNNIN--RAIDQAF--NSKVQTE----	EKTEIVGKRVVHNN	ADQFETMNVQDGRQG	NI	SOIVND	QDELA	SHKHPKIN
Pante1 5:	DLNKRAVENEQN--KETISELKTDKDLQQA----	EQVIAQNVNHT	AGIPTMEVQDGTVENASGIT	KQ	VVDANN	PHAL	AAHPKINP
MGR_bin156:	DLSSRTFVQSDVG--KEKISLAKM--KPETEVH----	KNISNGNEVHT	QDSFKMDVSPSSSK	V	FS	ELDPD	QHHTSEKHPVQA
UWPOB_RICK2:	NLGGVRSYLEETQG--KQAVVETLQ--DPKIQD----	QTIISGKRVVQSO	QDSFKDLSKSGSDSK	T	VAVDDO	CVTVL	SHKHPVNNP
MegNEIS296 1:	DPQQRRKVLTSDFG--KEEPAKVA--KPEIQTA----	NKISVGGKRVVHAE	SDSFKNVNTPDQVVAAGPKT	TS	ETIMED	GLVAK	SHKHPHDIAP
MegNEIS296 2:	SMEQKDKLKNHQK--LVTDALE--NPAPKKERAAQ--QQAIVAGKPKFNOE----	AKVA	RPVVVDGPFSTAS	E	TOVVNKA	QEV	CAIKKPAASS
MegNEIS296 3:	SMEQKDKLKNHQK--LVTDALE--NPAPKKERAAQ--QQAIVAGKPKFNOE----	AKVA	RPVVVDGPFSTAS	E	TOVVNKA	QEV	CAIKKPAASS
UWPOB_RICK1:	SMEQKDKLKNHQD--VVVKSLE--NPAPKQDIEAQ--QQAIVAGKPKFNOE----	AKVA	RPVAVDGFSTAS	G	TOVVNKA	QEV	CAIKKPAASS
OtsuBor:

<i>R. typhi</i> :	--LIVAKQDETO--VQINSYREND--PIKIDK--ADCSVHLSSMVALKADGTPKPKDAVVFPAHDEG--PNGKPOLKLEISS--POPTRVGTGDDAVAVIISHG--	EG	EG	EG	EG	EG	EG
<i>R. peacockii</i> :	--FTLAKQDETO--VQINSYREND--PIKIDK--ADCSVHLSSMVALKADGTPKPKDAVVFPAHDEG--PNGKPOLKLEISS--PKPLKACTGDDAVAVIISHG--	EG	EG	EG	EG	EG	EG
pRRP:	--LTLKQDEST--TIYSSYRIND--PVKTEKFASTVHLSLVARNKDGNAHLEKAVVFPAHDEG--PNGKPOLKLEISS--POPTRVGTGDDAVAVIISHG--	EG	EG	EG	EG	EG	EG
Pante1 1:	--TKVILEDEVTEKESVYRNINLPLTKP--SGTAVHLSFVQNEKGENETSALVFTTHNEQ-----GKLVETINPLSKVETEDDKSISGILQNGK--	NI					
Pante1 2:	--ALVLSLDETL--KQHSNYRIND--PTEVLS--KTCPVHLSDAVKDENGRNTALDRAVVFPAHDSQ-----GKLVETINPLSKVETEDDKSISGILQNGK--	OV					
Pante1 3:	--ALVLSLDETL--KQHSNYRIND--PTEVLS--KTCPVHLSDAVKDENGRNTALDRAVVFPAHDSQ-----GKLVETINPLSKVETEDDKSISGILQNGK--	OV					
Pante1 4:	--ALVLSLDETI--KQHSNYRIND--PTEVPS--KTSVHLSDAVKDENGRNTALDRAVVFPAHDSQ-----GKLVETINPLSKVETEDDKSISGILQNGK--	OV					
Pante1 5:	--PHTVQKSDETN--VAHNNRIND--PITLEN--QNPVHLSDAVKDENGRNTALDRAVVFPAHDDD-----GKLVETINPLSKVETEDDKSISGILQNGK--	QT					
MGR_bin156:	--TOVLLDESM--RSIKSYRIND--PKOLEG--GNCPAHPVMAVDENGRNTALDRAVVFPAHNDN-----GKLVETINPLSKVETEDDKSISGILQNGK--	KV					
UWPOB_RICK2:	--QS--KLNDETT--KQVSGYRSVNP--PKKDDT--GTPVHFSMAVDENGRNTALDRAVVFPAHDDK-----GKLVETINPLSKVETEDDKSISGILQNGK--	KI					
MegNEIS296 1:	--LAVTLDNCDK--VNVKSYRIND--PTEVET--GKCPVHLSDAVKDENGRNTALDRAVVFPAHDEQ-----GKLVETINPLSKVETEDDKSISGILQNGK--	EV					
MegNEIS296 2:	--QS--TAANCIT--KQVST--RSHAFPPS--KE--GSQVHASFPAVDENGRNTALDRAVVFPAHDKS-----GKLVETINPLSKVETEDDKSISGILQNGK--	EV					
MegNEIS296 3:	--QS--TAANCIT--KQVST--RSHAFPPS--KE--GSQVHASFPAVDENGRNTALDRAVVFPAHDKS-----GKLVETINPLSKVETEDDKSISGILQNGK--	EV					
UWPOB_RICK1:	--QT--TDPD--EST--KQVST--RSHAFPPS--KE--GSQVHASFPAVDENGRNTALDRAVVFPAHDKT-----GKLVETINPLSKVETEDDKSISGILQNGK--	EV					
OtsuBor:	--QLSASVNV--VT--KI--TKRIND--IPRAVEE--NKCPDIALVAQDITGKNNMSEKAVVLPVHNOE-----GKLVETINPLSKVETEDDKSISGILQNGK--	EV					

<i>R. typhi</i> :	YTLAVTRGKIKKAMKEVALNNGQSVDLSS--QTI AEDLTKVQG	[677]	1019
<i>R. peacockii</i> :	YTLAVTRGKIKKEMKREVELNQGQSVDLSS--Q--AEDIIIGQG	[665]	1018
pRRP:	YTLAVTRGKIEEMREVARNRQAVDLSLEVERKAQDLIR...	[213]	489
Pante1 1:	YTLAVTRGKQESMKEVAKNKGGINLSS--QTI	[51]	813
Pante1 2:	YSLVPTQGVREMMLEAKNKGQBITDLS--QSMA	[104]	395
Pante1 3:	YSLVPTQGVREMMLEAKNKGEBINLS--QSMA	[130]	420
Pante1 4:	YSLVPTQGVREMMLEAKNKGQBITDLS--QNITTAQDM	[219]	515
Pante1 5:	YTLVPTQEKIKKAMKEVAKNLGQGVDLSS--PSIEAPDLT	[294]	718
MGR_bin156:	YTLVPTQGVREMMREAVNKGGMANVSS--QSL	[57]	776
UWPOB_RICK2:	FITLVTKGKIDEMVQEVADNKGGLAANLSS--YVV	[44]	980
MegNEIS296 1:	YTLVPTQGVREMMREAVKNGMAVDLSS--QEVAAQE	[189]	899
MegNEIS296 2:	YTLVPTKKAENEMVAVSUSKVOGCMGINLSS	[165]	869
MegNEIS296 3:	YTLVPTKKAENEMVAVSUSKVOGCMGINLSS	[164]	1391
UWPOB_RICK1:	YTLVPTKKTINEMVAVSUSKVOGCMGINLSS	[160]	1419
OtsuBor:	YTLVPTREKIDQITREHSON	[222]	759

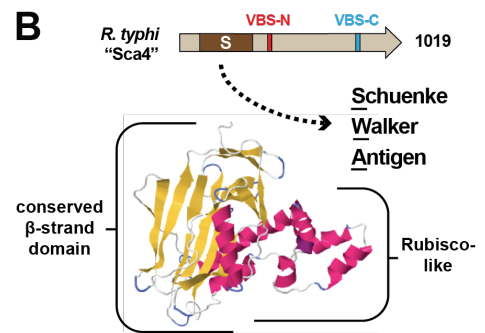


Fig. S5

