FIG S7. The evolutionary trajectory of six *Rickettsia* biosynthetic pathways that contain holes, or “missing enzymes”.

Our *Rickettsia* metabolic reconstruction identified six holes occurring in pathways for the synthesis of CMP-3-deoxy-β-D-manno-octulosonate (CMP-Kdo), Diaminopimelate (DAP), CDP-diacylglycerol (CDP-DG), terpenoid backbones, ubiquinone (CoQ_8), and queuosine (see manuscript for further details). These pathways were analyzed in the genomes of Rickettsiales and Holosporales to determine the presence/absence of the missing enzymes. If present, phylogenies were estimated to determine if the genes encoding these enzymes were present in the Rickettsiales/Holosporales common ancestor.

(A) *E. coli* enzymes corresponding to the holes in the *Rickettsia* CMP-Kdo (KdsC), DAP (DapC), CDP-DG (PlsX, PlsY or PlsB), terpenoid backbones (IspA), CoQ_8 (UbiC, UbiI), and queuosine (QueG) pathways were used as queries in blastp searches against the Rickettsiales and Holosporales databases at NCBI. For significant matches, only top hits with coverage to the query greater than 85% were considered. The four obtained rickettsial homologs were then used as queries in blastp searches against the *Alphaproteobacteria* (excluding Rickettsiales and Holosporales) and the NR (excluding *Alphaproteobacteria*) databases at NCBI. Again, for significant matches, only top hits with coverage to the query greater than 85% were considered.

(B) Phylogenomics analysis of CMP-Kdo, DAP, CDP-DG, terpenoid backbones, CoQ_8, and queuosine pathways across selected Holosporales and Rickettsiales genomes. The presence/absence of enzymes corresponding to the holes in the *Rickettsia* pathways are shown at right. Genome-based phylogeny was estimated on 105 orthologous groups (OGs) of proteins (single copy CDS in over 95% of genomes). We sampled 53 Rickettsiales/Holosporales and three outgroup *Alphaproteobacteria* genomes: IDs for sequences retrieved from PATRIC (P) and NCBI (N) are shown for each taxon. Several genomes were specifically annotated for this project: *Caedibacter varicaedens* (NCBI, NZ_BBVC00000000.1); “*Candidatus* Arcanobacter lacustris” str. SCGC (NCBI, JYHA00000000.1); “*Candidatus* Paracaedibacter symbiosus” (NCBI, NZ_JQAK00000000.1); "*Candidatus* Xenolissoclinum pacificiensis" str. L6 (NCBI, AXCJ00000000.1); *Rickettsia tamurae* str. AT-1 (NCBI, CCMG01000000.1); "*Candidatus* Hepatobacter penaei" str. NHPB (NCBI, JQAJ00000000.1); *Rickettsia* endosymbiont of *Adalia bipunctata* (unpublished); *Rickettsia* endosymbiont of *Ichthyophthirius multifiliis* (unpublished); *Rickettsia* sp. MEAM1 str. *Bemisia tabaci* (unpublished). Data for Rickettsiales endosymbiont of *Trichoplax adhaerens* is described elsewhere (1). OGs were generated using FastOrtho, a modified version of OrthoMCL (2). Multiple sequence alignment of each OG was performed using MUSCLE (default parameters) (3), with regions of poor alignment (length heterogeneous regions) masked using Gblocks (4). All modified alignments were concatenated into a single dataset for phylogeny estimation. Using PhyloBayes MPI (5), we analyzed the dataset with the CAT model of substitution, a nonparametric method for modeling site-specific features of sequence evolution (6, 7). Given the strong base compositional bias of rickettsial genomes (~30 %GC), the ability of the CAT model to accommodate saturation due to convergences and reversions (8) is of substantial importance for estimating rickettsial phylogeny, as demonstrated by us and others (1, 9–11). Two independent Markov chains were run in parallel using PhyloBayes MPI v.1.2e under the CAT-GTR model, with the bipartition frequencies analyzed at various time points using the bpcomp program. For tree-building, appropriate burn-in values were determined by plotting the log likelihoods for each chain over sampled generations (time). Analyses were considered complete when the maximum difference in bipartition frequencies between the two chains was less than 0.1. Ultimately, a burn-in value of 1000, with sampling every 2 trees, was used to build a consensus tree.

(C-F) Phylogeny estimations of DapC (C), PlsX (D), PlsY (E), and IspA (F) proteins. Datasets for each protein were constructed as follows: the rickettsial protein (panel A) was used in blastp queries against several taxon-specific databases: 1) "Rickettsiales", 2) "Holosporales", 3) "*Alphaproteobacteria* (minus Rickettsiales and Holosporales)", 4) "*Proteobacteria* (minus *Alphaproteobacteria*)", 5) "Bacteria (minus *Proteobacteria*)", and 6) "minus Bacteria". The top 5-

10 (query-dependent) subjects from each search resulting in significant (> 40 bits) alignments were all compiled and aligned using MUSCLE v3.8.31 (default parameters). Protein phylogenies were estimated under maximum likelihood with RAxML v8.2.4 (12), using a gamma model of rate heterogeneity and estimation of the proportion of invariant sites. Both the Lee and Gascuel (LG) and Blocks of Amino Acid Substitution Matrix (BLOSUM62) models of amino acid substitution were used, and branch support was assessed with 1,000 pseudo-replications.

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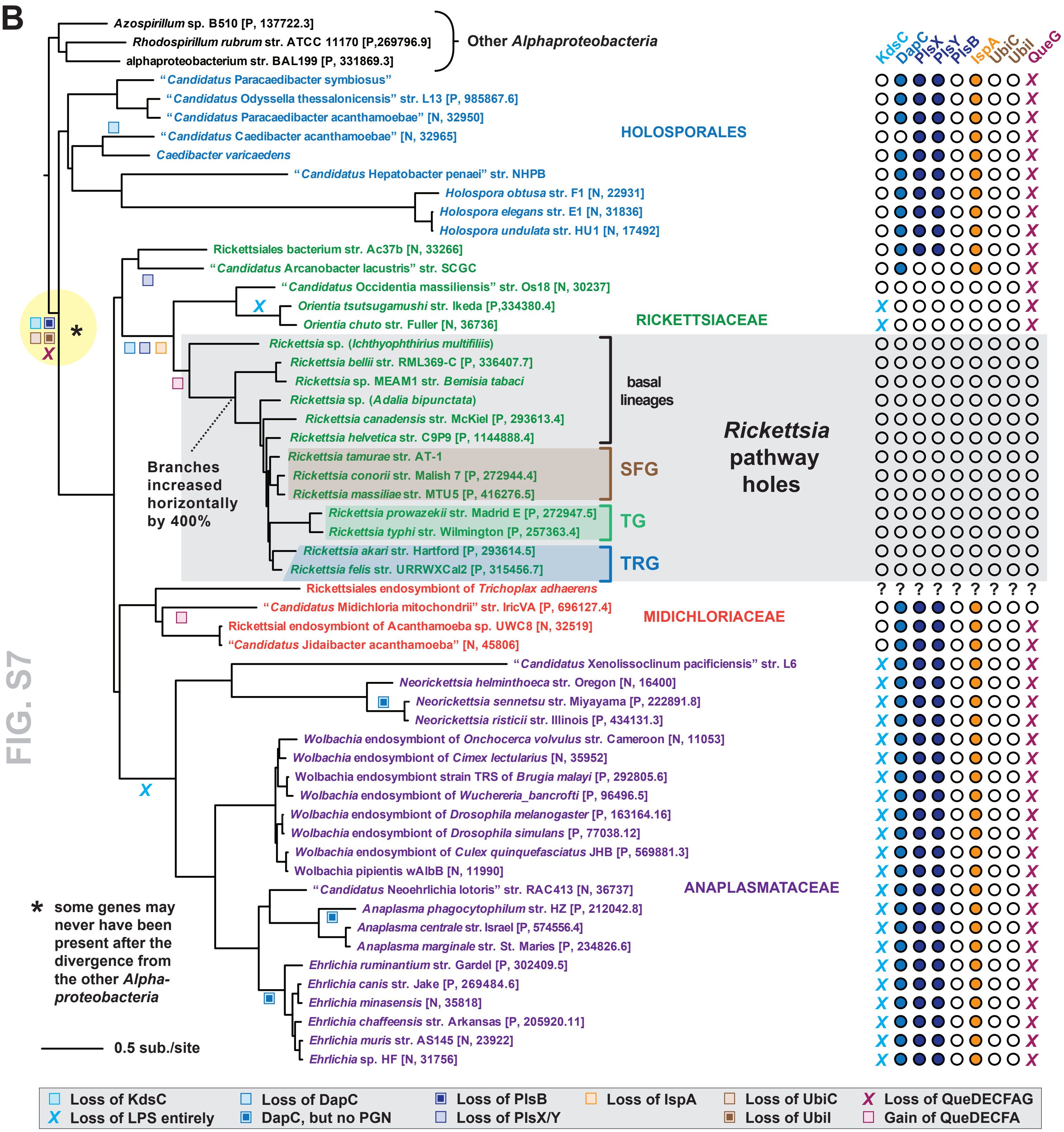
A

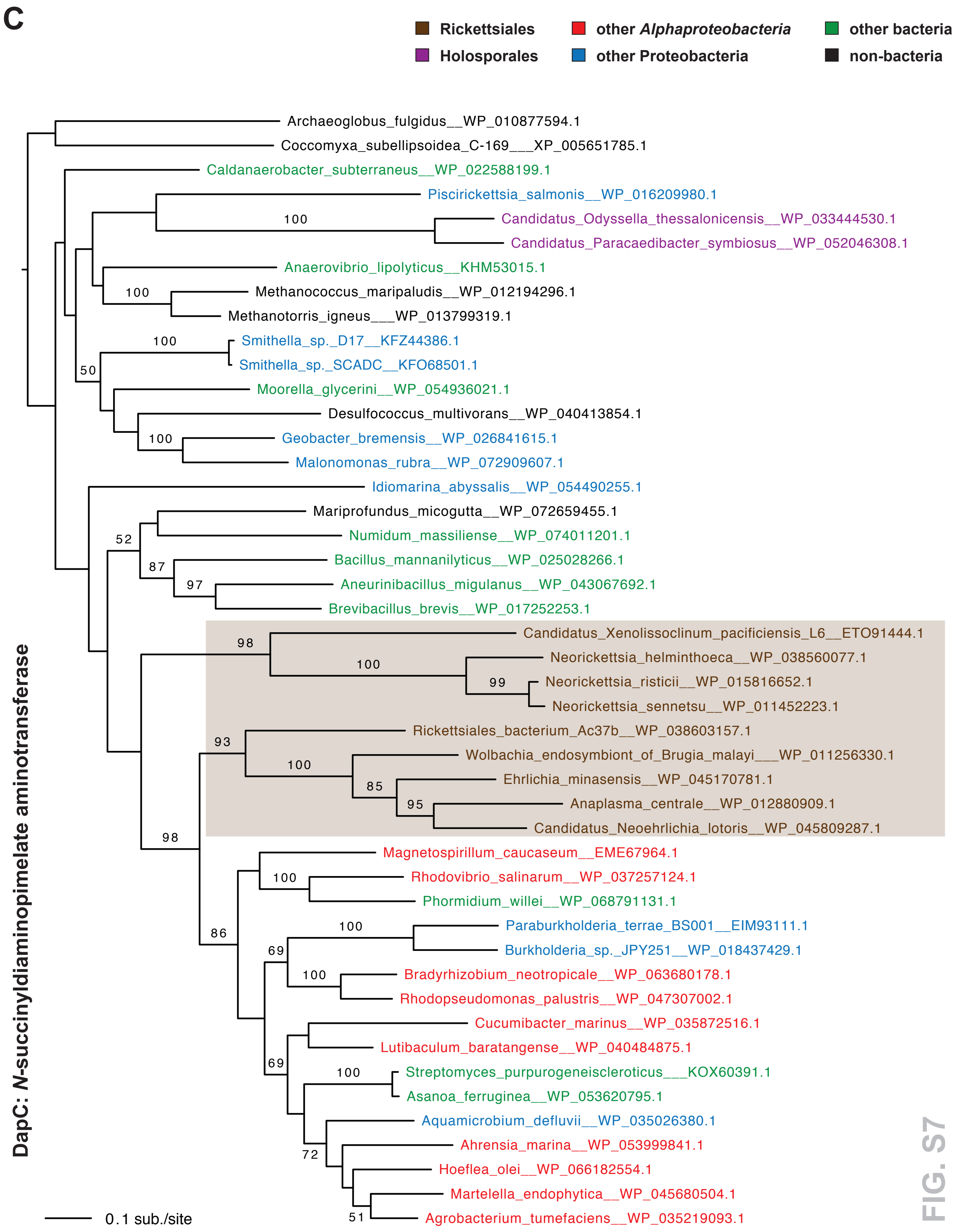
Hole	Reference protein	Top Rick (% ID)		Top *Alpha* E value; % ID		Top non-*Alpha* E value; % ID	
KdsC	P0ABZ4: *E. coli* str. K12	---		---		---	
DapC	P18335: *E. coli* str. K12	WP_070064655:	*Wolbachia pipientis* (42%)	4e-130;	50%	1e-122;	48%
PlsX	P27247: *E. coli* str. K12	WP_011452016:	*Neorickettsia sennetsu* (45%)	9e-94;	47%	4e-77;	45%
PlsY	P60782: *E. coli* str. K12	KKB96285:	"*Cand.* Arcanobacter lacustris" (40%)	5e-50;	49%	2e-36;	49%
PlsB	P0A7A7: *E. coli* str. K12	---		---		---	
IspA	P22939: *E. coli* str. K12	WP_039459403:	"*Cand.* Jidaibacter acanthamoeba" (44%)	7e-109;	59%	2e-72;	55%
UbiC	P26602: *E. coli* str. K12	---		---		---	
UbiI	P25535: *E. coli* str. K12	---		---		---	
QueG	P39288: *E. coli* str. K12	---		---		---	

B



Azospirillum sp. B510 [P, 137722.3]
Rhodospirillum rubrum str. ATCC 11170 [P,269796.9]
alphaproteobacterium str. BAL199 [P, 331869.3]
Other *Alphaproteobacteria*
KdsC DapC PlsX PlsY PlsB IspA UbiC UbiI QueG
"*Candidatus* Paracaedibacter symbiosus"
"*Candidatus* Odyssella thessalonicensis" str. L13 [P, 985867.6]
"*Candidatus* Paracaedibacter acanthamoebae" [N, 32950]
"*Candidatus* Caedibacter acanthamoebae" [N, 32965]
Caedibacter varicaedens
"*Candidatus* Hepatobacter penaei" str. NHPB
Holospora obtusa str. F1 [N, 22931]
Holospora elegans str. E1 [N, 31836]
Holospora undulata str. HU1 [N, 17492]
HOLOSPORALES
Rickettsiales bacterium str. Ac37b [N, 33266]
"*Candidatus* Arcanobacter lacustris" str. SCGC
"*Candidatus* Occidentia massiliensis" str. Os18 [N, 30237]
Orientia tsutsugamushi str. Ikeda [P,334380.4]
Orientia chuto str. Fuller [N, 36736]
RICKETTSIACEAE
Rickettsia sp. (*Ichthyophthirius multifiliis*)
Rickettsia bellii str. RML369-C [P, 336407.7]
Rickettsia sp. MEAM1 str. *Bemisia tabaci*
Rickettsia sp. (*Adalia bipunctata*)
Rickettsia canadensis str. McKiel [P, 293613.4]
Rickettsia helvetica str. C9P9 [P, 1144888.4]
basal lineages
Rickettsia tamurae str. AT-1
Rickettsia conorii str. Malish 7 [P, 272944.4]
Rickettsia massiliae str. MTU5 [P, 416276.5]
SFG
Rickettsia prowazekii str. Madrid E [P, 272947.5]
Rickettsia typhi str. Wilmington [P, 257363.4]
TG
Rickettsia akari str. Hartford [P, 293614.5]
Rickettsia felis str. URRWXCal2 [P, 315456.7]
TRG
Rickettsia pathway holes
Branches increased horizontally by 400%
Rickettsiales endosymbiont of *Trichoplax adhaerens*
? ? ? ? ? ? ? ? ?
"*Candidatus* Midichloria mitochondrii" str. IricVA [P, 696127.4]
Rickettsial endosymbiont of Acanthamoeba sp. UWC8 [N, 32519]
"*Candidatus* Jidaibacter acanthamoeba" [N, 45806]
MIDICHLORIACEAE
"*Candidatus* Xenolissoclinum pacificiensis" str. L6
Neorickettsia helminthoeca str. Oregon [N, 16400]
Neorickettsia sennetsu str. Miyayama [P, 222891.8]
Neorickettsia risticii str. Illinois [P, 434131.3]
Wolbachia endosymbiont of *Onchocerca volvulus* str. Cameroon [N, 11053]
Wolbachia endosymbiont of *Cimex lectularius* [N, 35952]
Wolbachia endosymbiont strain TRS of *Brugia malayi* [P, 292805.6]
Wolbachia endosymbiont of *Wuchereria_bancrofti* [P, 96496.5]
Wolbachia endosymbiont of *Drosophila melanogaster* [P, 163164.16]
Wolbachia endosymbiont of *Drosophila simulans* [P, 77038.12]
Wolbachia endosymbiont of *Culex quinquefasciatus* JHB [P, 569881.3]
Wolbachia pipientis wAlbB [N, 11990]
"*Candidatus* Neoehrlichia lotoris" str. RAC413 [N, 36737]
Anaplasma phagocytophilum str. HZ [P, 212042.8]
Anaplasma centrale str. Israel [P, 574556.4]
Anaplasma marginale str. St. Maries [P, 234826.6]
ANAPLASMATACEAE
Ehrlichia ruminantium str. Gardel [P, 302409.5]
Ehrlichia canis str. Jake [P, 269484.6]
Ehrlichia minasensis [N, 35818]
Ehrlichia chaffeensis str. Arkansas [P, 205920.11]
Ehrlichia muris str. AS145 [N, 23922]
Ehrlichia sp. HF [N, 31756]
X
*
* some genes may never have been present after the divergence from the other *Alpha-proteobacteria*
0.5 sub./site
□ Loss of KdsC
X Loss of LPS entirely
□ Loss of DapC
▣ DapC, but no PGN
■ Loss of PlsB
□ Loss of PlsX/Y
□ Loss of IspA
□ Loss of UbiC
■ Loss of UbiI
X Loss of QueDECFAG
□ Gain of QueDECFA

FIG. S7


C
🟫 Rickettsiales
🟥 other *Alphaproteobacteria*
🟩 other bacteria
🟪 Holosporales
🟦 other Proteobacteria
⬛ non-bacteria
DapC: *N*-succinyldiaminopimelate aminotransferase
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Neorickettsia_risticii__WP_015816652.1
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Rhodovibrio_salinarum__WP_037257124.1
Phormidium_willei__WP_068791131.1
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100
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100
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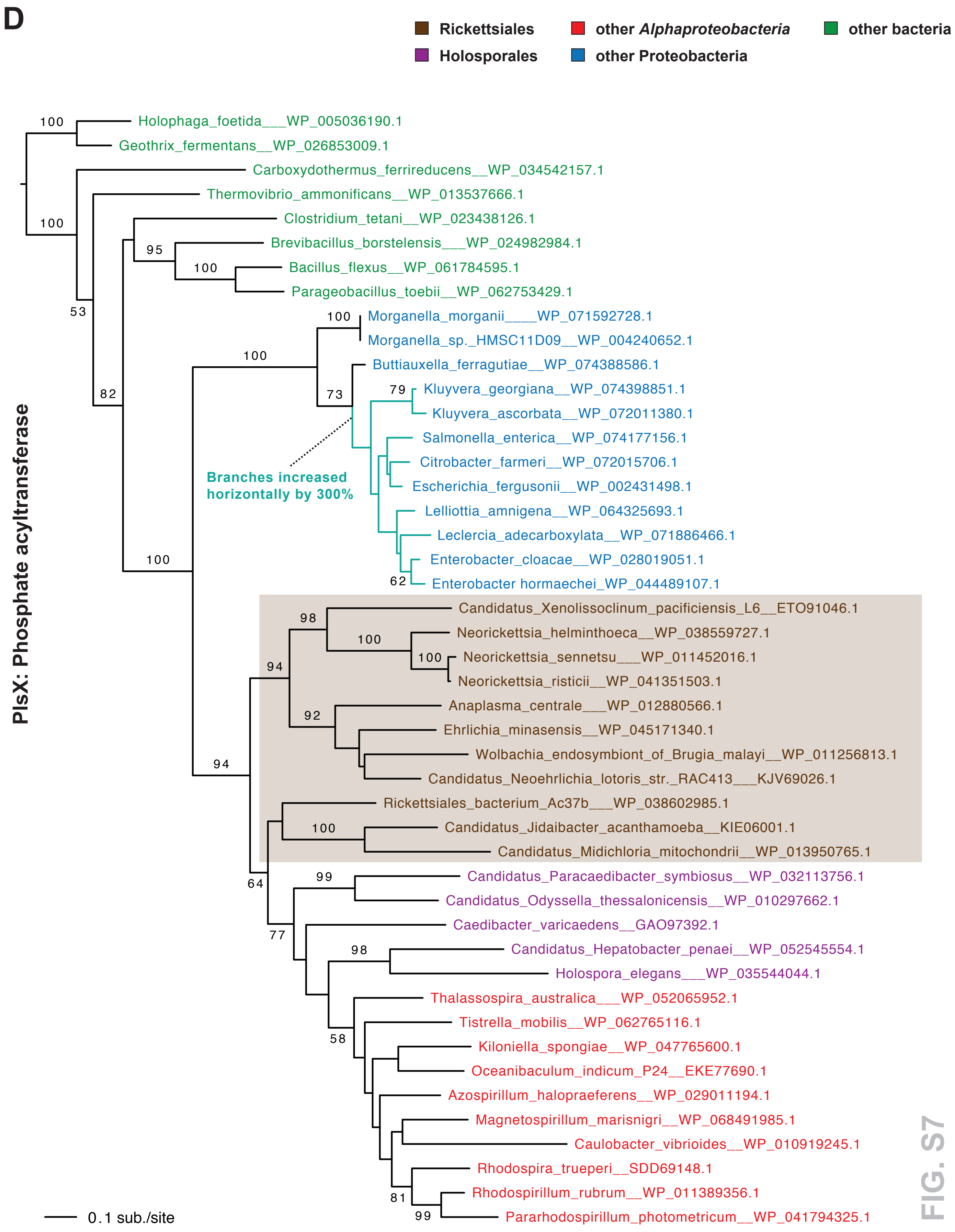
FIG. S7


D
🟫 Rickettsiales
🟥 other *Alphaproteobacteria*
🟩 other bacteria
🟪 Holosporales
🟦 other Proteobacteria
PlsX: Phosphate acyltransferase
100
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Anaplasma_centrale___WP_012880566.1
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Ehrlichia_minasensis__WP_045171340.1
Wolbachia_endosymbiont_of_Brugia_malayi__WP_011256813.1
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FIG. S7

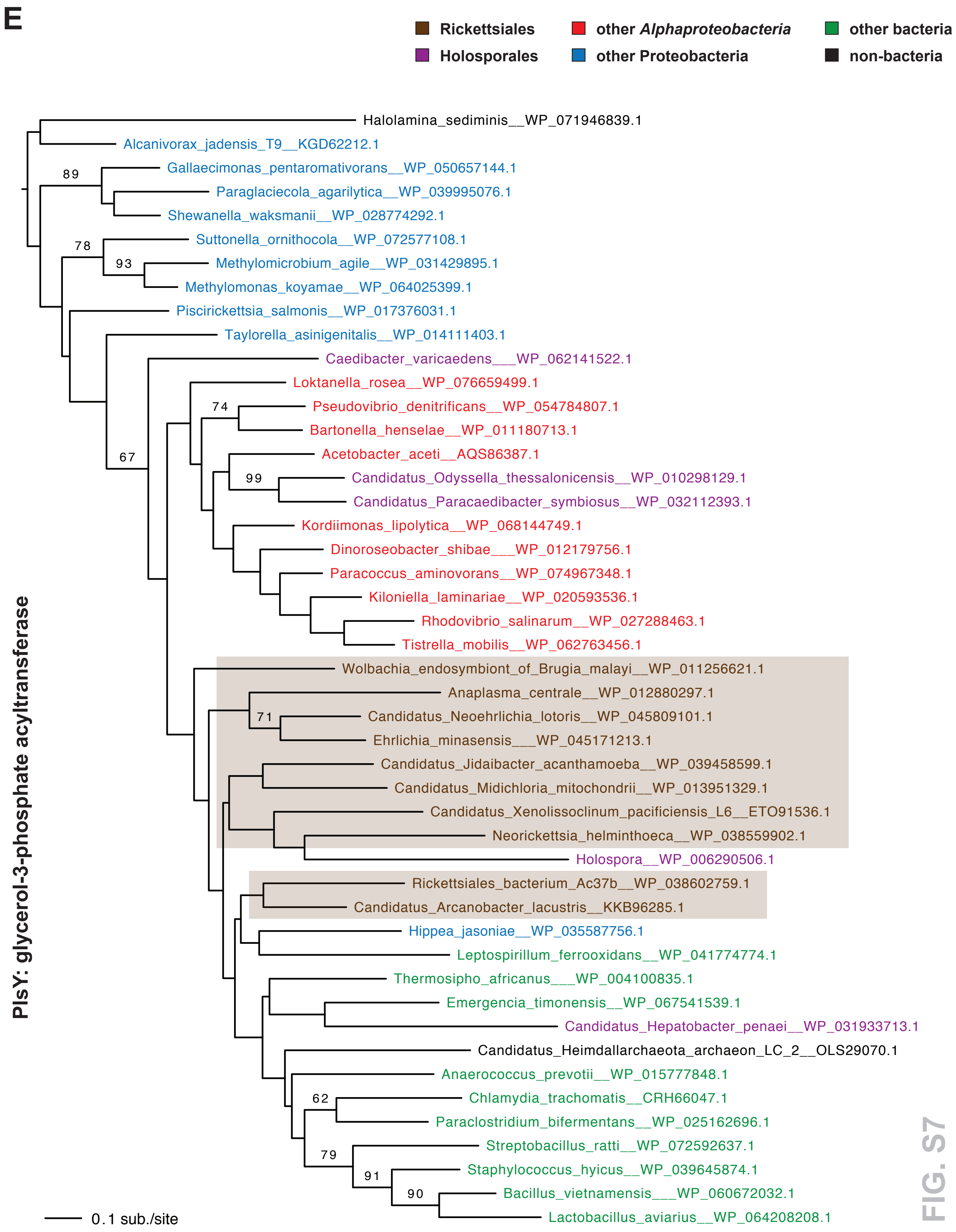
E
■ Rickettsiales
■ Holosporales
■ other *Alphaproteobacteria*
■ other Proteobacteria
■ other bacteria
■ non-bacteria
PlsY: glycerol-3-phosphate acyltransferase
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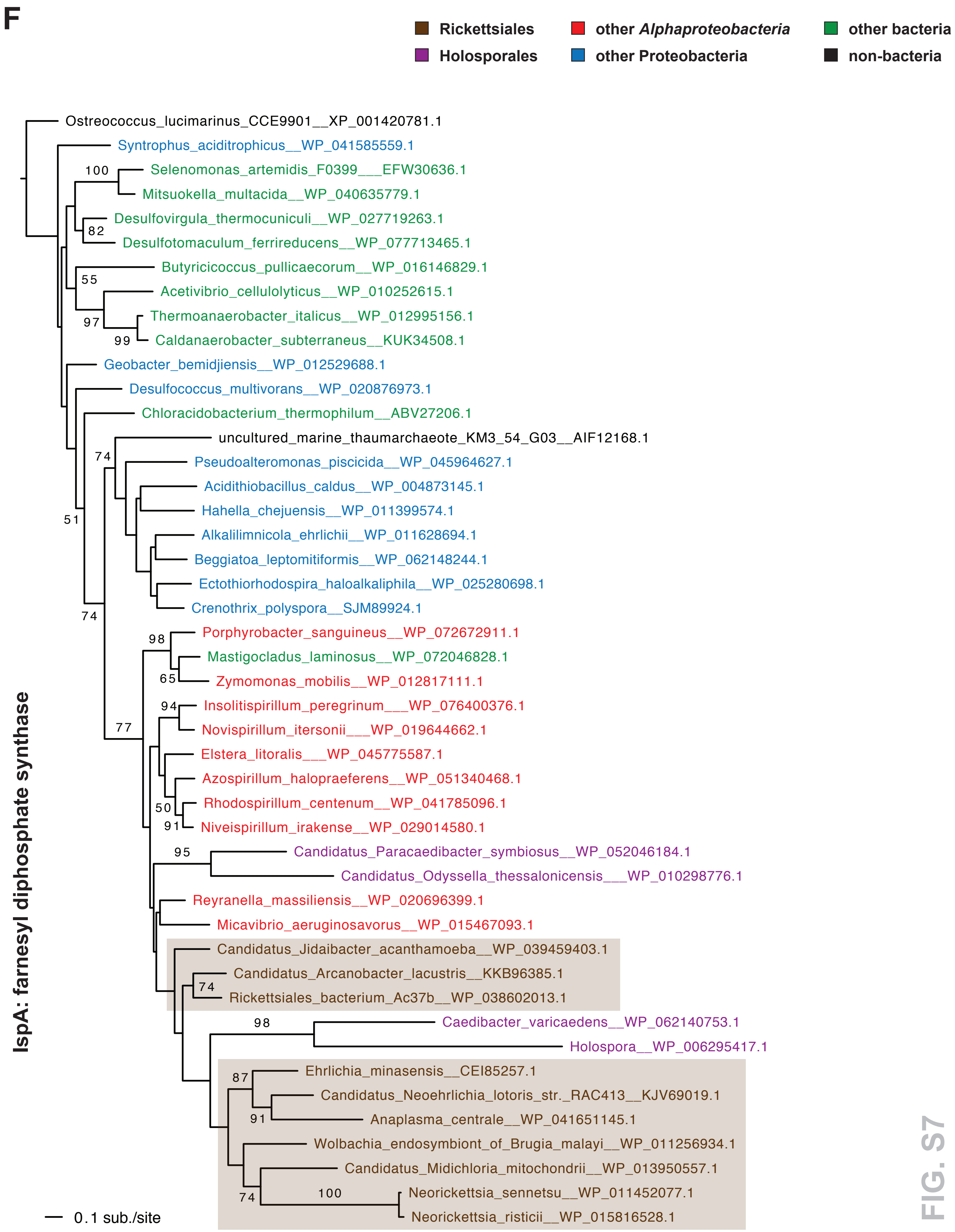
FIG. S7


F
Rickettsiales
Holosporales
other Alphaproteobacteria
other Proteobacteria
other bacteria
non-bacteria
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Syntrophus_aciditrophicus__WP_041585559.1
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Alkalilimnicola_ehrlichii__WP_011628694.1
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Crenothrix_polyspora__SJM89924.1
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98
Porphyrobacter_sanguineus__WP_072672911.1
Mastigocladus_laminosus__WP_072046828.1
65
Zymomonas_mobilis__WP_012817111.1
94
Insolitispirillum_peregrinum___WP_076400376.1
77
Novispirillum_itersonii___WP_019644662.1
Elstera_litoralis___WP_045775587.1
Azospirillum_halopraeferens__WP_051340468.1
Rhodospirillum_centenum__WP_041785096.1
50
91
Niveispirillum_irakense__WP_029014580.1
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Candidatus_Paracaedibacter_symbiosus__WP_052046184.1
Candidatus_Odyssella_thessalonicensis___WP_010298776.1
Reyranella_massiliensis__WP_020696399.1
Micavibrio_aeruginosavorus__WP_015467093.1
Candidatus_Jidaibacter_acanthamoeba__WP_039459403.1
Candidatus_Arcanobacter_lacustris__KKB96385.1
74
Rickettsiales_bacterium_Ac37b__WP_038602013.1
98
Caedibacter_varicaedens__WP_062140753.1
Holospora__WP_006295417.1
87
Ehrlichia_minasensis__CEI85257.1
Candidatus_Neoehrlichia_lotoris_str._RAC413__KJV69019.1
91
Anaplasma_centrale__WP_041651145.1
Wolbachia_endosymbiont_of_Brugia_malayi__WP_011256934.1
Candidatus_Midichloria_mitochondrii__WP_013950557.1
100
74
Neorickettsia_sennetsu__WP_011452077.1
Neorickettsia_risticii__WP_015816528.1
0.1 sub./site

IspA: farnesyl diphosphate synthase

FIG. S7