

# Two Bacterial Genera, *Sodalis* and *Rickettsia*, Associated with the Seal Louse *Proechinophthirus fluctus* (Phthiraptera: Anoplura)

Bret M. Boyd,<sup>a,b,c</sup> Julie M. Allen,<sup>a</sup> Ryuichi Koga,<sup>d</sup> Takema Fukatsu,<sup>d</sup> Andrew D. Sweet,<sup>a</sup> Kevin P. Johnson,<sup>a</sup> David L. Reed<sup>b</sup>

Illinois Natural History Survey, University of Illinois at Urbana-Champaign, Champaign, Illinois, USA<sup>a</sup>; Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA<sup>b</sup>; Genetics and Genomics Graduate Program, University of Florida, Gainesville, Florida, USA<sup>c</sup>; Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan<sup>d</sup>

## ABSTRACT

Roughly 10% to 15% of insect species host heritable symbiotic bacteria known as endosymbionts. The lice parasitizing mammals rely on endosymbionts to provide essential vitamins absent in their blood meals. Here, we describe two bacterial associates from a louse, *Proechinophthirus fluctus*, which is an obligate ectoparasite of a marine mammal. One of these is a heritable endosymbiont that is not closely related to endosymbionts of other mammalian lice. Rather, it is more closely related to endosymbionts of the genus *Sodalis* associated with spittlebugs and feather-chewing bird lice. Localization and vertical transmission of this endosymbiont are also more similar to those of bird lice than to those of other mammalian lice. The endosymbiont genome appears to be degrading in symbiosis; however, it is considerably larger than the genomes of other mammalian louse endosymbionts. These patterns suggest the possibility that this *Sodalis* endosymbiont might be recently acquired, replacing a now-extinct, ancient endosymbiont. From the same lice, we also identified an abundant bacterium belonging to the genus *Rickettsia* that is closely related to *Rickettsia rickettsii*, a human pathogen vectored by ticks. No obvious masses of the *Rickettsia* bacterium were observed in louse tissues, nor did we find any evidence of vertical transmission, so the nature of its association remains unclear.

## IMPORTANCE

Many insects are host to heritable symbiotic bacteria. These heritable bacteria have been identified from numerous species of parasitic lice. It appears that novel symbioses have formed between lice and bacteria many times, with new bacterial symbionts potentially replacing existing ones. However, little was known about the symbionts of lice parasitizing marine mammals. Here, we identified a heritable bacterial symbiont in lice parasitizing northern fur seals. This bacterial symbiont appears to have been recently acquired by the lice. The findings reported here provide insights into how new symbioses form and how this lifestyle is shaping the symbiont genome.

The sucking lice (Anoplura) are obligate and host-specific ectoparasites of mammals. These lice have specialized piercing-sucking mouthparts for feeding exclusively on mammal blood (1). One of these louse species, the human body louse *Pediculus humanus*, has been shown to rely on endosymbiotic bacteria for normal development that supply the lice with B vitamins deficient in vertebrate blood (reference 2, as interpreted by Perotti et al. [3]).

Heritable endosymbiotic bacteria (all belonging to the *Enterobacteriaceae* in the *Gammaproteobacteria*) are known for many species of mammalian sucking lice (4–17). These bacteria are housed in specialized organs, known as bacteriomes, and are vertically transmitted from mother to offspring (10, 11, 16, 18, 19). However, the location, general characteristics, and tissue type constituting the bacteriomes may differ across louse lineages (4, 10). On the basis of these observations, Buchner (10) suggested that the bacteriomes had originated independently in different louse lineages and were not derived from an ancestral structure. Later, molecular phylogenetics showed that the bacteria inhabiting these bacteriomes are distantly related to each other (14–17; see also reference 20 for a review). These patterns suggest that louse-bacterium endosymbiotic associations have originated multiple times and/or that the endosymbionts have been replaced repeatedly.

Despite the growing knowledge of mammal-louse endosymbioses, little is known about the endosymbionts of sucking lice parasitizing marine mammals. In total, four genera of sucking lice

are known to parasitize walruses and seals (21). We obtained specimens of the seal louse, *Proechinophthirus fluctus*, from the northern fur seal, *Callorhinus ursinus*, a marine mammal found in the Bering Sea, Pacific Ocean, and Sea of Japan (22). These lice are specialized for parasitizing marine mammals and, unlike lice on land animals, may have limited opportunities to feed (23). Upon initial inspection, these lice exhibited no obvious bacteriomes like those found, for example, in human lice.

The goals of this study were to determine whether these fur seal lice harbor endosymbionts and, if so, to identify these bacterial associates. In order to characterize potential bacterial endosymbionts of the northern fur seal louse, *P. fluctus*, we adopted a variety

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Address correspondence to Bret M. Boyd, [bretboyd@illinois.edu](mailto:bretboyd@illinois.edu).

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of techniques, including molecular phylogenetics using multiple markers, genome sequencing, *in situ* hybridization targeting bacterial 16S rRNA, and assessment of pathogenicity, B-vitamin synthesis, and motility as predicted by genome annotation data.

## MATERIALS AND METHODS

**Sample collection.** *Proechinophthirus fluctus* Osborne (Phthiraptera: Anoplura) fur seal lice were collected from *Callorhinus ursinus* Linnaeus (Otariidae) fur seals in St. Paul Island rookery, Pribilof Islands, Alaska, USA. Lice were preserved in 95% ethanol and stored at  $-80^{\circ}\text{C}$ .

**DNA extraction and sequencing.** Genomic DNA was extracted and isolated from whole lice using a phenol-chloroform method (24). DNA extracts from four lice were pooled, and total genomic DNA was used in library construction. The library was constructed using the Illumina TruSeq sample preparation kit with a targeted insert size of 300 to 400 bp. DNA fragments were sequenced on one-half lane of the Illumina HiSeq 2000 platform using the TruSeq SBS sequencing kit, yielding 100-bp paired-end reads. Library preparation and sequencing were done at the W. M. Keck Center, University of Illinois at Urbana-Champaign.

**Genome assembly.** We largely followed the symbiont genome assembly methods described by Boyd et al. (24). This included removing suspect base calls in the Illumina paired-end read library by quality trimming. To do this, we trimmed the first five bases from the 5' end of each read and seven bases from the 3' end to remove read positions that had elevated AT content. The reads were then soft trimmed from the 3' end to remove base calls with a phred score of less than 28 using a sliding window of 1 nucleotide. Reads that had fewer than 75 bp after quality trimming were removed from the library along with their mates. Remaining reads were assembled *de novo* into contigs using ABySS genome assembler ( $k = 64$ , paired-end) (25). To identify contigs representing the endosymbiont genome, all contigs were compared to a library of bacterial genomes, including gamma- and alphaproteobacterial endosymbionts and Gram-positive bacterial species, using blastn (including “*Candidatus* Riesia pediculicola” strain USDA gi295698239 and gi292493920; *Sodalis glossinidius* strain morsitans gi85057978, gi85060411, gi85060466, and gi85060490; *Wigglesworthia glossinidia* gi32490749, gi19225058, and gi19225058; *Photorhabdus luminescens* subsp. *laumondii* gi37524032; *Yersinia pestis* gi31795333; *Bacillus subtilis* subsp. *subtilis* gi223666304; *Buchnera aphidicola* strain APS gi15616630, gi10957103, and gi10957099; “*Candidatus* Blochmannia floridanus” gi33519483; and *Rickettsia conorii* strain Malish 7 gi15891923) (26). Contigs showing significant similarity (determined by an expected value approaching zero) to these bacterial genomes were considered to be part of an endosymbiont genome that had assembled into contigs independently of the louse genome.

Our initial alignment of contigs to bacterial genomes suggested that two species of bacteria were present in the lice. One was an alphaproteobacterium belonging to the genus *Rickettsia*, and the other was a gamma-proteobacterium that shared high sequence similarity to *Sodalis glossinidius*. Before reconstruction of these bacterial genomes, we needed to separate sequence data belonging to each bacterial genome. We isolated all reads that were incorporated into the assembly of each bacterial contig identified in the *de novo* assembly described above. This was done by aligning reads to the contigs using bowtie2 v.beta.2.6 alignment options –end-to-end and –sensitive and printing aligned reads to the file using the –al-conc option (27). This subset of the original reads was assembled independently of the louse sequence data using ABySS. The resulting contigs were then compared to representative genomes of *Rickettsia* (*R. conorii* strain Malish 7 gi:15891923) and *Sodalis* (*S. glossinidius* strain morsitans gi:85057978) using blastn. Contigs were assigned either to *Rickettsia* or *Sodalis* based on blast scores (i.e., those contigs with a significantly better alignment score to the *R. conorii* genome and lower E value were considered to belong to the *Rickettsia* genome and vice versa). Reads that went into the construction of these contigs were then again identified using read mapping and assigned to either the *Rickettsia* genome or the *Sodalis* genome. From these reads, both genomes were assembled inde-

pendently using ABySS. Draft genome assemblies were annotated using the rapid annotation subsystem pipeline (RAST) available at rast.nmpdr.org (submission date for *Sodalis* genome, 21 November 2012; submission date for *Rickettsia* genome, 27 November 2012) (28, 29). The resulting annotated genomes were downloaded from RAST as GenBank flat files and as general feature format files. The general feature files along with fasta files of the contigs from the *Sodalis* endosymbiont were loaded into SyMAP for a whole-genome comparison with other *Sodalis* genomes (30, 31).

To evaluate sequencing depth and genome variation in the *Sodalis* genome, we again aligned the reads to the assembled genomic contigs using bowtie2 (same alignment settings as described above). The results were output to a sequence alignment map (SAM) file and converted to its binary equivalent (BAM file) using the SAMtools view function (32, 33). The BAM file was manually viewed in Geneious (Biomatters). Awk and Geneious were used to determine the genomic mean and standard deviation of sequencing coverage as well as to identify significantly high and low coverage regions. SAMtools (mpileup) and bcftools (view) were used to generate a VCF file, and this file was filtered to include single nucleotide polymorphism (SNP) calls only.

Subsequent investigation identified that the *Rickettsia* bacterium was closely related to *Rickettsia peacockii*. *R. peacockii* carries a 26,406-bp plasmid that was not in our assembly (34). To determine if the plasmid was present in our read library but failed to assemble, we aligned reads to paralogs of the *dnaA* gene found in the primary chromosome and the plasmid in *R. peacockii*. The sequences of these two genes are considerably different; if the plasmid was present, we would expect to see variation in sequence reads containing the gene. Therefore, reads would align to each paralog using Bowtie2 vbeta.2.6 using options –end-to-end and –sensitive, demonstrating that two copies of the gene were present in the library. If the sequence data aligned to only one gene, we assumed that only one copy was present. *Sodalis* species may also harbor plasmids, and we used blastn (v. 2.2.28) to search our genome assembly against known *Sodalis* plasmids (gi85060490, gi85060466, gi85060411, gi749309516).

**Identifying *Rickettsia* genes involved in pathogenicity.** Felsheim et al. (34) identified genes whose products may be involved in host cell invasion and virulence in the spotted fever group of *Rickettsia* species. On the basis of their report, we obtained sequences of these genes from the *Rickettsia rickettsii* Sheila Smith (SS) genome using the gene identifiers reported by Felsheim et al. (34). We used a tblastx search to find potential orthologs shared between *R. rickettsii* SS and genes annotated by RAST in our *Rickettsia* bacterium. BLAST results were filtered for bidirectional best hits to the virulence genes in *R. rickettsii* identifiers reported by Felsheim et al. (34). These genes were aligned to their respective orthologs in *R. rickettsii* SS using Muscle, and alignments were viewed in Geneious to visually identify differences in gene sequences between the species (35).

***Sodalis* insertion sequence search.** To find known *Sodalis* insertion sequences (IS) in this endosymbiont genome, we searched for known IS identified in two other *Sodalis* genomes. We downloaded IS from NCBI for *Sodalis* endosymbiont strain PSPU IS1 (gi612149276 locus tag AB849119) and “*Candidatus* *Sodalis* pierantonius” strain SOPE ISSope1 to -4 (gi218090034 locus tags AM921789.1, AM921790.1, AM921791.1, and AM921792.1, respectively). The *Sodalis* endosymbiont contigs were used to build an NCBI blast database in Geneious. We then searched for the IS in the *Sodalis* endosymbiont contigs using blastn. To search for novel IS, we compared the genome to itself using SynMap implemented in CoGe (<https://genomeevolution.org>) (36, 37). The genome was loaded on both the *x* and *y* axes, and the syntenic path assembly option was selected. The results were visualized in CoGe, searching for sequences with hits in multiple locations across the contigs.

To determine if the IS were present in our read library but failed to assemble as part of the *Sodalis* genome, we used aTRAM (38). This software uses a reference sequence to search for similar reads using blast and conducts a *de novo* assembly of those reads. We used the Illumina HiSeq

TABLE 1 *Sodalis* species used in phylogenetic analysis

<i>Sodalis</i> taxonomic classification	Strain	Host classification		Data availability			
		Host	Host common name	Genome	<i>groEL</i>	<i>dnaJ</i>	<i>ftsZ</i>
<i>Sodalis glossinidius</i>	Morsitans	<i>Glossinia morsitans</i>	Tsetse fly	Yes	Yes	Yes	Yes
“Ca. <i>Sodalis melophagi</i> ”		<i>Melophagus ovinus</i>	Sheep ked fly	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Proechinophthirus fluctus</i>	Northern fur seal louse	Yes	Yes	Yes	Yes
<i>Sodalis</i> endosymbiont		<i>Columbicola exilicornis</i>	Cuckoo-dove louse	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Columbicola columbae</i>	Rock dove louse	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Columbicola macroura</i>	Mourning dove louse	No	Yes	No	No
“Ca. <i>Sodalis pierantonius</i> ”	SOPE	<i>Sitophilus oryzae</i>	Rice weevil	Yes	Yes	Yes	Yes
<i>Sodalis</i> endosymbiont	PSPU	<i>Philaenus spumarius</i>	Meadow froghopper	Yes	Yes	Yes	Yes
<i>Sodalis</i> endosymbiont		<i>Aphrophora quadrinotata</i>	Four-spotted spittlebug	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Philaenarcys bilineata</i>	Prairie spittlebug	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Mesoptylus fascialis</i>	Spittlebug	No	Yes	No	No
<i>Sodalis</i> endosymbiont	NRF1	<i>Antestiopsis thunbergii</i>	Stinkbug	No	Yes	No	No
<i>Sodalis</i> endosymbiont		<i>Cantao ocellatus</i>	Shield bug	No	Yes	No	No
<i>Sodalis praecaptivus</i>	HS1	NA <sup>a</sup> : free-living/pathogenic	NA <sup>a</sup>	Yes	Yes	Yes	Yes

<sup>a</sup> NA, not applicable.

read library as the short read sequences and the five IS sequences described above as the references and chose ABySS as the *de novo* assembler.

**Predicted B-vitamin synthesis by *Sodalis*.** Human louse endosymbionts are believed to provide B vitamins to their louse hosts (2, 3). To determine if the *Sodalis* genome has genes encoding proteins involved in B-vitamin synthesis, we built two complete metabolic models for this organism. A metabolic model was first constructed using the RAST pipeline (29). RAST uses an initial automated annotation to identify features within a new genome, establishes a phylogenetic context for the organism, and finally compares the annotation to a set of manually curated genes from closely related organisms for annotation validation and correction (29). These data were compiled into an initial metabolic reconstruction for the organism. We viewed cofactor and vitamin subsystems within this complete model using the SEED Viewer (28, 39). We then used SEED tools to compare the genome of the new endosymbiont to the *S. glossinidius* genome (the only other *Sodalis* genome available for comparison at the time). Genes present in the *S. glossinidius* genome associated with B-vitamin synthesis but not found in the initial metabolic model for our new endosymbiont were manually searched for by using blast tools within SEED. Next, we built a second metabolic model for this endosymbiont using the KAAS server (40). To do this, we exported all amino acid sequences from the RAST annotation server to a fasta file. These data served as the starting data for creating the new KAAS model. This was necessary, as KAAS does not include tools for gene discovery. Instead of using phylogenetic estimations (like those done by RAST) for comparison and annotation of genes, KAAS uses a default set of genomes for gene comparison (40). We added genome data from *B. aphidicola*, “Ca. *Riesia pediculicola*,” *S. glossinidius*, and *W. glossinidia* to the default genome list of compared genomes. We then compared resulting metabolic models for pantothenate, thiamine, folate, riboflavin, pyridoxine, and biotin synthesis to the corresponding models generated by the RAST subsystem. Results from the two methods were viewed by painting present functions onto KEGG pathway maps.

Upon review of the results, we identified significant differences in the metabolic prediction for this endosymbiont from that of the newly published and closely related *Sodalis praecaptivus* genome. Gene sequences whose products were involved in B-vitamin biosynthesis in *S. praecaptivus* were downloaded from KEGG using WebDBGET (41–46). This included predicted genes *ilvG*, *ilvM*, *ilvH*, *ilvI*, *ilvE*, *ilvD*, *ilvC*, *panE*, *panB*, *panC*, *panD*, *coaA*, *dfp*, *coaD*, *coaE*, *folC*, *pdxA*, *pdxY*, *fabB*, *fabF*, *fabG*, *fabZ*, *fabI*,

*bioH*, *bioF*, *bioA*, *ynfK*, *bioB*, *thiC*, *thiE*, *thiF*, *thiS*, *thiG*, and *thiH*. We then used blastn and tblastx searches to identify candidate genes in the fur seal louse endosymbiont genome. The results were then used to manually identify and develop annotations for these genes. The gene *coaA* was expected to be present but could not be identified in the genome assembly. Therefore, an independent aTRAM assembly (38) was conducted for this gene to determine if it was absent or missing due to an assembly error.

***Sodalis* motility.** *In situ* hybridization of *Sodalis* cells in fur seal lice, described below, identified extracellular cells with large comet-like tails. Two structures could result in this phenotype, flagella or actin filaments. *S. praecaptivus* genes encoding flagellar structural and regulatory proteins were downloaded from KEGG using WebDBGET. These genes were searched for in the fur seal *Sodalis* genome using both blastn and tblastx. BLAST results were edited manually and used to annotate *S. praecaptivus* flagellum-associated gene products present in the fur seal *Sodalis* endosymbiont (see Table S1 in the supplemental material). Those genes that were found to span regions of a contig estimated by the genome assembly software using n’s or spanning two contigs were assumed to be intact. The other structure that could appear as comet tails is actin filament and is known from more distantly related bacteria, such as *Listeria* (47). We used tblastx to search for a gene, *actA* (from *Listeria*), involved in actin assembly, in the *S. praecaptivus* and seal louse-associated *Sodalis* genomes.

**Phylogenetic reconstruction.** To determine the phylogenetic relationship of the *Sodalis* endosymbiont within the gammaproteobacterial family *Enterobacteriaceae*, we built a maximum likelihood tree based on three protein coding genes (*groEL*, *dnaJ*, and *ftsZ*) from representative *Enterobacteriaceae* (including *Sodalis* species described in Table 1) and two outgroup taxa from *Pasteurellaceae*. We selected *dnaJ* and *ftsZ*, because they are single orthologs present in >80% of *Enterobacteriaceae* genomes on orthoDB v8 (48) and could be retrieved from sequenced genomes. The gene *groEL* was chosen because this gene had been sequenced for additional *Sodalis* species for which there were no genome data available. Gene sequences for all three genes were retrieved from EnsembleBacteria release 26 or NCBI for taxa that had publically available genomes. Additional *groEL* sequences were obtained from nine *Sodalis* species (Table 1) and a chewing louse endosymbiont not closely related to *Sodalis* (clade C endosymbiont, described in reference 49). The gene sequences were aligned using Muscle, implemented in Geneious. The sequences were concatenated by taxon, and missing genes were coded as

gaps. PartitionFinder (v1.1.1) was used to find the best model of sequence evolution for each gene from all possible models using the AICc correction (50). A maximum likelihood tree was constructed using RAxML (VI-HPC) using a GTR+ $\Gamma$ +I model of sequence evolution for each partition (51). Support for the most likely tree was determined by percentage of 1,000 rapid bootstrap replicates implemented in RAxML. The tree was rooted to *Pasteurella* and *Haemophilus* and viewed in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). Due to much higher taxon sampling within *Sodalis* with the *groEL* gene, a second maximum likelihood tree was built using only the aligned *groEL* sequences with RAxML. We used a GTR+ $\Gamma$ +I model and determined support by percentage of 1,000 bootstrap replicates. The tree was rooted to *Pasteurella* and *Haemophilus* and viewed in FigTree.

To determine the phylogenetic placement of the *Rickettsia* bacterium associated with the fur seal louse, we used three protein coding genes, *atpA*, *coxA*, and *gltA*, to build a phylogenetic tree of *Rickettsia* species. These genes were identified by Weinert et al. (52) as useful phylogenetic markers for *Rickettsia* species and were available in sequenced genomes. The three protein coding genes were identified in our draft genome sequence and in other *Rickettsia* and *Orientia* genomes using tblastx through the CoGe platform (accessed through <http://genomeevolution.org>) (36, 37). Gene sequences were aligned using Muscle, implemented in Geneious. Aligned gene sequences were then concatenated to form a single matrix. As with the *Enterobacteriaceae* analysis, a maximum likelihood tree was constructed in RAxML (VI-HPC) from this matrix, under a GTR+ $\Gamma$  model of sequence evolution. Support for the tree was assessed from percentage of 1,000 rapid bootstrap replicates implemented in RAxML. The tree was rooted to *Orientia* and viewed in FigTree.

**In situ identification of endosymbionts.** The 16S rRNA gene was isolated and resequenced for both bacterial species from three additional lice using global primers 27F, 1525R, and 1329R (53). PCR was done using Stratagene high-fidelity master mix. PCR products were cloned using an Invitrogen cloning kit, and 96 colonies were sequenced using Sanger sequencing. Alignment of Illumina reads to the resulting Sanger sequence verified that they were identical. These Sanger-derived 16S rRNA genes were used to construct probes to identify both endosymbionts using *in situ* fluorescent hybridization of tissue sections.

Acetone-preserved lice (54) were rehydrated in Dulbecco's phosphate-buffered saline (PBS; Sigma), and their heads, legs, and posterior ends of the abdomens were removed to facilitate infiltration of reagents. Then, the tissues were fixed in PBS containing 4% formaldehyde (Wako Chemicals) at 4°C overnight. After several washes with PBS, the fixed tissues were dehydrated with several washes with acetone, followed by a 1-h incubation. The dehydrated tissues were embedded in a resin of the Technovit 8100 embedding kit (Kulzer). Thin sections of 2- $\mu$ m thickness were cut by using an RM2165 (Leica) rotary microtome. The sections were fixed on MAS-coated glass slides (Matsunami) and kept at -20°C until labeling.

The fluorescence-labeled probe Al555-Sod181R (5'-CAC TTT GGT CTT GCG ACA T-3') specific to the *Sodalis* endosymbiont was designed manually with assistance from the Silva database (55) and Probebase (56) and was labeled with Alexa Fluor 555 (Al555) at its 5' end. Hybridization of the tissue sections was conducted using a buffer containing 10 nM Al555-Sod181R and also 10 nM Alexa Fluor 647-labeled universal probe EUB917 (5'-GGG YCC CCG YCA ATT C-3'). This buffer was applied to the tissue sections on a slide glass and incubated at 25°C overnight in a humidified chamber. Then, the sections were subjected to three successive washes with PBS containing 0.1% Tween 20 (PBST) for 10 min each and were mounted in ProLong gold antifade reagent (Life Technologies). The mounted sections were observed and photographed on an Axiophot2 (Zeiss) microscope system. Digital images were merged, and the contrast was adjusted using Photoshop CS5.1 (Adobe).

**Nucleotide sequence accession numbers.** Genome assemblies referenced in the manuscript have been deposited in DDBJ/ENA/GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), with *Sodalis* sp. strain SPI-1 genome contigs

deposited under accession no. [LECR000000000](https://www.ncbi.nlm.nih.gov/nuclseq/LECR000000000) (version [LECR010000000](https://www.ncbi.nlm.nih.gov/nuclseq/LECR010000000)) and *Rickettsia* sp. strain SPI-2 genome contigs deposited under accession no. [LECS000000000](https://www.ncbi.nlm.nih.gov/nuclseq/LECS000000000) (version [LECS010000000](https://www.ncbi.nlm.nih.gov/nuclseq/LECS010000000)).

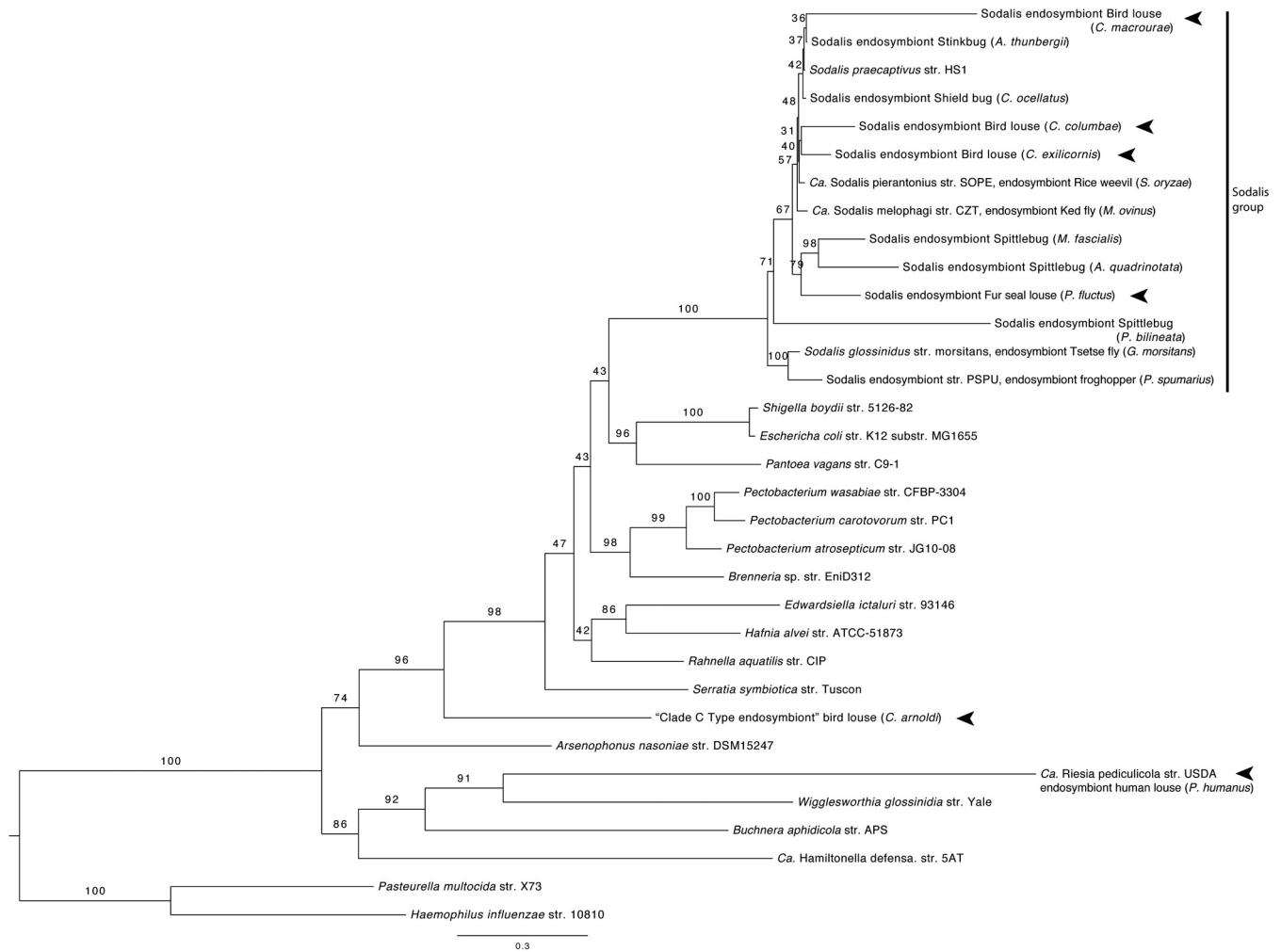
## RESULTS

**Phylogenetic placement.** Phylogenetic reconstruction of representative *Enterobacteriaceae* including the new fur seal louse endosymbiont found that this new endosymbiont was a member of the *Sodalis* and allied endosymbiont clade. This was true for both the concatenated three-gene matrix (*groEL*, *dnaJ*, and *ftsZ*) and the *groEL* sequences analyzed alone (Table 1 and Fig. 1; see Fig. S1 in the supplemental material). Support for this clade was high, with 100% of 1,000 bootstraps recovering this clade. The relationships among species within the *Sodalis* clade were largely unresolved, and bootstrap support for branches within the clade was moderate to low (most nodes were supported with <75% of bootstrap replicates). The other mammal louse endosymbiont included in this analysis, the human louse endosymbiont (*Ca. Riesia pediculicola*), was found to be distantly related to *Sodalis* species. It was more closely related to *W. glossinidia* and *B. aphidicola* in the tree built from the three-gene matrix and to *A. nasoniae* when a tree was constructed using *groEL* alone.

Phylogenetic reconstruction of *Rickettsia* species based on three protein coding genes supported the fur seal louse-associated *Rickettsia* bacterium as a member of the spotted fever species group. Specifically, it was placed in a clade that includes *Rickettsia rickettsii*, *Rickettsia philipii*, and *Rickettsia peacockii* (Fig. 2). It was not supported as belonging to the typhus clade species that has been previously found in human head lice. When estimating phylogenetic relationships from protein coding genes, we found strong support (>90% of 1,000 bootstrap replicates) for this relationship.

**Genome assemblies.** The draft genome assembly of the *Sodalis* endosymbiont totaled 2,179,576 bp, and 50% of bases were G or C. The assembly resulted in 99 scaffolds, with the largest being 171,838 bases long. The mean genome-wide read coverage was 29 $\times$ , with a standard deviation (SD) of 16. A detailed review of the longest contig revealed regions of high and low coverage. Mean read coverage for this contig was slightly higher than the genomic average (mean = 32 $\times$ , SD = 15), but 4.2% of the contig bases had very high coverage and 1.6% of the bases had very low coverage (more than 2 SD). The length of high- and low-coverage regions was smaller than the read and insert sizes (mean size = 50 bp, range = 1,339 bp; read length = 75,100 bp; insert target range for paired reads = 300 to 400); therefore, these are regions that could be estimated from read associations during genome assembly. This suggests that genomic build may be fragmented due to inconsistencies in genome sequencing depth and that the contigs are likely separated by assembly gaps greater than 400 bp. Despite potential gaps in the genome assembly, the size of our genome build is within the range of other sequenced *Sodalis* genomes (*Sodalis* sp. strain PSPU at 1.4 Mbp, 54% GC; *S. glossinidius* strain moristans at 4.5 Mbp, 55% GC; "*Candidatus Sodalis pierantonius*" strain SOPE at 4.5 Mbp, 56% GC; and the free-living *S. praecaptivus* strain HS1 at 4.7 Mbp, 57% GC) (57–60). This is also considerably larger than previously sequenced AT-rich genomes of human and chimpanzee louse endosymbionts ("*Candidatus Riesia*" species: size = 0.577 Mbp and 0.582 Mbp, respectively; % GC = 37% and 35%, respectively) (24, 61).

Another issue that may cause the genome assembly to be frag-



**FIG 1** Fur seal louse endosymbiont supported as a member of the *Sodalis* clade in the most likely tree based on maximum likelihood analysis of combined *groEL*, *dnaJ*, and *ftsZ* sequences from *Sodalis* and allied endosymbionts, representative *Enterbacteriaceae*, and outgroup taxa from *Pasteurellaceae*. Numbers at nodes are percentages of 1,000 bootstrap replicates. A vertical bar delineates the *Sodalis* group of bacteria, and arrowheads indicate louse endosymbionts. The clade C type endosymbiont from bird lice (*Columbicola arnoldi*) represents an *Enterbacteriaceae* endosymbiont distantly related to *Sodalis* described by Smith et al. (49). Other bird louse endosymbionts were described by Smith et al. (49) as belonging to clade A from *Columbicola*, a *Sodalis* clade.

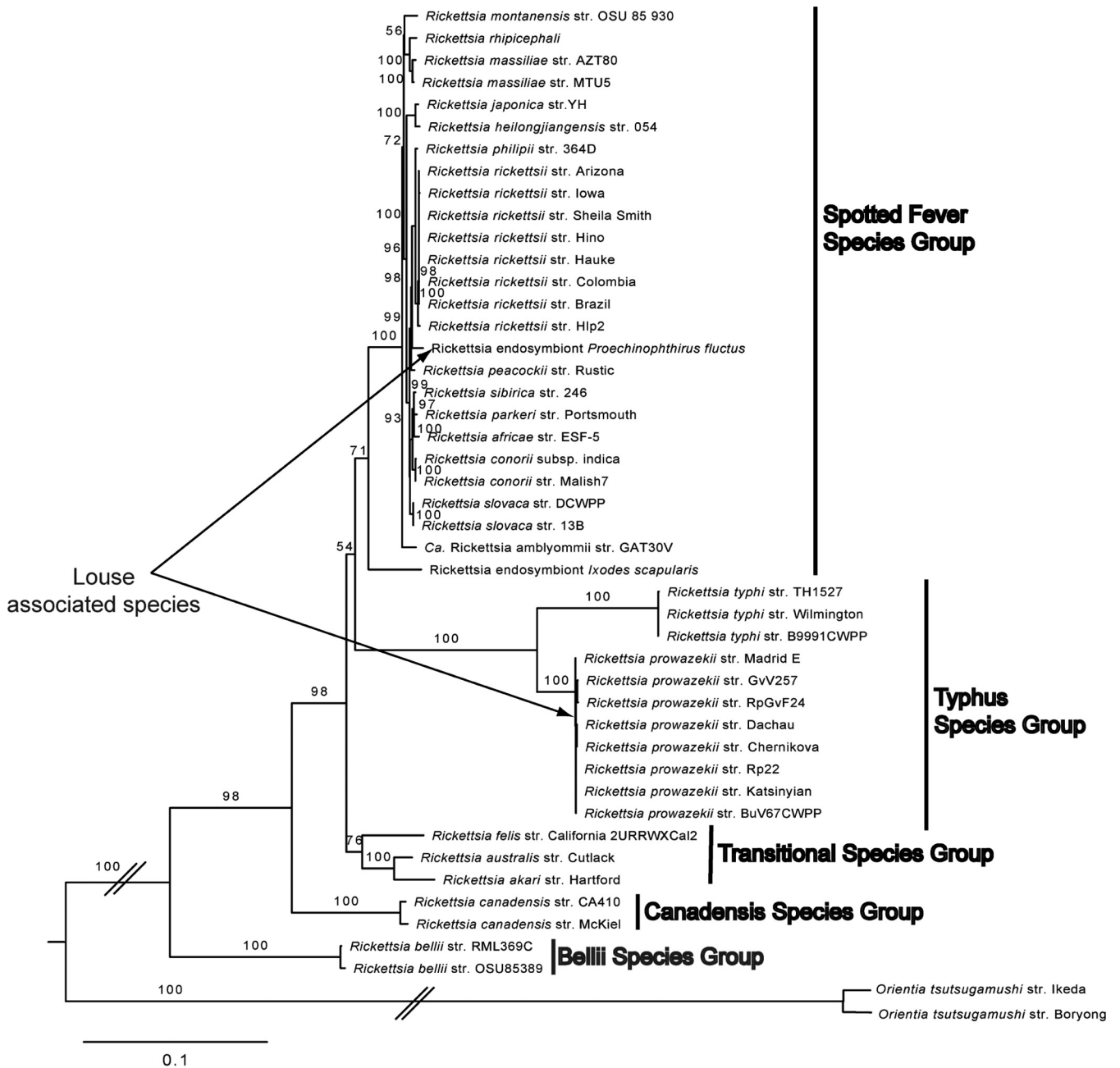
mented is the presence of genomic variation in the bacteria sampled for sequencing. In order to obtain enough DNA for genome sequencing, we had to pool extracts from four lice collected from the same mammalian host. Upon review of the data, we identified the presence of SNPs in the *Sodalis* genome data. In total, we identified 2,034 candidate SNPs spread out through the contigs. However, these did not appear to have affected the quality of the assembly, as many SNPs were found within accurately assembled genome regions and were called with either one of the alternative alleles or by an ambiguous base by the assembly software.

Nearly all of the contigs from the *Sodalis* endosymbiont genome showed genomic synteny with the *Sodalis praecaptivus* strain HS1 genome (Fig. 3). Genomic syntenies between the fur seal louse *Sodalis* endosymbiont and the “*Candidatus Sodalis pierantonius*” strain SOPE and *S. glossinidius* strain morsitans endosymbionts were slightly less complete (Fig. 3). None of the contigs showed a similarity to the plasmids known from other *Sodalis* species. We failed to detect insertion sequences previously known from “*Candidatus Sodalis pierantonius*” strain SOPE and *S.*

*glossinidius* strain morsitans either in our assembly or in the original sequencing library.

The draft genome sequence of our *Rickettsia* bacterium totaled 1,251,943 bp and was 32% GC. The genome was assembled into 3 larger scaffolds that contained most of the genome and 10 smaller scaffolds. We found no evidence of the pRPR plasmid known from the closely related species *R. peacockii*. Alignment of our scaffolds showed that gene order was largely conserved between our new louse-associated *Rickettsia* bacterium and *R. rickettsii*, with the exception of two inversions (Fig. 4). One was a large inversion that occurred in the common ancestor of our new *Rickettsia* bacterium, *R. rickettsii*, and their sister species, *R. parkerii*, with a subsequent inversion within the original inversion in *R. rickettsii*. The second inversion appeared unique to the new seal louse *Rickettsia* bacterium.

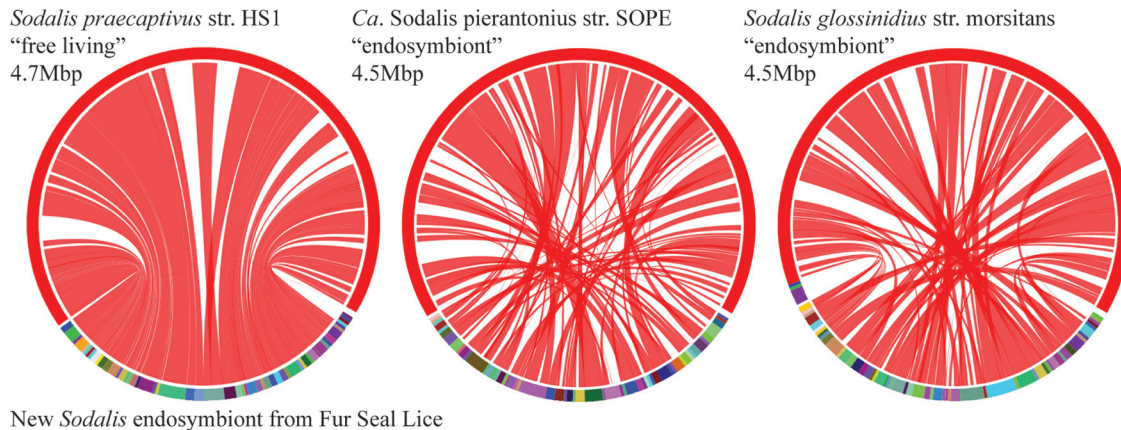
**Endosymbiont characteristics.** The *Sodalis* endosymbiont cells were rod shaped like *S. praecaptivus* but, at  $\sim 6 \mu\text{m}$  in length and  $\sim 1 \mu\text{m}$  in diameter, were larger than *S. praecaptivus* cells (Fig. 5A). These endosymbiont cells were densely packed in bac-



**FIG 2** The fur seal louse-associated *Rickettsia* bacterium supported a member of the spotted fever species group in the most likely tree based on maximum likelihood analysis of *atpA*, *coxA*, and *gltA*, showing relationships of *Rickettsia* and *Orientia* (*Rickettsiaceae*). Numbers at nodes are percentages of 1,000 bootstrap replicates. Vertical bars delineate *Rickettsia* species groups. Louse-associated *Rickettsia* species are indicated by arrows. The slashes indicate branch lengths that were shortened for publication.

terocytes found throughout the wall of the abdomen, while lower concentrations of endosymbiont cells were observed in abdominal fatty tissues (Fig. 5B to D). Additional infections were found in the lateral oviducts of adult female lice (Fig. 5E and F). In one of the specimens, *Sodalis* endosymbiont cells were found to have escaped the tissues of the lateral oviduct and were migrating to the posterior pole of a developing egg. These extracellular cells exhibited comet-like tails (Fig. 6A and B). Large masses of *Sodalis* endosymbiont cells were found in the posterior pole of eggs (Fig. 5G and H). This is consistent with maternal inheritance of *Sodalis*

endosymbionts, which is the primary method of transmission in other *Sodalis* species (62–68). After migrating to the oocytes, the *Sodalis* cells invade and form a large mass at the posterior pole of the developing eggs (Fig. 5G and H). This is similar to chewing louse (*Sodalis* species) and human louse (“*Ca. Riesa pediculicola*”) endosymbionts that also invade through the posterior pole (11, 12). Perotti et al. (12) found that the pores in the hypople provided the route for human louse endosymbiont invasion at the posterior pole. This *Sodalis* endosymbiont may also be utilizing these same pores for oocyte invasion. We were unable to locate



New *Sodalis* endosymbiont from Fur Seal Lice

**FIG 3** Comparison of *Sodalis* genomes highlighting syntenic regions. The lower half of the circle represents the fur seal louse endosymbiont contigs, and the upper half represents other *Sodalis* genomes. Shading between genomes indicates shared syntenic blocks based on coding regions. Colors delineate different contigs or chromosomes, and color choice is arbitrary. Seal louse endosymbiont contigs are ordered by their synteny with the genome to which they are compared, but the true order and orientation of the louse contigs are not known because of the fragmented nature of the genome assembly. Images were generated using SyMAP (30, 31).

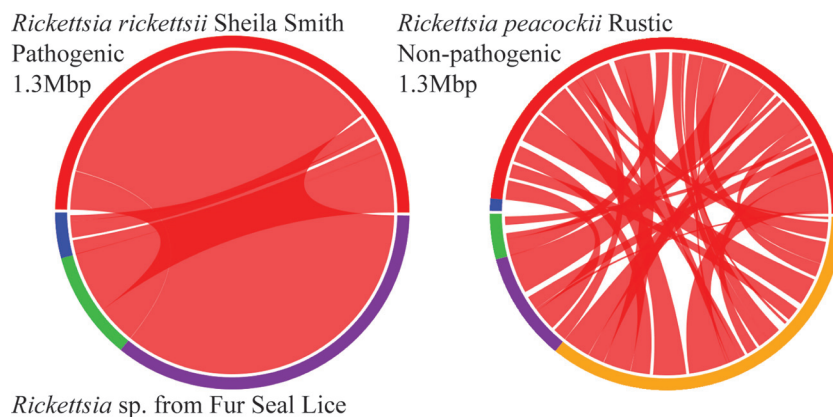
*Rickettsia* cells using the same techniques, suggesting that they may be more diffusely associated with the louse tissues or enteric.

**Motility by *Sodalis*.** As described above, the *Sodalis* endosymbionts exhibit large comet-like tails. Either flagella or actin filaments could result in this observed phenotype. The related free-living *S. praecaptivus* endosymbionts possess two larger genomic regions that both contain all of the genes needed to form flagella and to regulate their production (58). We identified a region in the fur seal louse *Sodalis* endosymbiont that was potentially orthologous to one of these regions. In this region, we identified *S. praecaptivus* genes that encode flagellar structural components and positive regulators of flagellar synthesis, including *flgA*, *flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgG*, *flgH*, *flgI*, *flgK*, *flgL*, *flhA*, *flhB*, *flhC*, *flhD*, *fliC1*, *fliC2*, *fliD*, *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliK*, *fliM*, *fliN*, *fliO*, *fliP*, *fliQ*, *fliR*, *fliS*, *motA*, and *motB* (Fig. 6; see Table S1 in the supplemental material). However, unlike this region in *S. praecaptivus*, the genes *flgM*, a negative regulator of flagellar synthesis, and *fliT* were absent (although candidate pseudogenes for each were

identified). We did find a potential ortholog of the *S. praecaptivus* gene *flgM2*, the negative regulator gene found in the second *S. praecaptivus* flagellum-encoding region. None of the other genes in this second region were detected. We failed to identify a candidate ortholog of the *actA* gene, a gene essential to actin filament formation (69), in either *S. praecaptivus* or the fur seal louse endosymbiont.

**B-vitamin synthesis in *Sodalis*.** Metabolic reconstruction using both RAST and KAAS models and manual annotation against the *S. praecaptivus* genome supported the *Sodalis* endosymbiont as possessing complete pathways for synthesis of pantothenate, folate, nicotinamide, riboflavin, biotin, and pyridoxine (Fig. 7; see Tables S1 and S2 in the supplemental material).

*De novo* biosynthesis of pantothenate (vitamin B<sub>5</sub>; transportable precursor of coenzyme A) from 3-methyl-2-oxobutanoate and β-alanine appeared complete, with the genes *panB*, *panC*, and *panE* being present. Synthesis of 3-methyl-2-oxobutanoate from either pyruvate or L-valine appeared complete; however, the *panD* gene required for synthesis of β-alanine was not predicted. Using



*Rickettsia* sp. from Fur Seal Lice

**FIG 4** Comparison of the fur seal louse-associated *Rickettsia* genome with the genomes of pathogenic *R. rickettsii* Sheila Smith and the endosymbiont *R. peacockii* Rustic. The lower halves of the circles represent the fur seal louse-associated *Rickettsia* genome, and the upper half represents previously described *Rickettsia* genomes. Shading between genomes indicates shared syntenic blocks, as determined by coding regions. Colors delineate different contigs or chromosomes, and color choice is arbitrary. Images were generated using SyMAP (30, 31).

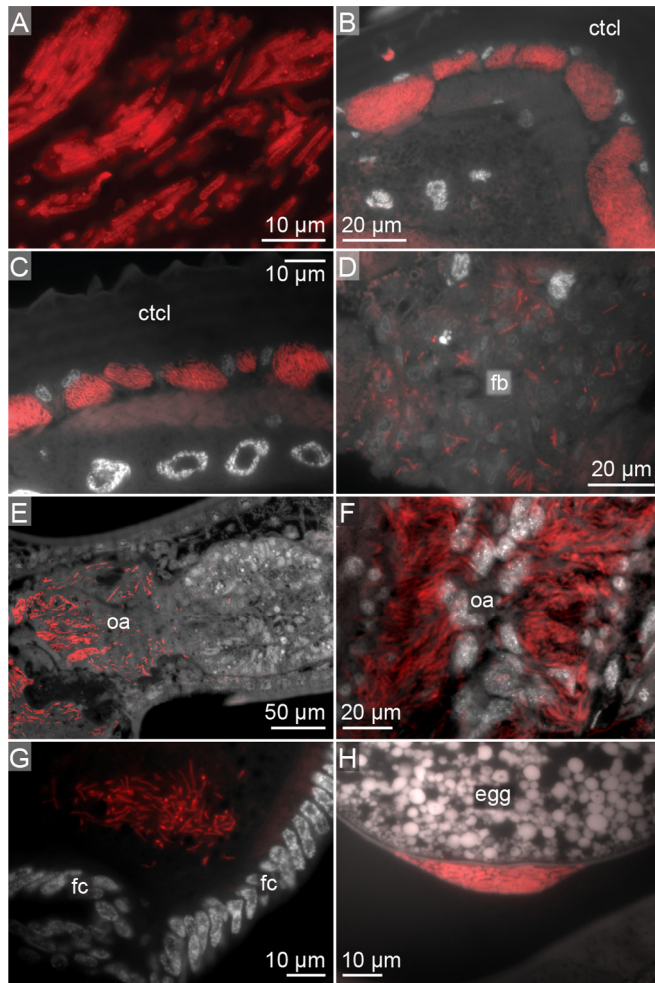


FIG 5 Storage and transmission of *Sodalis* endosymbionts. Red indicates *in situ* hybridization of 16S rRNA in cross sections of lice. (A) *Sodalis* endosymbiont cells; (B and C) intracellular masses of *Sodalis* cells in bacteriocytes along the abdominal wall; (D) diffuse *Sodalis* cells in the fat body; (E and F) *Sodalis* cells in lateral oviducts; (G and H) masses of *Sodalis* cells in posterior pole of oocytes. ctcl, cuticle; fb, fat body; fc, follicle; oa, ovary.

blast results of *panB*, *panC*, *panD*, and *panE* genes, we identified a genomic region containing candidate *panB*, *panC*, and *panD* orthologs located at a distance from *panE*, an arrangement similar to that of *S. praecaptivus*. The *panD* gene was found in blast searches but was not predicted in the RAST model. By aligning the *S. praecaptivus panD* sequence to this region, we isolated the endosymbiont *panD* ortholog and translated it to find two stop codons within the gene (see data in the supplemental material). This suggests that the endosymbiont has lost the ability to synthesize  $\beta$ -alanine through loss of the *panD*-encoded function. RAST and KAAS predicted that synthesis of coenzyme A from pantothenate was largely intact with *dfp*, *coaD*, and *coaE* present but with *coaA* missing. Subsequent blast searches using the *S. praecaptivus coaA* gene sequence also failed to find this missing gene. However, an independent aTRAM assembly yielded much of this gene sequence (see data in the supplemental material). Thus, it is likely that *coaA* is present but appears to be missing due to assembly error.

Synthesis of folate (vitamin B<sub>9</sub>) from chorismate or 7-8-dihy-

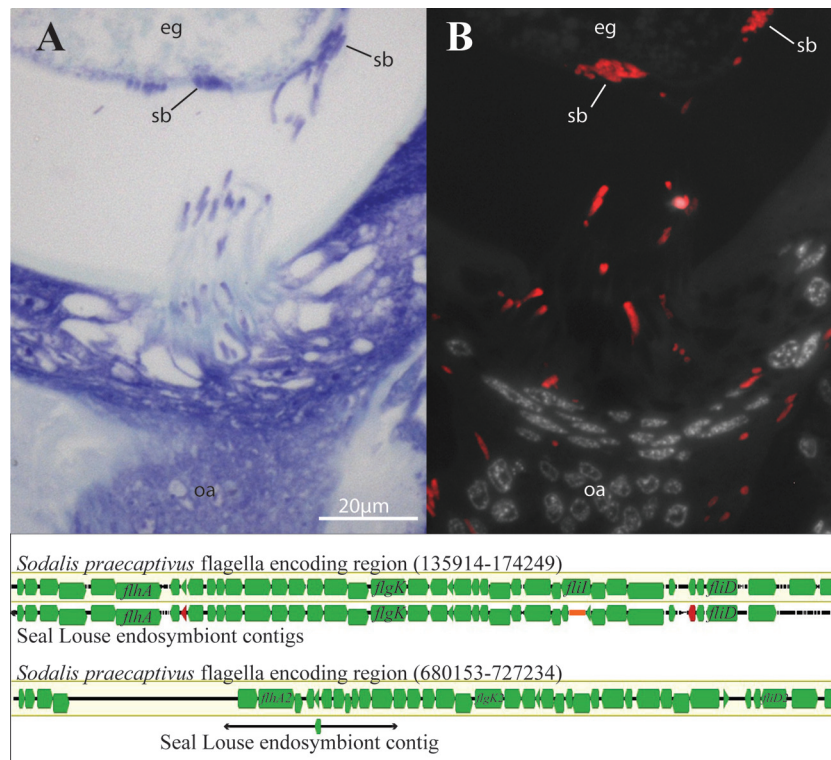
drofolate appeared to be present, but synthesis of folate from GTP appeared incomplete, with the genes *folB*, *folC*, and *folK* absent. An additional blast search for *folC* failed to identify this gene, but given the completeness of the pathway, it may also be missing due to an assembly error. Elements of the phosphorylation pathway to produce cofactors NAD and NADP from nicotinamide D-ribonucleotide (vitamin B<sub>3</sub>) were present, though limited in comparison to other *Sodalis* species, with only the genes *nadE*, *nadR*, and *ppnK* present. Synthesis of riboflavin (vitamin B<sub>2</sub>) was complete, with metabolism of riboflavin to flavin mononucleotide and flavin adenine dinucleotide also present. Synthesis of pyridoxine phosphate (vitamin B<sub>6</sub>) from D-erythrose 4-phosphate was almost complete; only a single gene in the pathway, *pdxA*, was missing. An additional blast search did not find this gene, but given that the remainder of the pathway was found, this gene may be missing due to assembly error.

The only pathway where the KAAS and RAST models disagreed was in the metabolism of biotin (vitamin B<sub>7</sub>), with only the RAST model having predicted biotin synthesis. KAAS predicted that most, but not all, of the gene products needed for biotin synthesis were present. Subsequent blastn and tblastx searches using *S. praecaptivus* genes from the biotin pathway found missing genes, but the genes were not complete. Part of one key gene, *bioF*, was identified on the end of a contig, suggesting that it was present but was not assembled completely. Two other genes (*fabG* and *ynfK*) were not found, but this is again likely due to assembly error. Despite the issues with assembly, the synthesis of biotin does appear similar to that in *S. praecaptivus*.

Both RAST and KAAS models predicted the conversion of thiamine diphosphate to thiamine phosphate. The conversion is consistent with exogenous scavenging of thiamine diphosphate rather than biosynthesis of thiamine. *S. praecaptivus* is predicted to have a complete pathway for thiamine synthesis, so we suspected that we may have failed to annotate this in the new endosymbiont genome. Therefore, we searched for a region similar to a genomic region in *S. praecaptivus* that contains multiple genes involved in thiamine biosynthesis. This region is flanked by two genes that we expected to be conserved between the two species, a 16S rRNA gene and *rpoC*. Using these genes as guides, we identified this region in the *Sodalis* endosymbiont genome. This region was assembled; however, it appears that the region between these two genes may be degraded in this endosymbiont, with many genes missing (see Fig. S2 in the supplemental material). This includes multiple genes whose products are required for thiamine biosynthesis. Therefore, it appears that this endosymbiont cannot synthesize thiamine.

**Pathogenicity genes in *Rickettsia*.** From our *de novo* genome assembly, we identified 18 genes possibly associated with virulence in vertebrates in *R. rickettsii* as identified by Felsheim et al. (34). We found that 5 of these 18 genes were potentially disrupted by mutation based on our assembly. Four of these genes possessed deletions, and one gene contained a frameshift mutation (Table 2). This included an ankyrin repeat-containing gene that was absent or disrupted in *R. peacockii* and in two attenuated strains of *R. rickettsii* (34; R. Felsheim, personal communication). A large deletion was found in the ankyrin repeat-containing gene in the fur seal louse-associated *Rickettsia* bacterium that almost perfectly matched a deletion in the same gene in the attenuated Iowa strain of *R. rickettsii*.





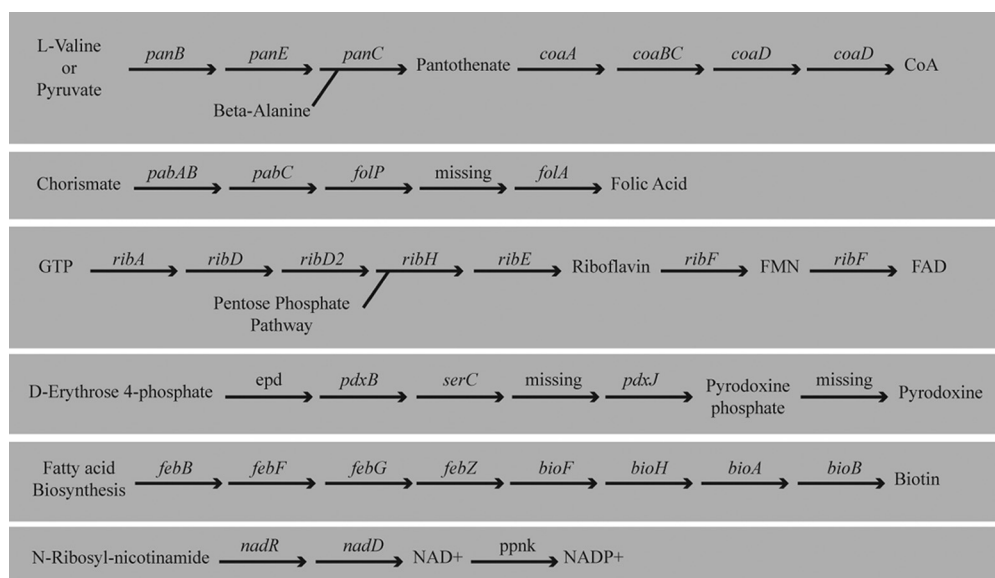
**FIG 6** Locomotion of *Sodalis* endosymbionts during infection of oocytes and comparison of predicted flagellum-encoding genomic regions in *Sodalis* species. (Top) Cross section of adult louse showing *Sodalis* cells with comet-like tails migrating from the lateral oviduct to the posterior pole of the oocyte with toluidine staining (A) and the same cross section with binding of the *Sodalis* 16S rRNA probe (B). (Bottom) Comparison of flagellum-encoding genomic regions between *S. praecaptivus* and the fur seal louse endosymbiont with genes involved in the production of flagella annotated in green (only representative genes identified by name) and candidate pseudogenes annotated in red. An orange bar represents the estimated gap between contigs. eg, egg; sb, symbiont ball; oa, ovary.

## DISCUSSION

We found that the lice parasitizing the northern fur seals harbor two bacterial species. One was a heritable gammaproteobacterium endosymbiont belonging to the genus *Sodalis*. The other bacterial

associate was identified as an alphaproteobacterium belonging to the genus *Rickettsia*.

***Sodalis* endosymbiont.** *Sodalis* and closely related endosymbionts form a diverse group of *Enterobacteriaceae* that have been



**FIG 7** Biosynthetic pathways for B vitamins and cofactors as predicted by the genomic data for the *Sodalis* endosymbiont of the fur seal louse.

**TABLE 2** Genes associated with virulence in *R. rickettsii* SS and their state in the new *Rickettsia* bacterium

Gene found in <i>R. rickettsii</i> <sup>a</sup>	State of orthologs in new <i>Rickettsia</i>
A1G_03950; methyltransferase	Intact
A1G_03470; NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	Intact
A1G_06990; <i>ompA</i>	Deletion of bases 138–140, 636–1075, and 1526–3307
A1G_02570; phosphoethanolamine transferase	Intact
A1G_00130; cell surface antigen Scal-like	Intact
A1G_0216; protease II	Deletion of bases 1051–1059
A1G_02820, A1G_02850, and A1G_02830; ABC transport	Intact
A1G_03355; protein-disulfide isomerase, <i>dsbA</i>	Intact
A1G_04605; <i>yhbC</i>	Intact
A1G_04620; transcription regulator <i>rirA</i>	Frameshift mutation at base 372
A1G_05015; <i>rickA</i>	Deletion in repeated region
A1G_04995-05010; succinyl-CoA:3-ketoacid-CoA transferase AB subunits	Intact
A1G_05165; ankyrin repeat protein (9 copies of repeat present)	1 copy of ankyrin repeat

<sup>a</sup> CoA, coenzyme A.

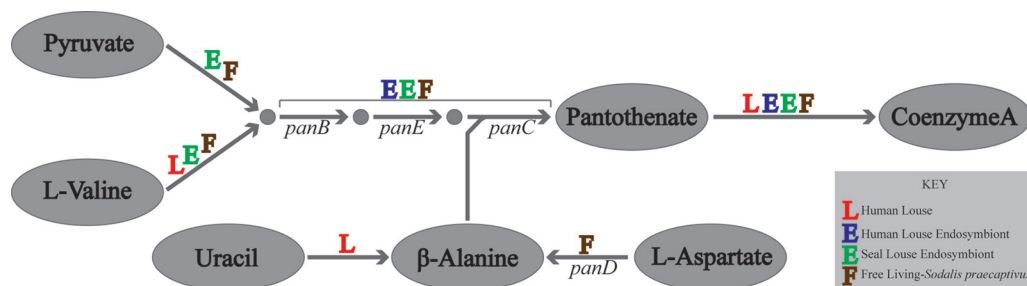
found in association with tsetse flies, ked flies, rice weevils, solitary bees, stinkbugs, spittlebugs, chewing lice, and, in this instance, blood-sucking lice (49, 62–64, 70–77). This is in addition to the recently described and free-living *Sodalis praecaptivus* (57, 78). The fur seal louse endosymbiont is more closely related to endosymbionts of chewing lice (that parasitize doves) than to other blood-sucking louse endosymbionts. As chewing lice are moderately closely related to sucking lice, it would seem logical to suspect that *Sodalis* represents the ancestral symbiont of lice. However, it appears more likely that *Sodalis* has invaded lice multiple times, likely replacing older endosymbionts.

The species-level relationships of *Sodalis* were mostly unresolved in our analysis. Smith et al. (49) interpreted this lack of resolution as evidence for repeated recent invasions of chewing lice by *Sodalis*. Repeated host invasions coupled with accelerated rates of nucleotide substitution in symbiotic species leave the evolutionary relationships of *Sodalis* difficult to resolve. The resulting star-like patterns (short internal branches with long tips) degrade confidence in the overall relationships between species (49). It does, however, support multiple recent invasions of lice by *Sodalis* bacteria. As this endosymbiont may have also been recently acquired, it may shed light on how new symbioses have formed with lice. We did not detect an organized bacteriome containing *Sodalis*, like that harboring “*Candidatus Riesia*” in human lice (10–12). This less organized storage may represent an early stage of symbiosis between sucking lice and bacteria.

If the seal louse endosymbiont was recently acquired, we might expect to see evidence that its genome is being reduced. By targeting specific orthologous genome regions between the seal louse

endosymbiont and the free-living *S. praecaptivus* genome, we found evidence of gene loss. This degradation has removed redundant functions within the endosymbiont genome and metabolic functions potentially shared with the louse. This includes the loss of genes needed for thiamine and  $\beta$ -alanine synthesis, as well as paralogs involved in flagellar synthesis. The loss of genes appears to be the result of substitutions or small mutations. We saw no evidence that this degradation is the result of insertional sequence proliferation that has been proposed for other *Sodalis* endosymbionts (e.g., *S. pierantonius* and *S. glossinidius*) (79, 80). Future studies should seek to identify pseudogenes throughout the genome for a more global perspective on genome reduction.

In human lice, endosymbionts (“*Candidatus Riesia pediculicola*”) have been implicated in provisioning B vitamins that are absent from the louse’s diet of blood (2, 3, 24, 61). Given that all sucking lice feed on mammal blood, we suspect that this seal louse endosymbiont may have a similar role. Metabolic models for the seal louse-associated *Sodalis* endosymbiont suggest it has the capacity to synthesize B vitamins similar to “*Ca. Riesia*” (Table 2). Metabolic complementation between insects and their endosymbionts in the biosynthesis of one of these vitamins, pantothenate, is present in many systems (81). In human lice, the endosymbiont does not appear to be able to synthesize a substrate needed for synthesis of pantothenate,  $\beta$ -alanine (Fig. 8). Instead, this precursor is potentially synthesized by the host and made available to the endosymbiont. In contrast, the free-living *S. praecaptivus* does retain functionality to produce  $\beta$ -alanine. The *S. praecaptivus* gene needed for biosynthesis of  $\beta$ -alanine from L-aspartate was identified as a pseudogene in the seal louse endosymbiont. The



**FIG 8** Comparison of pantothenate and CoA biosynthesis by the free-living *S. praecaptivus*, the fur seal louse *Sodalis* endosymbiont, the human head louse endosymbiont (“*Ca. Riesia pediculicola*”), and the human louse showing metabolic complementation between endosymbionts through loss of *panD*.

loss of this gene suggests a dependence on the host for  $\beta$ -alanine by the *Sodalis* endosymbiont, but the remaining pathway for synthesis of pantothenate appears complete. This is consistent with metabolic complementation between seal lice and the *Sodalis* endosymbiont. As we were able to identify a candidate pseudogene, this gene may have been disrupted recently.

**Rickettsia.** The fur seal *Rickettsia* associate was found to belong to the spotted fever species group of *Rickettsia*. This group includes the arthropod-transmitted pathogenic species *R. philipii* and *R. rickettsii* and a symbiotic species, *R. peacockii* (52, 82, 83). *Ixodes* ticks are the typical hosts for these *Rickettsia* species (52). The typical tick vectors for spotted fever group *Rickettsia* are not known to parasitize the northern fur seals (84–86). This may suggest that lice facilitate transmission of the *Rickettsia* bacteria, either vertically (from mother to offspring) and/or horizontally between seals they are parasitizing. However, we were unable to detect any evidence of vertical transmission of *Rickettsia* in the lice. It is possible that the *Rickettsia* cells were much less abundant than the *Sodalis* endosymbiont and were not detected or that *Rickettsia* cells were extracellular and wash away during sample preparation. Taken together, this may suggest this *Rickettsia* is either a pathogen that is transferred via the alimentary canal between vertebrate hosts or an organism that is accidentally and transiently infecting the lice.

This *Rickettsia* bacterium has a genome similar to that of its close phylogenetic relative, the pathogenic *R. rickettsii*, but unlike that of the tick endosymbiont *R. peacockii* (Fig. 4). The genome of *R. peacockii* has been extensively rearranged by the presence of a mobile element (34). This rearrangement may be associated with a shift in the life history of this species to obligate endosymbiosis (82). The fact that the seal louse *Rickettsia* genome is similar to the pathogenic spotted fever group *Rickettsia* genome could be informative about its life history, suggesting it is a pathogen. However, the genes that may be needed for horizontal transmission by *Rickettsia* appear to be disrupted. This included five genes identified by Felsheim et al. (34) that appear to be necessary for host invasion and coopting of host cell machinery. This may suggest that this seal louse-associated *Rickettsia* bacterium is not pathogenic.

Human lice can be infected by *Rickettsia prowazekii*, and those infections are lethal to the lice due to subsequent rupture of the gut wall (87). This rupture of the gut causes its contents to leak into the hemolymph, giving the lice a visible red color (88). Three additional species of *Rickettsia* (*Rickettsia conorii*, *R. rickettsii*, and *Rickettsia typhi*) have been shown to infect parasitic lice under laboratory conditions (88, 89). These experimental infections also resulted in rupture of the louse gut similar to that which occurs with *R. prowazekii* infections (88, 89). Despite the presence of a *Rickettsia* species in fur seal lice, we saw no evidence of the pathology in any of the lice during visual examination. This observation along with potential loss-of-function mutations in genes important to pathogenicity could suggest this is an attenuated strain of *Rickettsia*. However, these conclusions are tentative, as we were unable to find evidence of vertical transmission and unable to confirm the gene deletions using PCR (available specimens were consumed in genome sequencing and *in situ* characterization). Future investigation should focus on obtaining additional specimens for confirmation of deletions in these genes and verifying the organism's phenotype in culture.

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