The Genome Sequence of *Rickettsia felis* Identifies the First Putative Conjugative Plasmid in an Obligate Intracellular Parasite

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We sequenced the genome of *Rickettsia felis*, a flea-associated obligate intracellular α-proteobacterium causing spotted fever in humans. Besides a circular chromosome of 1,485,148 bp, *R. felis* exhibits the first putative conjugative plasmid identified among obligate intracellular bacteria. This plasmid is found in a short (39,263 bp) and a long (62,829 bp) form. *R. felis* contrasts with previously sequenced *Rickettsia* in terms of many other features, including a number of transposases, several chromosomal toxin–antitoxin genes, many more spoT genes, and a very large number of ankyrin- and tetratricopeptide-motif-containing genes. Host-invasion-related genes for patatin and RickA were found. Several phenotypes predicted from genome analysis were experimentally tested: conjugal pili and mating were observed, as well as β-lactamase activity, actin-polymerization-driven mobility, and hemolytic properties. Our study demonstrates that complete genome sequencing is the fastest approach to reveal phenotypic characters of recently cultured obligate intracellular bacteria.


Introduction

*Rickettsiae* are obligate intracellular small gram-negative bacteria associated with different arthropod hosts. Many *Rickettsia* species infect human beings and are responsible for mild to severe diseases. *Rickettsia felis*, the agent of the flea-borne spotted fever rickettsiosis, exhibits several specificities among the currently recognized *Rickettsia* species. After being identified in fleas in 1990 [1], *R. felis* has been found worldwide in flea species such as *Ctenocephalides felis*, parasitizing cats and dogs, and *Pulex irritans*. *R. felis* is transovarially transmitted in these insects [2]. Several cases of human infection caused by *R. felis* have been reported [3,4]. *Rickettsia* species are phylogenetically classified into two groups: the typhus group and the spotted-fever group (SFG). *R. felis* belongs to the SFG, together with tick-associated *Rickettsia* species such as *R. conorii*, *R. sibirica*, and *R. rickettsii*. However its lifestyle resembles that of *R. typhi* (typhus group), which is also hosted and transovarially transmitted by fleas. Furthermore, *R. felis* is known to coinfect fleas with *Bartonella henselae*, *B. quintana*, and *Wolbachia pipiens* [5]. The culture conditions of *R. felis* were established in 2001 using *Xenopus laevis* tissue culture (XTC) cells at relatively low temperatures (optimally at 28 °C) [3]. Besides these features, little is known about this pathogen. To date, six *Rickettsia* genome sequences are available. These are from two typhus group species (*R. prowazekii* [6] and *R. typhi* [7]) and four SFG species (*R. conorii* [8], *R. sibirica* [9], *R. rickettsii*, and *R. akari*). To further identify the specificities of *R. felis*, we determined its genome sequence.

Results

General Genome Features

The genome of *R. felis* comprises three replicons: a 1,485,148 bp circular chromosome and two circular plasmids identified for the first time in the genus *Rickettsia* (Figure 1). The predicted total complement of 1,512 protein-coding genes (open reading frames [ORFs]) is the largest among currently sequenced *Rickettsia* genomes (Table 1). Of these, 1,402 (92.7%) exhibited homologs in the nonredundant database and 1,080 (71.4%) were assigned putative functions. The *R. felis* chromosome exhibits a long-range (24–277 kbp) colinearity relative to other *Rickettsia* genomes, although it is more frequently interrupted by inversions/translocations than is observed between other *Rickettsia* genomes (Figure 2A). This colinearity allowed the precise assessment of orthologous relationships between ORFs of five *Rickettsia* species (*R. felis*, *R. conorii*, *R. sibirica*, *R. prowazekii*, and *R. typhi*). On this basis, we identified 530 *R. felis*-specific ORFs, that were either absent or degraded (split or fragmented) in the other four *Rickettsia* genomes (Tables 2 and 3). Consistently, the *R. felis* genome exhibited a much higher number of gene families than other *Rickettsia* species (see Table 1). The *R. felis*-specific ORFs...
 included a remarkably high number of paralogs for transposases, surface cell antigens (sca), global metabolism regulators (spoT), and proteins containing protein–protein interaction motifs such as ankyrin repeats and tetratricopeptide repeats (TPRs). Furthermore, we identified many other ORFs putatively associated with the adaptations of R. felis to its host environment or with its pathogenesis.

Plasmids

The two R. felis plasmids, named pRF and pRFΔ, are 62,829 bp and 39,263 bp long, respectively. Their topologies and sizes were confirmed experimentally (Figures S1 and S2). The pRF plasmid contains 68 ORFs, of which 53 (77.9%) exhibited homologs in public databases and 44 (64.7%) were associated with functional attributes. The nucleotide sequences of pRFΔ and pRF are identical, except for an additional 23,566-bp segment that contains 24 ORFs (pRF15–pRF38) in pRF (see Table 3). These plasmids are likely to be R. felis specific since all attempts to detect specific plasmid sequences by polymerase chain reaction (PCR) from DNA of available reference rickettsial species were unsuccessful. In contrast, the same assays against 30 fleas naturally infected by R. felis resulted in amplification of the plasmid sequences in all cases.

Plasmids are referred to as conjugative or nonconjugative. The former are disseminated by conjugation from cell to cell, while the latter are only vertically transmitted. The pRF plasmid encodes several homologs of proteins involved in the different conjugative steps (see Table 3; Figure S3). First, it exhibits a split gene (pRF38/pRF39) homologous to the traA of the Agrobacterium tumefaciens tumor-inducing plasmid [10]. TraATi is thought to be a DNA-processing machinery with nickase and helicase activities to generate the transfer strand from the origin of transfer (oriT) [10]. Second, the pRF encodes another split gene (pRF43/pRF44) homologous to the traD of the Escherichia coli F plasmid. TraDF is a ‘‘coupling protein’’ that connects the DNA-processing machinery (and transfer strand) to the mating pair formation (Mpf) apparatus, a type IV secretion system (T4SS) [11]. Finally, pRF exhibits an ORF (pRF47) similar to TraGF, a protein involved in the F-pilus assembly and aggregate stabilization [12].

Despite the presence of these ORFs linked to the initiation of plasmid transfer, the pRF sequence lacks clear homologs for the proteins involved in the Mpf apparatus found in other bacteria. Nevertheless, the R. felis chromosome (as well as other Rickettsia genomes) encodes most of the components of T4SS, which are highly similar to the vir genes of A. tumefaciens. Since the R. felis T4SS components (virB2

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**Figure 1.** Circular Representation of R. felis, R. conorii, and R. prowazekii Genomes

The three outer circles represent the chromosomes of R. felis, R. conorii, and R. prowazekii, respectively, with specific ORFs colored in red and nonspecific ORFs colored in black. Colinear genome fragments are highlighted by a shared background color, with their relative orientations indicated by arrows. The two inner circles represent two R. felis plasmids (pRF and pRFΔ), with ORFs in the region unique to pRF colored in red.

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Table 1. Comparison of R. felis and Other Published Rickettsia Genomes

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>Size (bp)</th>
<th>G+C (%)</th>
<th>Coding (%)</th>
<th>ORFs</th>
<th>RNAs</th>
<th>Repeat (%)</th>
<th>RPEs</th>
<th>Number of Gene Families</th>
<th>Transposases</th>
<th>Toxin-Antitoxin</th>
<th>Ankyrin</th>
<th>TPR</th>
<th>Vector</th>
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<tbody>
<tr>
<td>SFG</td>
<td>R. felis</td>
<td>1,587,240</td>
<td>32.5</td>
<td>83.6</td>
<td>1,512</td>
<td>39</td>
<td>4.3</td>
<td>728 (88)</td>
<td>145 (122)</td>
<td>82</td>
<td>30</td>
<td>22</td>
<td>11</td>
<td>Flea</td>
</tr>
<tr>
<td>Chromosome</td>
<td></td>
<td>1,485,148</td>
<td>32.5</td>
<td>83.8</td>
<td>1,400</td>
<td>39</td>
<td>4.4</td>
<td>725 (88)</td>
<td>66</td>
<td>30</td>
<td>18</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRF plasmid</td>
<td></td>
<td>62,829</td>
<td>33.6</td>
<td>80.3</td>
<td>68</td>
<td>0</td>
<td>4.8</td>
<td>1 (0)</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRF6 plasmid</td>
<td></td>
<td>39,263</td>
<td>33.2</td>
<td>80.6</td>
<td>44</td>
<td>0</td>
<td>1.6</td>
<td>1 (0)</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. conorii</td>
<td></td>
<td>1,268,755</td>
<td>32.4</td>
<td>81.5</td>
<td>1,374</td>
<td>39</td>
<td>0.65</td>
<td>559 (78)</td>
<td>74</td>
<td>0</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>Tick</td>
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<td>Typhus group</td>
<td>R. prowazekii</td>
<td>1,111,523</td>
<td></td>
<td>76.2</td>
<td>834</td>
<td>39</td>
<td>0.30</td>
<td>120 (22)</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Typhus group</td>
<td>R. typhi</td>
<td>1,111,496</td>
<td>28.9</td>
<td>76.3</td>
<td>838</td>
<td>39</td>
<td>0.29</td>
<td>121 (25)</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>Flea</td>
</tr>
</tbody>
</table>

*R. conorii and R. prowazekii exhibit two and one transposases, respectively, above the specified PSI-BLAST E-value threshold (10^-10).*

**ORFs for toxin-antitoxin systems.**

**mRNA-repeat-containing ORFs.**

**hTPR-containing ORFs.**

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[RF1075], *virB3* [RF0087], *virB4* [RF0088]. *virB6* [RF0089], RF0909, RF0991, RF0992, and RF0993, *virB8* [RF0463 and RF0465], *virB9* [RF0462 and RF0466], *virB10* [RF0467], *virB11* [RF0468], and *virD4* [RF0469] are conserved in all sequenced *Rickettsia* genomes that lack plasmids, the primary suspected role of the *R. felis* T4SS is to translocate virulence factors to hosts. However, the T4SS of *R. conorii* and *R. prowazekii* have been shown to function both as DNA-transfer machineries and as effector translocators [13]. Thus, the *R. felis* T4SS may also promote the transfer of DNA as in *A. tumefaciens*. We also noticed that the *R. felis* chromosome exhibits a DNA primase gene (RF0786) similar to Trac found in the *E. coli* IncP plasmid. TraC initiates the replication of transferred DNA strands in the recipient cells. Finally, the *R. felis* chromosome encodes a protein (RF0020) similar to competence protein ComE3, a protein (RF0964) similar to the F-pilin acetylation protein TraX, and a split gene (RF0705/RF0706) homologous to the Pil-pilus assembly protein FimD. In conclusion, the presence of those putative conjugative transfer genes suggests that the *R. felis* plasmids have been acquired by conjugation and that *R. felis* may still retain the capacity of transferring plasmids.

**Genome Plasticity**

We identified 333 repeated DNA sequences (50 to 2,645 bp long) in the *R. felis* genome, accounting for 4.3% of the sequence, a proportion markedly higher than in other sequenced *Rickettsia* genomes (see Table 1; Figure 2B). The major source of those repeats is the proliferation of transposase genes, for which we identified 82 copies (or inactive derivatives). Among other obligate intracellular bacteria, only *W. pipientis* aMel [14] and *Parachlamydia* sp. UWE25 [15] exhibit such a high number of large mobile genetic elements. The occurrence of highly similar transposase sequences appears to play a major role in the plasticity of the *R. felis* genome (see Figure 2A). Transposase ORFs were identified at most extremities of the *R. felis* genomic segments colinear with the *R. conorii* genome, suggesting that the *R. felis* chromosome has been rearranged many times through recombination mediated by these mobile sequences. With the use of the GRAPPA software inferring the most parsimonious genome-rearrangement scenario, we estimated at least 11 inversion events between *R. felis* and *R. conorii*. In contrast, only four inversions are required to associate more distantly related *R. conorii* and *R. prowazekii* genomes. In addition to transposases, we identified eight phage-related ORFs (see Table 2). The *R. felis* genome thus appears to have been invaded more frequently by such foreign DNAs than other *Rickettsia* species. Besides long repeats, *Rickettsia* genomes are known to contain a number of small palindromic repeats (*Rickettsia* palindromic elements [RPEs]) capable of invading both coding and noncoding regions [16]. We identified 728 RPEs in the *R. felis* genome. Of these RPEs, 85 were found within ORFs and three were found in RNA-coding genes.

The *R. felis* chromosome and plasmids share several homologs, suggesting gene exchanges between these replicons. Of 68 ORFs in pRF, 11 have a close homolog (>50% amino acid sequence identity) in the chromosome; these are seven transposases, patatin-like phospholipase (pRF11), thymidylate kinase (pRF13), and two small heat-shock proteins (pRF51 and pRF52). Among these, patatin-like proteins exhibit the most intriguing phylogeny (Figure S4). The genomes of five *Rickettsia* species (*R. prowazekii*, *R. typhi*, *R. conorii*, *R. sibirica*, and *R. felis*) exhibit chromosomal patatin-like phospholipase gene (*pat1*). Gene organization around *pat1* is similar between these *Rickettsia*. Interestingly, a phylogenetic analysis for these Pat1 and the plasmid-encoded Pat2 indicates a close relationship between Pat1 (RF0360) and Pat2 of *R. felis*, together being an outgroup of Pat1 sequences of other *Rickettsia*, suggesting a gene replacement of the chromosomally encoded *pat1* by the plasmid-encoded *pat2* in the lineage leading to *R. felis*.

Most *R. felis* genes with orthologs in other *Rickettsia* have probably been inherited vertically from a common ancestor. On the other hand, genes without orthologs in other *Rickettsia* may have been acquired by lateral gene transfer. To test this hypothesis, we analyzed the taxonomic distribution of
BLASTP best hits of *R. felis* ORFs against the nonredundant database (excluding rickettsial sequences) (Figure S5). *R. felis* ORFs with orthologs in other *Rickettsia* matched preferentially (64%) with sequences from the same taxonomic group as *R. felis* (i.e., α-proteobacteria). In contrast, the BLAST best hits for the chromosomal ORFs lacking orthologs in other *Rickettsia* were found preferentially in γ-proteobacteria (31%; 58 ORFs) and cyanobacteria (18%; 33 ORFs). The taxonomic distributions of the best matches for these two ORF sets were significantly different (*p* < 0.001; χ² test). This result suggests that many *R. felis*-specific genes may originate from distantly related organisms by lateral transfer. However, methods based on nucleotide composition bias failed to identify unambiguous candidates for lateral gene acquisition in *R. felis*.

**Surface Antigens**

The *sea* family is one of the largest paralogous gene families in *Rickettsia* [8]. Five *sea* members have been identified in the previously published *Rickettsia* genomes. Several Sca proteins are known to account for major antigenic differences between *Rickettsia* species [17] and may play important roles in adhesion to host cells [18]. Sca proteins are characterized by highly variable N-terminal sequences and a conserved C-terminal auto transporter β-domain, which translocates the N-terminal part outside the outer membrane. The *R. felis* genome exhibits the highest number of *sea* genes among currently available *Rickettsia* genomes. We identified nine intact *sea* paralogs (sca1, sca2, sca3, sca4, sca5/ompB, sca8, sca9, sca12, and sca13) as well as four fragmented or split paralogs (sca6/ompA, sca7, sca10, and sca11). Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments demonstrated that, under mild log growth phase, all *R. felis* *sea* paralogs were transcribed, including split ones. Phylogenetic analyses suggest that ancient duplication events gave rise to these paralogs before the divergence of *Rickettsia* species. We noticed that *sea* genes exhibit highly different patterns of presence/absence across different *Rickettsia* species (Table S1). Only *ompB* and *sea4* are conserved in all available *Rickettsia* genomes [19], remaining members being degraded or absent in one or more species. Together with the accelerated amino acid changes, differential gene degradation of *sea* paralogs probably contributes to the intra-species variation of those cell-surface proteins and might be linked with their adaptation to different host environments.

*R. felis* is genetically and serologically classified into the SFG of *Rickettsia* [20]. However, cross-reactivities caused by both proteins and lipopolysaccharides have been found with *R. typhi* using mouse sera [2] and human sera (Figure S6). *R. conorii* rarely cross-reacts with *R. typhi*. We therefore suspected that genes found in both *R. felis* and *R. typhi*, but missing in *R. conorii*, might be responsible for the cross-reactivities of *R. felis* and *R. typhi*. A list of such genes includes a *sea* family gene (sca3), encoding a protein with a predicted molecular weight of 319 kDa, and *rfaj* for the lipopolysaccharide 1,2-glucosyltransferase (Table 4).

**Adaptation to Environment**

Transcriptional regulation may be of critical importance in *R. felis*, as the numbers of *spoT*, the gene regulating “alarmone,” and chromosomal toxin–antitoxin modules are higher in the *R. felis* genome than in any other sequenced bacterial genome.

SpoT and RelA are two hallmark enzymes regulating global cellular metabolism of *E. coli* in response to starvation [21]. These enzymes control the concentration of alarmone, (p)ppGpp (guanosine tetra- and pentaphosphates), which in turn acts as an effector of transcription. Remarkably, *R. felis* exhibits 14 *spoT* (*spoT1–13 and 15*) paralogs (Figure S7). Using RT-PCR, we examined the transcription status of 14 *R. felis* *spoT* genes. All the *spoT* ORFs were transcribed. We classified these ORFs into two groups, based on their alignment against the sequence of the *Streptococcus dysgalactiae* RelAeq that possesses both (p)ppGpp hydrolase and synthetase activities [22]. The first group (*SpoT1–10, 14, and 15*) was aligned with...
Table 2. *R. felis*-Specific Genes Encoded in the Chromosome

<table>
<thead>
<tr>
<th>Category</th>
<th>ORFs</th>
<th>Gene Name</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large gene families</td>
<td>65 ORFs</td>
<td>tnp</td>
<td>Transposase or inactivated derivative</td>
</tr>
<tr>
<td></td>
<td>16 ORFs</td>
<td>ank</td>
<td>Ankyrin-repeat-containing gene</td>
</tr>
<tr>
<td></td>
<td>10 ORFs (five genes)</td>
<td>sca</td>
<td>Surface cell antigen homolog (Sca7–9, 11, 13)</td>
</tr>
<tr>
<td></td>
<td>7 ORFs</td>
<td>tpr</td>
<td>TPR-containing protein</td>
</tr>
<tr>
<td></td>
<td>7 ORFs</td>
<td>spoT</td>
<td>Guanosine polyphosphate pyrophosphohydrolases/synthetase homolog (SpoT5–10, 13)</td>
</tr>
<tr>
<td></td>
<td>7 ORFs</td>
<td>MFS-type transporter</td>
<td></td>
</tr>
<tr>
<td>Toxin–antitoxin system</td>
<td>6 ORFs</td>
<td>relE</td>
<td>Cytotoxic translational repressor of toxin–antitoxin system RelE</td>
</tr>
<tr>
<td></td>
<td>5 ORFs</td>
<td>parE</td>
<td>Toxin of toxin–antitoxin system ParE</td>
</tr>
<tr>
<td></td>
<td>3 ORFs</td>
<td>vapC2</td>
<td>Toxin of toxin–antitoxin system, containing PIN domain</td>
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<td></td>
<td>3 ORFs</td>
<td>phd</td>
<td>Antitoxin of toxin–antitoxin system Phd</td>
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<tr>
<td></td>
<td>3 ORFs</td>
<td>Probable antitoxin of toxin–antitoxin stability</td>
<td></td>
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<td></td>
<td>5 ORFs</td>
<td>stkD</td>
<td>Antitoxin of toxin–antitoxin system StkD</td>
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<tr>
<td>Drug resistance</td>
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<td>Cytotoxic translational repressor of toxin–antitoxin system RelE</td>
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<tr>
<td></td>
<td>5 ORFs</td>
<td>Probable toxin of toxin–antitoxin system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 ORFs</td>
<td>Probable antitoxin of toxin–antitoxin stability</td>
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<td>Phage-associated genes</td>
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<td>Phage portal protein</td>
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<td>7 ORFs</td>
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<td>Phage protease (HK97 family) and phage major capsid protein (HK97 family)</td>
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<td>Excinuclease ABC, C subunit, N-terminal</td>
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<td>7 ORFs</td>
<td>RF0137</td>
<td>Site-specific DNA methylase (EC 2.1.1.72)</td>
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<td>7 ORFs</td>
<td>RF0259</td>
<td>Protein phosphatase</td>
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<td>Site-specific recombination (cassette chromosome recombinase B)</td>
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<td>rRNA methylase (partial)</td>
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<td>7 ORFs</td>
<td>RF0915</td>
<td>Methylation- DNA–protein-cysteine methyltransferase (EC.2.1.1.63)</td>
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<td>Transporters</td>
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<td>ABC transporter, ATP-binding protein</td>
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<td></td>
<td>7 ORFs</td>
<td>RF0643</td>
<td>RND efflux system, outer-membrane protein</td>
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<td></td>
<td>7 ORFs</td>
<td>RF0862</td>
<td>Similar to amino acid permeases</td>
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<td></td>
<td>7 ORFs</td>
<td>RF0790</td>
<td>Na⁺/proline symporter, signal transduction histidine kinase</td>
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<td></td>
<td>7 ORFs</td>
<td>RF1381</td>
<td>Na⁺/H⁺ antiporter NhA</td>
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<td>Regulatory functions</td>
<td>7 ORFs</td>
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<td>Tryptophan-repressor-binding protein</td>
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<td>7 ORFs</td>
<td>RF0537</td>
<td>Transcriptional regulator, AbgB family</td>
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</table>
the sequence of the hydrolase domain, and the second group (SpoT11, 12, and 13) with the sequence of the synthetase domain. Being consistent with the previous observation [23], our phylogenetic analyses suggest that each paralogous gene group originated in early duplication events before the divergence of Rickettsia species. Notably, every sequenced Rickettsia genome encodes at least one ORF exhibiting hydrolase catalytic residues and one ORF exhibiting synthetase catalytic residues, suggesting that both hydrolase and synthetase functions are required for Rickettsia. We also found that seven spoT (spoT1–4 and 7–9) genes were located in the R. felis chromosome next to a gene encoding a transporter of the major facilitator superfamily (MFS) including proline/betaine transporters. MFS is also a large paralogous gene family composed of at least 23 ORF members in R. felis.

Toxin–antitoxin systems are composed of tightly linked toxin and antitoxin gene pairs and ensure stable plasmid inheritance when they are encoded in plasmids. In these systems, the toxic effect of a long-lived toxin is continuously inhibited by a short-lived antitoxin only when whole systems are maintained. The toxin–antitoxin modules have also been found on the chromosomes of many free-living prokaryotes, but have rarely been found in obligate intracellular bacteria [24,25]. In the R. felis chromosome, we identified 16 toxin genes (RF0016, RF0095, RF0271, RF0456, RF0490, RF0602, RF0701, RF0732, RF0787, RF0792, RF0898, RF0911, RF0956, RF1272, RF1286, and RF1368) and 14 antitoxin genes (RF0015, RF0094, RF0272, RF0457, RF0489, RF0601, RF0702, RF0731, RF0779, RF0788, RF0899, RF0910, RF0957, and RF1369), comprising at least 13 modules in operon structures. It is suggested that toxin–antitoxin systems, when encoded on the bacterial chromosome, might be involved in selective killing (a primitive form of bacterial apoptosis) or reversible stasis of bacterial subpopulations during periods of starvation or other stress [26,27]. It is also tempting to speculate that the toxin–antitoxin system could be targeted to the eukaryotic host cells. In this case, this system may help to maintain the presence of bacteria in the host. Notably, in the chromosomally encoded mazEF system of E. coli, the toxin action is regulated by (p)ppGpp. The large number of toxin–antitoxin modules in R. felis, as well as a number of spoT paralogs, might thus be linked to the synchronization of its multiplication within eukaryotic hosts.

It is probable that five R. felis–specific ORFs are related to its capacity of antibiotic resistance. We identified a streptomycin resistance protein homolog (RF0774), a class C β-lactamase, AmpC (RF1367), a class D β-lactamase (RF1275), a penicillin acylase homolog with conserved catalytic residues (RF1137), and an ABC-type multidrug transport-system protein, MdlB (RF0981). AmpC β-lactamase is known to be induced by AmpG of the MFS, which was also identified in the R. felis genome (RF0265, RF0608, RF0834, and RF1247). In vivo β-lactamase activity of R. felis was measured using high-performance liquid chromatography (see below).
**Table 3. R. felis ORFs Encoded in pRF Plasmid**

<table>
<thead>
<tr>
<th>Category</th>
<th>ORFs</th>
<th>Gene Name</th>
<th>Annotation</th>
<th>Presence in pRF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene families</strong></td>
<td>7</td>
<td>trp</td>
<td>Transposase or inactivated derivative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>trp</td>
<td>Transposase or inactivated derivative</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ank</td>
<td>Ankyrin-repeat-containing gene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>tpr</td>
<td>TPR-containing gene</td>
<td>−</td>
</tr>
<tr>
<td><strong>Conjugative transfer proteins</strong></td>
<td></td>
<td>trA</td>
<td>Conjugative transfer protein similar to A. tumefaciens Ti plasmid TraA</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trD</td>
<td>Conjugative transfer protein similar to A. tumefaciens Ti plasmid TraD</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tAO</td>
<td>Conjugative transfer protein similar to E. coli F plasmid TraO</td>
<td>+</td>
</tr>
<tr>
<td><strong>Partitioning and DNA metabolism</strong></td>
<td></td>
<td>parA</td>
<td>Chromosome-partitioning ParA family protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>tmg</td>
<td>Type I restriction-modification system, R (restriction) subunit</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tmk</td>
<td>Type I restriction-modification system, M (modification) subunit</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trpR</td>
<td>TnpR resolvase (plasmid-encoded site-specific recombinase)</td>
<td>−</td>
</tr>
<tr>
<td><strong>Host invasion</strong></td>
<td></td>
<td>pat2</td>
<td>Patatin-like phospholipase</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hyal</td>
<td>Hyaluronidase</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sca12</td>
<td>Similar to CheY-like receiver domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>lon</td>
<td>ATP-dependent protease L (TPR-containing)</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hspP2</td>
<td>Small heat-shock protein</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hspP1</td>
<td>Small heat-shock protein</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19 ORFs</td>
<td>Hypothetical proteins</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 ORFs</td>
<td>Hypothetical proteins</td>
<td>−</td>
</tr>
</tbody>
</table>

**Adaptation to Eukaryotic Hosts**

*R. felis* may have developed a specific mechanism to cross-talk with its eukaryotic hosts. It exhibits 22 ankyrin-repeat-containing proteins and 11 TPR-containing proteins. These two protein motifs are frequently found in eukaryotic proteins, but their distributions are rather limited in viruses and bacteria, in both of which they appear to be linked with pathogenicity.

The ankyrin repeat is a protein–protein interaction motif, involved in transcription initiation, cell cycle regulation, cytoskeletal integrity, and cell-to-cell signaling [28]. *Anaplasma phagocytophilum*, a closely related intracellular *α*-proteobacterium, exhibits a protein containing ankyrin repeats (AnkA), which was detected in the cytoplasm and the nucleus of infected eukaryotic cells (human leukemia-60) [29]. According to the Superfamily database [30], only 15 bacterial species possess more than three ankyrin-repeat-containing proteins, and two species exhibiting the highest number of ankyrin repeats are obligate intracellular bacteria, *W. pipientis* (21 proteins) and *Coxiella burnetii* (20 proteins), although Wu et al. [14] reported slightly different numbers of ankyrin-repeat-containing proteins for these species. A recent genome analysis of a facultative intracellular bacterium, *L. pneumophila*, revealed 20 proteins with ankyrin repeats [31]. Ankyrin repeats were also found in more than 30 ORFs of the giant virus *Acanthamoeba polyphaga* Mimivirus [32].

TPR, composed of a motif of 34 amino acids organized in tandem, is also recruited by different proteins and facilitates protein–protein interactions [33]. Its role in the adaptation of parasites to their hosts has been suggested. The *R. felis* genome exhibits 11 TPR-containing ORFs (seven in the chromosome and four in the pRF plasmid). Only *Leptospira interrogans* (the agent of leptospirosis), *Treponema* species (including the agent of syphilis), and *L. pneumophila* [31] exhibit a high number of both TPR and ankyrin repeats. These organisms are eukaryotic parasites. The cryptococcal crooked neck 1 gene of *Cryptococcus neoformans* (a yeast), containing 16 copies of TPR, appears associated with its virulence [34].

**Host Invasion/Pathogenesis**

Plasmids often carry out functions that benefit bacteria in their survival or expression of virulence. pRF exhibits two ORFs that are possibly associated with the pathogenesis of *R. felis*: a hyaluronidase and a patatin-like protein. The hyaluronidase homolog (pRF56) exhibits a significant homology to hyaluronidase NagI (1,297 aa) of *Clostridium perfringens*. Hyaluronidas, which depolymerize hyaluronic acid—an unbranched polysaccharide ubiquitously present in the extracellular matrix of animal tissues—are known as “spreading factors” [35]. Another ORF (pat2) exhibits a significant homology to patatin-like phospholipases. Its paralog (pat1) was also identified in the chromosome, as already mentioned. Patatin is the major storage glycoprotein found in potato tubers, but also exhibits phospholipase A2 activity for protection from infection. Proteins containing patatin-like domains are more frequently found in pathogenic than in...
nonpathogenic bacteria. McLeod et al. [7] suggested that patatin-like proteins might be responsible for the phospholipase A₂ activity identified some years ago in rickettsiae [36].

Potential host-invasion capacity is also provided by *R. felis*-specific ORFs found on the chromosome, for instance, a chitinase homolog (RF0413) and a chitin-binding protein homolog (RF0710). Chitin is a homopolymer of N-acetylgalcosamine and a major component of the exoskeleton of arthropods and of the peritrophic envelope of insects, a lining layer of the midgut. These genes may facilitate the access of bacteria to the insects’ gut epithelial cells. *R. felis* may also use chitin as a nutrient source, as does *E. coli* [37]. We identified a homolog (RF0268) for ecotin, an enzyme capable of using the actin cytoskeleton to disseminate through eukaryotic cells, a method exploited by other SFG species [40].

Phenotypic Post-Genomics Analysis

The obligate intracellular nature of *R. felis* hindered progress in the detailed characterization of its phenotypic diversity. Here, we envisaged post-genomics as a way of associating in vivo phenotypes of these bacteria to genomic features. The presence of pili-associated genes prompted us to investigate, by electron microscopy, the presence of such appendages on the cell surface. This approach led to the first characterization of pili on the surface of a *R. felis*; we observed two forms of pili at the surfaces of *R. felis* (Figure 3). One form of pili establishes direct contact between bacteria, providing a very typical figure of Mpf apparatus; these pili are probably specialized in conjugal transfer. The other form of pili forms small hair-like projections emerging out from the cell surface; these pili are probably involved in the attachment of the bacteria to other cells. Without pili, many disease-causing bacteria lose their invasion capability. The latter type of pili might be considered as virulence factors, as described for *Francisella tularensis* [41,42].

As previously mentioned, we also found a RickA homolog in the *R. felis* genome [40]. Based on this finding, we performed immunofluorescence assays. The orientations of actin filaments beside bacteria are distinct from the stress fibers of the host. This further suggests that *R. felis* is probably capable of using the actin cytoskeleton to disseminate through eukaryotic cells, a method exploited by other SFG species [40]. The presence of this domain in other bacterial ORFs is limited to several lineages, such as cyanobacteria and *Streptomyces*, and their functions are unknown.

*Figure 3*. Visualization of *R. felis* Pili by Transmission Electron Microscopy

Bacteria collected from the supernatant of *R. felis*-infected XTC cells were negatively stained. (A) Sexual pilus observed between two bacteria. (B) *R. felis* also possesses small appendages likely to be fimbriae pili.

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and Wigglesworthia glossinidia, a possible conjugative plasmid. Of the nine previously has yet been observed for those plasmids and genomic island. However, no conjugation encoded in a genomic island [43]. Moreover, the phylogenetic relationship and hosts of both differ significantly from their immediate neighbors with those for R. prowazekii (transmitted by lice) are comparable with their non-flea-infecting neighbors. They both differ significantly from their immediate neighbors [45,46]. Moreover, the phylogenetic relationship and hosts of R. felis and R. prowazekii (transmitted by lice) are comparable with those for B. quintana (transmitted by lice) and B. henselae (transmitted by fleas) [47]. B. henselae exhibits a larger genome with more repeats and integrases than B. quintana. Y. pestis, transmitted by fleas, also exhibits many more insertion sequences than its close relative, Y. pseudotuberculosis [48]. Altogether, flea-infecting bacteria appear to exhibit a specific evolution (i.e., more repeats, transposases, and/or integrases) compared with their non-flea-infecting neighbors.

For obligate intracellular bacteria such as rickettsiae, few phenotypic characters have been observed. To date, four intracellular bacterial genomes have been entirely sequenced, the procedure being completed in 7 y or less after their first identification or culture, including R. felis [14,15,49,50]. In the present study, the genome sequencing of R. felis provided evidence of the presence of conjugative plasmids, two types of pili, hemolytic activity, β-lactamase activity, and intracellular motility. We believe that for such recently identified/fascinating organisms, complete genome sequencing is a very potent and timesaving strategy to identify unrecognized phenotypic properties.

Materials and Methods

Bacterial purification and DNA extraction. R. felis (strain California 2) was cultivated on XTC cells growing on RPMI with 5% fetal bovine serum, supplemented with 5 mM L-glutamine. The purification of the bacteria was performed by different steps. First, the bacteria were treated in the presence of 1% trypsin in K36 buffer for 1 h at 37 °C, then centrifuged and digested by DNaseI for 1 h at 37 °C to reduce the eukaryotic DNA contamination. The sample was loaded on a renografin gradient and the bands of the purified bacteria were washed in K36, treated again by DNaseI. After inactivation with EDTA (50 mM), the bacteria were resuspended in TE, dispatched in 150-μl tubes and stored at −80 °C. Depending on this initial concentration, one or two tubes were diluted in 1 ml of TNE (10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA) and incubated for 5 h at 37 °C in the presence of lysozyme (2 mg/ml). Lysis was performed for 2 h at 57 °C by adding 1% SDS and RNaseA (25 μg/ml). Overnight treatment with 1 mg/ml of proteinase K followed at 37 °C. After three phenol–chloroform extractions and alcoholic precipitation, the DNA was resuspended in 30 μl of TE and its concentration was estimated by agarose gel electrophoresis.

Pulsed-field agarose gel electrophoresis. The concentrated bacterial suspension was included in 1% (vol/vol) Incert agarose gel blocks (BMA, Rockland, Maryland, United States). The agarose blocks were digested by Proteinase K (1 mg/ml) (EurhoBio Laboratories, Paris, France) in 1% lauroylsarcosine and 0.5 M EDTA (pH 8) (Sigma-Aldrich, St. Louis, Missouri, United States) for 24 h at 50 °C. Fresh Proteinase K was then added and the incubation was continued for 24 h. The blocks were then washed twice in TE (pH 7.6) for 30 min at room temperature. Proteinase K inactivation was performed through incubation in a 4% phenylmethylsulfonyl fluoride (MBI Fermentas, Burlington, Canada) solution for 1 h at 50 °C. This inactivation step was carried out twice. The blocks were then washed two to three times in TE and stored in 0.5 M EDTA (pH 8) at 4 °C. Before restriction enzyme digestion, the agarose blocks were equilibrated twice with TE with 15 min. Digestion was carried out for 4 h, then fresh enzyme was added and the incubation was continued overnight. The digested agarose blocks and molecular-weight markers (Low Range PFG Marker, Lambda Ladder PFG Marker [New England Biolabs, Beverly, Massachusetts, United States]) were electrophoresed in 0.5% TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA).

Each agarose block was laid in a 1% PFGE agarose (Sigma-Aldrich) solution in 0.5× TBE. Pulsed-field gel electrophoresis was carried out on a CHEF-DR II device (Bio-Rad, Hercules, California, United States) under different electrophoresis conditions. The 1% agarose gel was run at 200 V using ramped pulse times from 1 to 5 s for 10 h to observe the pattern of small DNA fragments (2–48 kb). The migration was taking place under the following two consecutive conditions: (i) a ramping time from 3 to 10 s at 200 V for 12 h, with the pattern representative for 48– to 242-kb fragments, then (ii) a ramping time from 20 to 40 s at 180 V for 15 h, with the pattern representative for 145- to 610-kb fragments.

Shotgun of R. felis genome and sequencing strategy. Three shotgun genomic libraries were constructed by mechanical shearing of the genomic DNA using a Hydro Shepard device (GeneMachine, http://genome.igbni.nih.gov/genemachine). DNA fragments were blunt-ended using T4 DNA polymerase (New England Biolabs) and ligated to the BstXI adapter. Fragments of 3, 4.5, and 7 kb were separated on a preparative agarose gel (FMC BioProducts, Rockland, Maine, United States), extracted with Qiaquick kit (Qiagen, Valencia, California, United States), and ligated into pCDNA2.1 (Invitrogen, Carlsbad, California, United States) for the two smaller inserts and into pCNS (a low copy number vector; C. R., unpublished data) for the largest one. DNA cloning was performed using electroporant E. coli DH10B Electromax cells (Invitrogen). Plasmid DNAs were purified and pools of 96 clones were analyzed by gel electrophoresis to validate the libraries. DNA sequencing of insert ends was carried out using Big Dye 3.1 terminator chemistry on an automated capillary ABI3700 sequencer (Applied Biosystems, Foster City, California, United States).
Sequences were analyzed and assembled into contigs using Phred, Phrap, and Consed software [51] taking all sequences into account. Sequences were considered valid when at least 75% of the nucleotides had a Phred score of more than 20. The finishing of the genome sequencing included only additional directed reactions that were performed on an ABI3100 sequencer. Two circular plasmid molecules of 45,846 and 38,486 bp were identified, respectively, with the use of RepeatFinder [58], by ignoring the redundant repeats. The number of inversions to associate a pair of Rickettsia genomes using GRAPPA release 2.0 [65].

Ultrasstructural characterization of pili by electronic microscopy. R. felis cells were carefully collected from the supernatant of XTC cells infected for 5 d and grown at 28 °C. Following centrifugation (400 g, 10 min), bacteria were fixed for 1 h at 4 °C in glutaraldehyde (2.5% in phosphate-buffered saline [PBS]). Cells were then washed in PBS and placed on a carbon–formvar-coated 400-mesh copper grid (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States) for 15 min. Cells were then negatively stained with 2% phosphotungstic acid for 10 s before analysis by electron microscopy (Philips Morgagni 268D, Philips Electronics, Eindhoven, the Netherlands).

Estimation of β-lactamase activity. To evaluate the level of β-lactamase activity, 10^4 R. felis cells grown on XTC cells and then sonicated were mixed with amoxicillin to a final concentration of 20 μg/ml, and incubated for 2 h at 28 °C. The concentration of amoxicillin was measured in the R. felis + amoxicillin suspension as well as in a suspension of XTC cells without bacteria + amoxicillin, at a final concentration high enough to completely inhibit the topogram. In addition, the amount of β-lactamase was determined by comparing the topogram with and without amoxicillin.

Detection of F-actin and immunofluorescence staining. Vero cells grown on glass coverslips were infected with R. felis for 24–48 h at 28 °C in a humidified CO_2_ incubator (5% CO_2). Infected cells were then fixed for 1 h at 4 °C with formaldehyde (3% w/v in PBS supplemented with 1 mM MgCl_2 and 1 mM CaCl_2), washed three times in PBS, and then made permeable with 0.2% Triton X-100 in PBS for 1 min. After three washings in PBS, the coverslips were incubated for 1 h with a monoclonal anti-R. felis antibody. Bacteria were visualized by staining with anti-mouse-Alexa 488 (Molecular Probes, Eugene, Oregon, United States) for 30 min. The coverslips were mounted using Fluoroprep (BioMériaux, Marcy-l’Étoile, France) and were examined with a confocal laser scanning microscope using a 100x oil immersion objective lens.

Hemolysis experiments. Human blood (10 ml) was centrifuged (1,500 g, 10 min), and after the PBS washes erythrocytes were suspended in 20 ml of PBS. This suspension (100 μl) was mixed with 800 μl of PBS and 100 μl of rickettsial suspension (10^8, 10^7, and 10^6 bacteria, respectively). In some experiments, rickettsiae were incubated for 1 h at 35 °C in the presence of 2 mM DTT. Complete hemolysis of the erythrocytes was determined by visual detection of pink supernatant, and spontaneous hemolysis corresponded to control without bacteria. Following 3 h of incubation at 35 °C, the samples were fixed using paraformaldehyde (0.5% final concentration) and centrifuged. Hemoglobin release was estimated by measurement of the optical density of the supernatant at 545 nm. This experiment was performed in duplicate.

Primers. The sequences of the primers for PCR and RT-PCR are provided in Table S3.
Supporting Information

**Figure S1.** Confirmation of Plasmid Topologies for pRF and pRF6 by PCR
(A) The locations of the three primer sets (pRFa–pRFb, pRFc–pRFd, and pRFa–pRFf) used to validate the presence of the two distinct plasmid forms are indicated.
(B) The result of the PCR assay with these primers. Two pairs of primers (pRFa–pRFf and pRFb–pRFc) used to obtain the probes for the Southern blot (see Figure S2), as well as another pair of primers (pRF17F/pRF1RI) used in plasmid detection in fleas infected by *R. felis*, are also indicated in (A).

Found at DOI: 10.1371/journal.pbio.0030248.sg001 (1.5 MB TIF).

**Figure S2.** Characterization of *R. felis* Plasmids by Southern Blot
The two membranes (A and B) loaded with *R. felis* genomic DNA (*51/4*) and *R. felis* DNA digested by *Pvu* (*293*) were hybridized either by the probe pRFa–pRFb or by pRFa–pRFf.

Found at DOI: 10.1371/journal.pbio.0030248.sg002 (39 KB PDF).

**Figure S3.** A Model for the Conjugative Plasmid Transfer of *R. felis*
This model is based on gram-negative bacterial conjugation systems involving T4SS. Homologs responsible for different steps of conjugation were identified in the *R. felis* genome. DNA-processing machinery, plasmid-encoded pRF38/39 (TraA); the coupling protein, plasmid-encoded pRF43/44 (TraD) or chromosomally encoded virB4 (RF0469); Mpf apparatus, chromosomal genes for virB2 (RF1075), virB3 (RF1075), virB4 (RF0468), virB6 (RF0908, RF0909, RF0901, RF0909, and RF0903), virB8 (RF0463 and RF0465), virB9 (RF0462 and RF0466), and virB10 (RF0467); and priming for DNA replication in the recipient cell, chromosomally encoded RF0786 (TraC).

Found at DOI: 10.1371/journal.pbio.0030248.sg003 (57 KB PDF).

**Figure S4.** Phylogenetic Trees for the Patain-Like Proteins, Thymidylate Kinases, and Small Heat-Shock Proteins
Phylogenetic trees were constructed using the neighbor-joining method with Jones-Taylor-Thornton model.

Found at DOI: 10.1371/journal.pbio.0030248.sg004 (16 KB PDF).

**Figure S5.** Taxonomic Distribution of BLAST Best Hits of *R. felis* ORFs
*R. felis* ORFs were searched against the nonredundant database of *R. felis* antigen; *R. conorii* and *R. typhi*; and *R. prowazekii* (experimental) support, supplied ideas, and coordinated the experiments to characterize the plasmids. CR constructed genomic libraries. GB performed most phylogenetic analyses. PEF performed β-lactamase activity assay and contributed to the bioinformatics experiments to support, supplied ideas, and coordinated the experimental aspects of the work. All authors contributed in drafting the manuscript.

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Found at DOI: 10.1371/journal.pbio.0030248.sg007 (17 KB PDF).

**Figure S8.** Confocal Laser Analysis of *R. felis*-Infected Vero Cells
Bacteria were stained by indirect immunofluorescence using a monoclonal anti-*R. felis* antibody followed by an anti-mouse-Alexa 594 antibody (red). F-actin was stained with FITC-phalloidin (green). Arrows indicate *R. felis* with actin tail.

Found at DOI: 10.1371/journal.pbio.0030248.sg008 (29 KB PDF).

**Table S1.** Distribution of *sea* Genes among *Rickettsia* Genomes
Found at DOI: 10.1371/journal.pbio.0030248.s001 (30 KB DOC).

**Table S2.** Comparison of Different Features of Bacteria Infecting Fleas with Their Close Relatives
Found at DOI: 10.1371/journal.pbio.0030248.s002 (32 KB DOC).

**Table S3.** Nucleotide Sequences of the Primers Used in the Present Study
Found at DOI: 10.1371/journal.pbio.0030248.s003 (28 KB DOC).

**Accession Numbers**

The genome sequence of *R. felis* is accessible via GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under the accession numbers: CP000053, CP000054, and CP000055. The EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl) accession number for Cl. perfringens Nq1 is Q5XM09. The Pfam (http://www.sanger.ac.uk/Software/Pfam/) accession number for ComE3 is PF03772. The Protein Data Bank (http://www.rcsb.org/pdb) accession number for *S. dysgalactiae* Relomp is 1V7J.

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**Competing interests.** The authors have declared that no competing interests exist.

**Author contributions.** HO performed genome annotation and coordinated the bioinformatics analyses. PR performed electron microscopic analysis of pili, immunofluorescence assay of actin-based motility, and hemolytic activity assay. SA and CR assembled genome sequences, performed sequence finishing, and carried out experiments to characterize the plasmids. CR constructed genomic libraries. GB performed most phylogenetic analyses. PEF performed β-lactamase activity assay and contributed to the bioinformatics analyses. HP contributed to the sequencing. JMC provided laboratory (computing) support and supplied ideas. DR provided laboratory (experimental) support, supplied ideas, and coordinated the experimental aspects of the work. All authors contributed in drafting the manuscript.
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