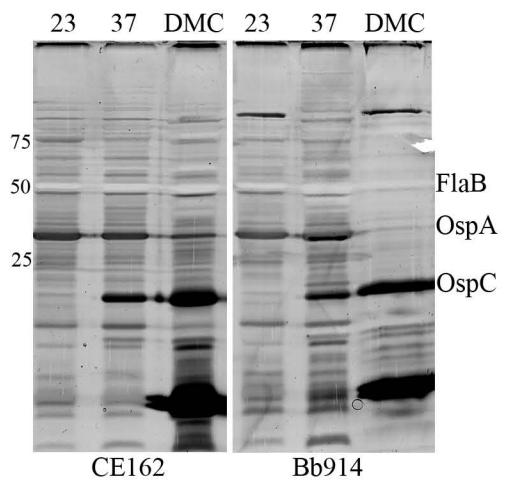
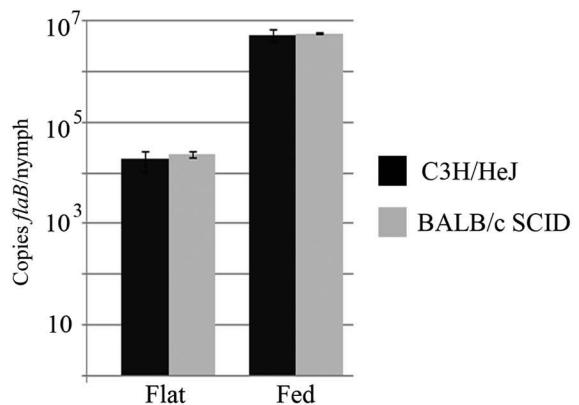


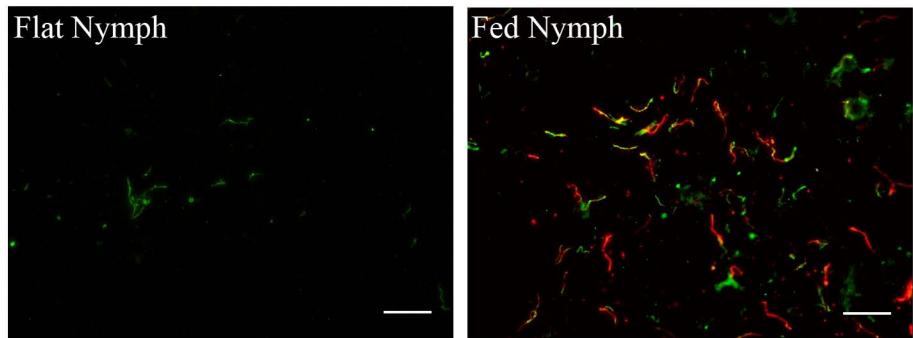
**Supplemental Figure 1. Generation of Bb914.** (A) Cloning strategy for the construction of pMC1916, the suicide vector used to insert the *gfp* reporter ( $P_{flaB}$ -*gfp*) and gentamycin-resistance cassette ( $P_{flgB}$ -*aacC1*) into the *bbb20*-*bbb21* intergenic region of cp26 in *B. burgdorferi* strain 297. (B) Cartoon depicting the double cross-over event between pMC1916 and cp26 in Bb914; gene designations for cp26 are based on the strain B31 genomic sequence (28).



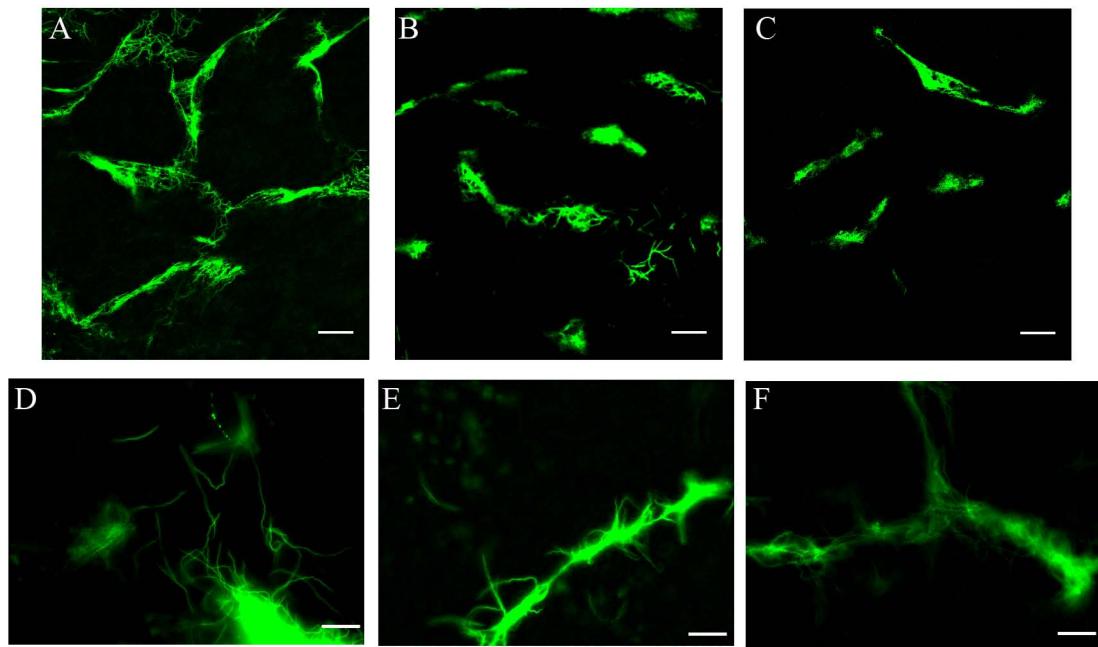
**Supplemental Figure 2. Bb914 upregulates expression of OspC in vitro and in vivo.** Polypeptide profiles of Bb914 and its parent, CE162, before and after temperature-shift in vitro and following cultivation within dialysis membrane chambers (DMCs). Whole cell lysates were separated on 12.5% SDS-polyacrylamide gels and stained with silver. All samples were separated on the same gel; the scanned gel image was cropped to remove an empty lane.



**Supplemental Figure 3. Bb914 burdens in *I. scapularis* nymphs following acquisition from syringe-inoculated C3H/HeJ or BALB/c SCID mice.** Spirochetes were acquired by larvae fed on either C3H/HeJ or BALB/c SCID mice that were syringe-inoculated with  $1 \times 10^4$  Bb914. Burdens in the resulting nymphs were analyzed before and after feeding on naïve C3H/HeJ mice. Bars represent the means  $\pm$  SDs from three independent experiments.



**Supplemental Figure 4. Induction of OspC expression in Bb914 during the nymphal blood meal.**  
Double-labeling indirect immunofluorescence analysis of Bb914 in nymphal midguts before (Flat Nymph) or 72 h post-placement (Fed Nymph) on naïve C3H/HeJ mice. Immunostaining was performed with mono-specific rat and rabbit antisera directed against OspC (red) and FlaB (green), respectively. Scale bars: 25  $\mu$ m.



**Supplemental Figure 5. Spirochetal networks form in fed tick midguts regardless of the mounting medium used.**

(A-C) Representative 5  $\mu$ m composite confocal micrographs of freshly isolated Bb914-infected tick midguts at 72h post-attachment on naïve C3H/HeJ mice mounted in (A) Vectashield (identical to Figure 4G with red channel removed), (B) immersion oil, or (C) CMRL. Representative epifluorescence micrographs of freshly isolated Bb914-infected midguts at 72h post-attachment on naïve C3H/HeJ mice mounted in (D) immersion oil, (E) CMRL, and (F) BSK-II. Scale bars (A-F): 25  $\mu$ m.

**Supplemental Table 1.** Primers used in this study

Primer	Sequence (5'- 3')	Purpose	Reference
BBB20ups 5'- <u>BssH II</u>	<u>TGCGCG</u> CTTGCTTATGTCGATTTAAA ATCAAATT	Clone upstream cp26 insertion fragment	This work
BBB20 3'- <u>Aat II</u>	AATTTGTTA <u>GACGTCT</u> ATTTAAATGC TTAAATTCAATTAT	Clone upstream cp26 insertion fragment	This work
BBB21 5'- <u>Aat II</u>	GCATTAAATAG <u>GACGTCT</u> AAACAAAAAT TGGGTGCTAG	Clone downstream cp26 insertion fragment	This work
BBB22downs 5'- <u>BssH II</u>	AAG <u>CGCG</u> CTATACTTTGGCAATTGCT CAGTC	Clone downstream cp26 insertion fragment	This work
pBS- <u>AatII</u> T7	CT <u>GACGT</u> CGTAATACGACTCACTATAG GGC	Subclone pBluescript polylinker	This work
pBS- <u>AatII</u> M13rev	<u>TGACGT</u> CCAGGAAACAGCTATGACCAT GATTACG	Subclone pBluescript polylinker	This work
flgB-aacC1 5'- <u>Sac I</u>	CG <u>GAGC</u> TCTTAATACCCGAGCTTCAAG GA	Subclone <i>gentamycin</i> -resistance marker	This work
flgB-aacC1 3'- <u>Sac I</u>	G <u>CGAGC</u> TCGATCTGGCTTGAACG	Subclone <i>gentamycin</i> -resistance marker	This work
BBB20 junc F	AAGTCAAGAAAGATTCTTGGC	Used to confirm orientation of <i>gfp</i> and <i>gentamycin</i> -resistance cassettes relative to the insertion site	This work
BBB21 junc R	CTCACATAAAACCAAAGAAACTAC	Used to confirm orientation of <i>gfp</i> and <i>gentamycin</i> -resistance cassettes relative to the insertion site	This work
GFP601-R	GGTAATGGTTGTCTGGTAAAGGACAG GGCC	Used to confirm orientation of <i>gfp</i> and <i>gentamycin</i> -resistance cassettes relative to the insertion site	(88)
flaB-rev	CTTTTCTCTGGTGAGGGAGCTC	qPCR	(67)
flaB-for	GCTCCTCCTGTTGAACACCC	qPCR	(67)
flaB-Taqman Probe	FAM-CTTGAACGGGTGCAGCC TGAGCA-BHQ1	qPCR	(67)

## Supplemental Movies

**Supplemental Movie 1. *Borrelia* within 72 h fed midguts are non-motile.** An intact midgut, freshly isolated from a Bb914-infected nymph 72 h post-placement, was mounted in Vectashield and imaged by time-lapse confocal microscopy for 1.5 hours (see the lower right hand corner for time stamp). The movie is arranged into segments depicting spirochetal networks within the epithelium and individual organisms in the luminal space. Identical results were obtained from midguts mounted in immersion oil, CMRL or BSK-II; a minimum of 7 midguts were imaged using each mounting medium. Movies presented here and elsewhere within the Supplement were generated using ImageJ (95) and QuickTime Pro software.

**Supplemental Movie 2. *Borrelia* within unfed midguts are non-motile.** An intact midgut, freshly isolated from a Bb914-infected flat nymph, was mounted in Vectashield and imaged by time-lapse confocal microscopy for 1.5 hours (see lower right hand corner for time stamp). Identical results were obtained from 7 flat nymphs.

**Supplemental Movie 3. *Borrelia* within unfed nymphal midguts respond poorly to culture medium.** Time-lapse epifluorescence microscopy was used to image Bb914 within intact and nicked, unfed midguts immersed in CMRL. The movie is arranged into three segments: (i) intact midgut; (ii) nicked midgut; and (iii) a spirochete released from a nicked midgut. Each segment shows the last 30 s of a 30 min viewing period (see the lower left hand corner for the time stamp). In the first two segments, a white line is used to delineate the periphery of the imaged diverticulum; in the second segment, an arrow indicates the location of the nick site. Identical results were obtained with midguts immersed in BSK-II; a minimum of 10 midguts were imaged in each medium.

**Supplemental Movie 4. *Borrelia* within fed nymphal midguts rapidly become motile upon exposure to culture medium.** Time-lapse epifluorescence microscopy was used to image Bb914 within intact and nicked 72 h fed midguts immersed in CMRL. The movie is arranged into three segments: (i) intact midgut; (ii) nicked midgut, near the nick site; and (iii) nicked midgut, ~5 mm away from the nick site. Each segment shows the first 30 s of a 30 min viewing period (see the lower left hand corner for the time stamp). In the first two segments, white lines are used to delineate the periphery of the imaged diverticula; in the second segment, arrows indicate the nick sites. Identical results were obtained with midguts immersed in BSK-II; a minimum of 10 midguts were imaged in each medium.

**Supplemental Movie 5. *Borrelia* within a gelatin matrix exhibit three distinct motility patterns: non-motile, motile/non-translational, and motile/translational.**

**Supplemental Movie 6. *Borrelia* in the hemolymph are vigorously motile.** Hemolymph collected from Bb914-infected nymphs at 72 h post-placement on naïve C3H/HeJ mice was imaged by time-lapse confocal microscopy for 30 min (see lower right hand corner for time stamp). Spirochete movement was noted throughout the entire viewing period and was consistently observed in samples collected from 8 nymphs.