Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN–RpoS regulatory pathway

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RpoS and RpoN are two alternative sigma factors typically associated with general stress responses in bacteria. To date, there has been no experimental evidence that RpoS and RpoN can directly control the expression of one another. Herein, using a combined strategy of gene disruption and genetic complementation targeting *rpoN* and *rpoS* in *Borrelia burgdorferi* strain 297, we describe a regulatory network for *B. burgdorferi*. In this network, RpoN controls the expression of RpoS, which, in turn, governs the expression of two important membrane lipoproteins, outer surface protein C and decorin-binding protein A, and likely other proteins of *B. burgdorferi*. Our findings provide a foundation for elucidating further key regulatory networks that potentially impact many aspects of *B. burgdorferi*'s parasitic strategy, host range, and virulence expression.

A remarkable feature of *Borrelia burgdorferi*, the agent of Lyme disease, is its ability to thrive in diverse arthropod (*Ixodes* ticks) and mammalian (rodent) hosts (1). Upon exposure to blood, *B. burgdorferi* migrates from the tick midgut to the salivary glands and then is injected into mammalian dermal tissue (2, 3). During these processes, dramatic adaptive changes occur that are reflected in altered protein profiles of the spirochete, such as the reciprocal down-regulation of outer surface (lipo)protein (Osp) A and the up-regulation of OspC (4, 5). OspC is a circular plasmid (cp26)-encoded (6), 22-kDa lipoprotein (7) that varies in sequence (8, 9) and may or may not be an immune target, depending on expression levels and the strain of *B. burgdorferi* (10, 11). Although intensively studied (12, 13), the function of OspC remains unknown, albeit its up-regulation during tick feeding and preponderance among spirochetes exposed to blood suggest that it facilitates *B. burgdorferi* migration to tick salivary glands and/or transmission into mammalian tissues (14–16). Another lipoprotein, decorin-binding protein A (DbpA), is purported to facilitate the adherence of *B. burgdorferi* to extracellular matrix as the spirochete invades mammalian tissue (17). Studies that address the regulation of these lipoproteins will assist in clarifying their roles in Lyme disease pathogenesis and perhaps in elucidating their physiological functions.

A number of environmental cues (e.g., temperature, pH, and spirochete cell density) have been implicated in influencing differential antigen expression in *B. burgdorferi* (4, 18–22). More recently, it has been shown that the *in vitro* cultivation of virulent *B. burgdorferi* strain 297 (297) at elevated temperature (37°C), reduced pH (pH 6.8), and increased spirochete cell density, parameters ostensibly that mimic conditions of tick engorgement, caused an up-regulation of OspC, DbpA, OspF, and Mlp-8 (group I proteins) with a concomitant down-regulation of OspA, P22, and Lp6.6 (group II proteins) (23). Conditions that induced the group I proteins also induced the synthesis of RpoS (σ^s; σ^{38}), one of two alternative sigma factors predicted to be present in *B. burgdorferi* B31 (B31) (24). Although conventionally associated with general stress responses (25), a role(s) for RpoS in the life cycle of *B. burgdorferi* remains unknown. However, the simultaneous induction of *rpoS* and the group I genes prompted the hypothesis that group I-like genes in 297 may be controlled through RpoS (23).

RpoN (σ^N; σ^{54}) is another important sigma subunit that (*i*) does not share obvious homologies with other sigma factors, (*ii*) directs the core enzyme to a class of distinct $-24/-12$ promoters, and (*iii*) mediates enhancer-dependent transcription (26). Most bacteria possess one copy of *rpoN*, which generally is constitutively expressed and not essential (26). Because *rpoN*-mediated transcription has been associated with bacterial pathogenicity (27–30), we examined the potential influence of *rpoN*, as well as *rpoS*, on patterns of adaptive gene expression in low-passage 297. Using a combined strategy of targeted gene disruption and genetic complementation, it was found that RpoN regulates the expression of RpoS, which, in turn, influences the expression of OspC and DbpA. These studies provide a foundation for elucidating further key regulatory networks that potentially impact many aspects of *B. burgdorferi*'s parasitic strategy.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. A virulent population of 297 (31) was recovered from needle-inoculated mice; generally, once-passaged organisms were used. Unless otherwise indicated, borreliae were cultivated at 34°C in complete Barbour-Stoenner-Kelly medium (BSK) (Sigma) (32) under an atmosphere of 1% CO_2. pBSK solid medium (33) supplemented with either erythromycin (Erm) (0.06 μg/ml) or kanamycin (Kan) (170 μg/ml) was used to select for transformants. All plasmids and recombinant constructs (Table 1) were propagated in *Escherichia coli* DH5α (GIBCO/Life Technologies, Grand Island, NY).

DNA Manipulations. All recombinant DNA experiments and the use of antibiotic resistance markers in 297 were reviewed and approved by the University of Texas Southwestern Biological and Chemical Safety Advisory Committee. Plasmid DNA used for electroporation of 297 was purified by using the StrataPrep EF Plasmid Midiprep Kit (Stratagene). The design of oligonucleotide primers (see Table 2, which is published as supporting information on the PNAS web site, www.pnas.org) to amplify 297 genes was based on published gene sequences for B31 (24); DNA amplifications were performed by using the Expand High Fidelity PCR System (Roche Diagnostics).

Construction of Suicide Plasmids Containing Disrupted Genes. pGEM-T [encoding ampicillin (Amp) resistance] was the basis for all suicide plasmids; pALH394 and pALH386 were con-

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Abbreviations: B31, *Borrelia burgdorferi* strain B31; 297, *B. burgdorferi* strain 297; BSK, Barbour-Stoenner-Kelly medium; Dbp, decorin-binding protein; Erm, erythromycin; Kan, kanamycin; Osp, outer surface protein; Amp, ampicillin; RT-PCR, reverse transcriptase–PCR.

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Table 1. Recombinant plasmids

Plasmid	Description	Source
pGEM-T	TA cloning vector, high copy number, Amp^r	Promega
pCR2.1	TA cloning vector, high copy number, Amp^r, Kan^r	Invitrogen
pUC4K	Aminoglycoside 3′-phosphotransferase gene from Tn*903* (*kan*), Amp^r, Kan^r	Amersham Pharmacia
pJRS233	Erm^r	K. McIver (Univ. of Texas Southwestern)
pJRS525	Spec^r, wide host range cloning vector	K. McIver (Univ. of Texas Southwestern)
pFAC	pGEM-T, for SP6 transcription of *ospC*- and *flaB*-specific competitor RNA	This study
pALH133	pGEM-T, *ermC* from pJRS233, Amp^r, Erm^r	This study
pALH227	pJRS525, Δ[spec, *Alw*NI-*Fsp*I(965 bp)] Ω[P_{flaB}-*kan*, *Fsp*I–*Alw*NI (1,158 bp)], Kan^r	This study
pALH251	pALH227Ω[P_{flgB}-*rpoS*, *Bam*HI–*Nco*I (1,224 bp)], Kan^r	This study
pALH293	pALH227Ω[P_{rpoN}-*rpoN*, *Bam*HI–*Nco*I (1,628 bp)], Kan^r	This study
pALH362	pGEM-T, 4,631-bp fragment (analogous B31 chromosome 810459–815090), Amp^r	This study
pALH364	pGEM-T, 5,156-bp fragment (analogous B31 chromosome 468016–473172), Amp^r	This study
pALH386	pALH362, except *rpoS*::*ermC* [*Bbs*I (1 kb)], Amp^r, Erm^r	This study
pALH394	pALH364, except *rpoN*::*ermC* [*Afl*II (1 kb)], Amp^r, Erm^r	This study
pALH400	pALH394, except *ermC*::P_{rpoN}-*rpoN*-P_{flaB}-*kan* [*Bcl*I (3 kb)], Amp^r, Erm^s, Kan^r	This study

structed with inactivated 297 gene homologs of *rpoN* (BB0450) and *rpoS* (BB0771), respectively (Fig. 1) (24). For *rpoN*, a 5.1-kb DNA fragment with flanking sequence was obtained by amplification (priXF01, priXF02) and cloned to yield pALH364 (Fig. 1*a*). For *rpoS*, pALH362 contained a 4.6-kb fragment that was obtained by using primers priXF03 and priXF04 (Fig. 1*b*). Unique restriction sites within each target structural gene were chosen as insertion sites for disruption.

The Erm resistance gene, *ermC*, was chosen as a selectable marker for the genetic manipulation of 297 based on its prior use in pGK12 for B31 (34). The source of *ermC* was pJRS233, a derivative of the *Staphylococcus aureus* plasmid pE194 (34, 35). *ErmC* was PCR-amplified with its predicted promoter by using primers to introduce appropriate restriction sites for insertion into *rpoN* (*Afl*II, priAH78 and priAH79) or *rpoS* (*Bbs*I, priAH133 and priAH134), yielding pALH394 (Fig. 1*b*) and pALH386 (Fig. 1*b*), respectively. To minimize potential polar effects, *ermC* was inserted opposite in orientation to the gene being disrupted, which was confirmed by PCR using primers complementary to *ermC* (priAH102 and priAH104) and primers flanking the insertion site of the resistance marker (see Fig. 3*a*); the latter primers were priAH59 and priAH60 for *rpoN* (see Fig. 3*b*) and priAH131 and priAH27 for *rpoS* (see Fig. 3*c*).

Electroporation of 297. The 297 cells were made electrocompetent essentially as described (33) except that spirochetes were harvested from the midlogarithmic phase of growth (about 1×10^7 spirochetes per ml). Transformation (34) was carried out by using 15–20 μg of circular plasmid DNA to electroporate ≈1×10^9 bacteria (in 60 μl of electroporation buffer). After recovery, aliquots of 0.5, 1.0, and 1.5 ml ($0.5–3.0 \times 10^8$ spirochetes) were added to ≈15 ml of pBSK (with antibiotic) and plated. Colonies appeared 12–20 days after plating. Transformation efficiencies tended to be about 2×10^{-8}, determined from the ratio of antibiotic-resistant colony-forming units (cfu) relative to the total number of cfu per transformed culture, in the context of an overall plating efficiency of about 70%.

PCR Confirmation of Gene Disruptions. To confirm marker exchange, Erm^r colonies were picked and propagated in 1.5 ml of

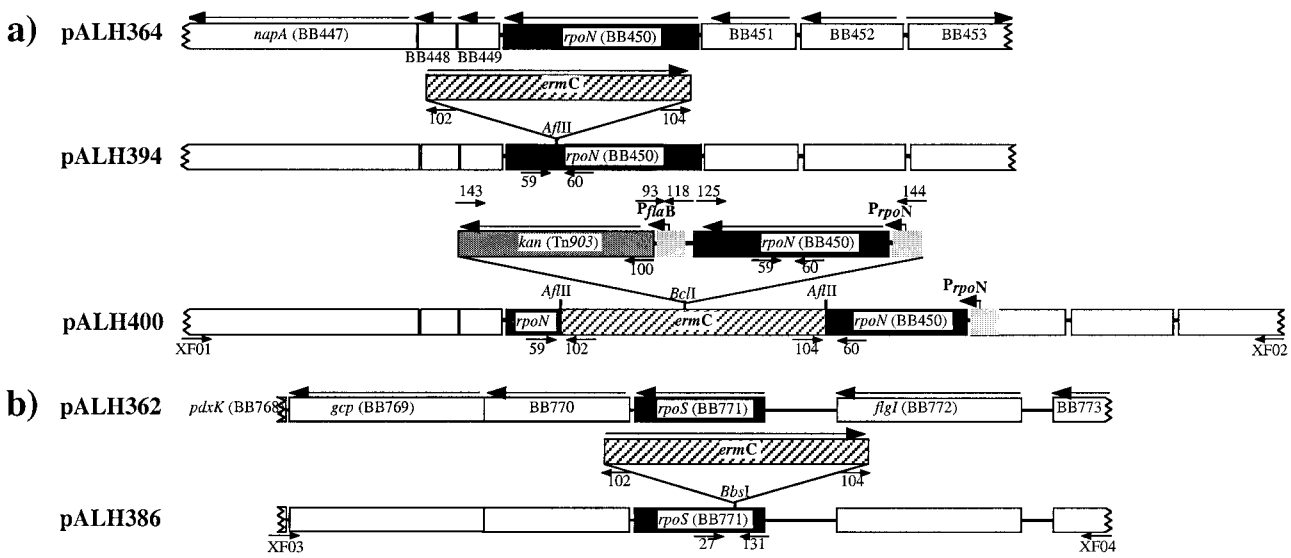

Fig. 1. Strategy for gene inactivation and complementation of *rpoN* (*a*) and *rpoS* (*b*) in 297. *rpoN* and *rpoS* (black solid boxes) were first cloned in pGEM-T (pALH364 and pALH362, respectively). Only the relevant portions of the plasmids are shown (labeled at the left). *rpoN* and *rpoS* were insertionally disrupted with *ermC* (diagonal stripes) (pALH394 and pALH386, respectively). For complementation of *rpoN*, suicide plasmid pALH400 was constructed as described in *Materials and Methods*. Relevant restriction sites are shown, and relevant promoters are indicated by light gray boxes. Short arrows denote primers used for PCR (Table 2).

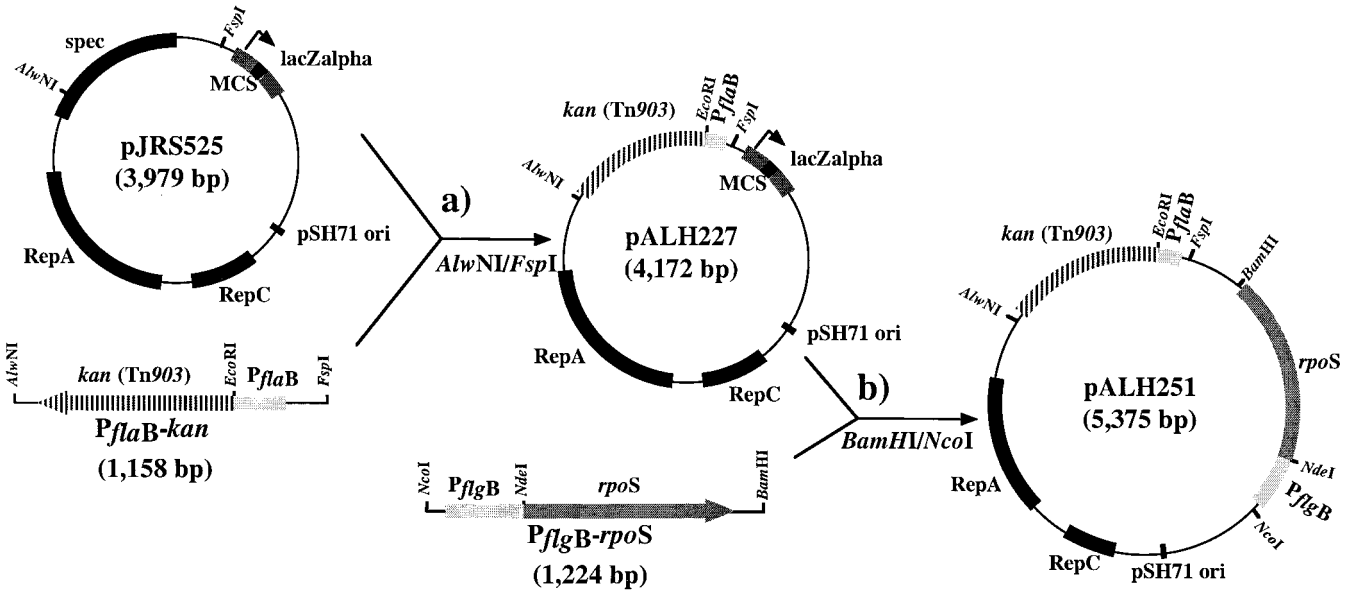

Fig. 2. Construction of pALH251 for complementation of inactivated *rpoN* with constitutively expressed *rpoS*. (*a*) A 965-bp fragment was excised from pJRS525 and a 1,158-bp fragment containing a P_{flaB}-*kan* promoter fusion was inserted into the *Alw*NI and *Fsp*I sites, yielding pALH227. (*b*) pALH251 was obtained on ligating a P_{flgB}-*rpoS* fusion construct (1,224 bp) into the *Bam*HI and *Nco*I sites of the multiple cloning site of pALH227.

BSK-H containing Erm. Spirochetes (200 μl of culture) were collected by centrifugation. The cell pellet was suspended in 20 μl of sterile water and 3-μl aliquots were microwaved in 200-μl thin-wall PCR tubes and subjected to PCR. The PCR strategy (see Fig. 3*c*) was as described for confirmation of plasmids pALH394 and pALH386 (Fig. 1). For all Erm^r clones tested, only PCR products indicative of disrupted genes (from double crossovers) were obtained.

Construction of Suicide and Shuttle Plasmids for Genetic Complementation. In each of two strategies for complementation, a Kan resistance gene (*kan*), derived from pUC4K containing the transposon Tn*903* (Amersham Pharmacia, accession number X06404), was used as a selectable marker; its use for the genetic manipulation of *B. burgdorferi* has been described (36, 37). Promoterless *kan* was linked to the constitutively expressed 297 *flaB* promoter (P_{flaB}) (36) via an *Eco*RI site; the *kan* gene and P_{flaB} were PCR-amplified with primers that introduced an *Eco*RI site upstream of *kan* (priAH100, priAH119) and downstream of P_{flaB} (priAH93, priAH118). The 224-bp P_{flaB} region and the 934-bp *kan* fragments were cloned separately into pGEM-T (lacks an *Eco*RI site). Both plasmids were linearized with *Eco*RI and combined in a ligation reaction. The ligation products were subjected to PCR by using the forward primer for P_{flaB} (priAH118) and the reverse primer for *kan* (priAH119), thereby selectively amplifying the desired product [1,158 bp with a junction of CAT**GGAG***GAATTC*GTT*ATG*$_{kan}$ (*Eco*RI site underlined, RBS_{flaB} bolded, and start codon of *kan* italicized)]. This amplicon was then inserted into *Fsp*I- and *Alw*NI-digested pJRS525 (38), which contains the same origin of replication as the recently described *B. burgdorferi* shuttle vector pGK12 (34). The resulting plasmid, pALH227 (Fig. 2*a*), confers Kan resistance in both *E. coli* and *B. burgdorferi*.

pALH227 served as a cloning vector for a wild-type copy of *rpoN*; a 1,628-bp fragment encompassing *rpoN* and its promoter (P_{rpoN}) was amplified (priAH145, priAH125) and cloned into the *Nco*I and *Bam*HI sites of pALH227, and the resulting plasmid (pALH293) served as a template for PCR amplification of the entire P_{rpoN}-*rpoN*-$(n)_{232}$-P_{flaB}-*kan* region (priAH144, priAH143). The resulting 3-kb *Bcl*I fragment was ligated into the single *Bcl*I site within *ermC* of pALH394, yielding pALH400 (Fig. 1*a*).

pALH227 also was used to construct a shuttle vector (pALH251) (Fig. 2*b*) for RpoN-independent (constitutive) expression of *rpoS* in a *rpoN* mutant of 297. To effect this, the structural *rpoS* gene was fused to the *flgB* promoter (P_{flgB}) by a strategy similar to the one described above. Briefly, the 410-bp P_{flgB} region and the *rpoS* ORF (805 bp) were obtained by PCR, whereby an *Nde*I site was introduced at the 3′ end of P_{flgB} (priAH122, priAH123) and at the 5′ end of *rpoS* (priAH126, priAH127). Both PCR products were individually cloned into pCR2.1 (lacks an *Nde*I site). The desired P_{flgB}-*rpoS* fusion [junction region: **GAGGGAGG**TTTC*CATATG*$_{rpoS}$ (*Nde*I site underlined, RBS_{flgB} bolded, start codon italicized)] was obtained by PCR (as described above) by using primers priAH122 (5′) and priAH127 (3′). This construct (1,224 bp) was ligated into the *Nco*I and *Bam*H sites within the multiple cloning site of pALH227, yielding pALH251 (Fig. 2*b*), which was sufficiently stable to allow qualitative expression of RpoS (see *Results*).

Reverse Transcriptase–PCR (RT-PCR). Total RNA from 297 or isogenic mutants was used in RT-PCRs (39). To detect transcripts for *rpoS* and confirm the disruption of *rpoN*, comparative RT-PCR was performed (*rpoN*, priAH59 and priAH60; *rpoS*, priAH131 and priAH132). To detect *ospC* and *flaB* transcripts, quantitative competitive RT-PCR was performed (39) by using primers ospC-5′, ospC-3′, flaB-5′, and flaB-3′ (39). Briefly, pFAC (Table 1) was constructed to allow SP6 RNA polymerase-dependent synthesis of an RNA with sequences for both *flaB*- and *ospC*-specific primers; *in vitro*-transcribed RNA served as a competitor for either natural transcript. About 1 μg of *Nco*I-linearized pFAC was used for *in vitro* transcription with SP6 RNA polymerase (SP6/T7 Transcription Kit; Roche Molecular Biochemicals). Competitor RNA was quantitated and 10-fold dilutions were used for RT-PCR in conjunction with constant quantities of test RNA (10 ng for *ospC* and 1 ng for *flaB*).

Results

Cloning and Disruption of 297 Genes Encoding Alternative Sigma Factors. To assess the influence of RpoS and RpoN on differential antigen expression in 297, each theoretical 297 regulatory

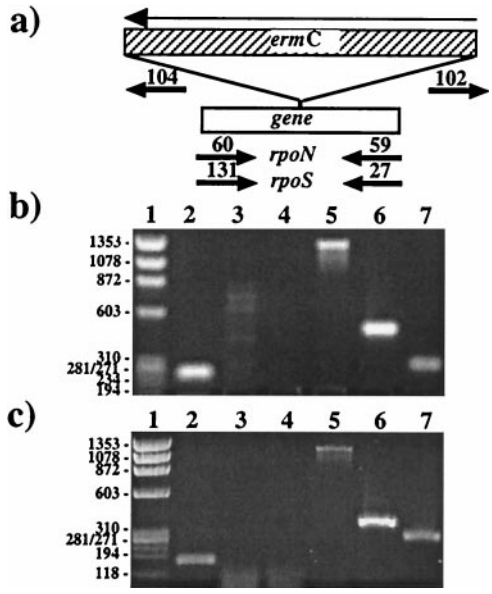

Fig. 3. PCR analysis of *rpoN* and *rpoS* mutants. (*a*) Schematic of PCR primer pairs (short arrows). (*b* and *c*) Agarose gel patterns of amplicons for wild type (lanes 2–4), (*b*) a *rpoN* mutant, or (*c*) a *rpoS* mutant (lanes 5–7). Lanes 1 contain DNA markers of ΦX174/*Hae*III. Gene disruption by *ermC* results in an increased size of the amplicons (compare lanes 2 and 5). A combination of *ermC*-specific and flanking primers yielded only products for the mutants (lanes 6 and 7), but not for 297 (lanes 3 and 4). Lanes 3 and 6: priAH102 (arrow 102) in combination with (*b*) priAH59 (arrow 59) or (*c*) priAH27 (arrow 27). Lanes 4 and 7: priAH104 (arrow 104) in combination with (*b*) priAH60 (arrow 60) or (*c*) priAH131 (arrow 131).

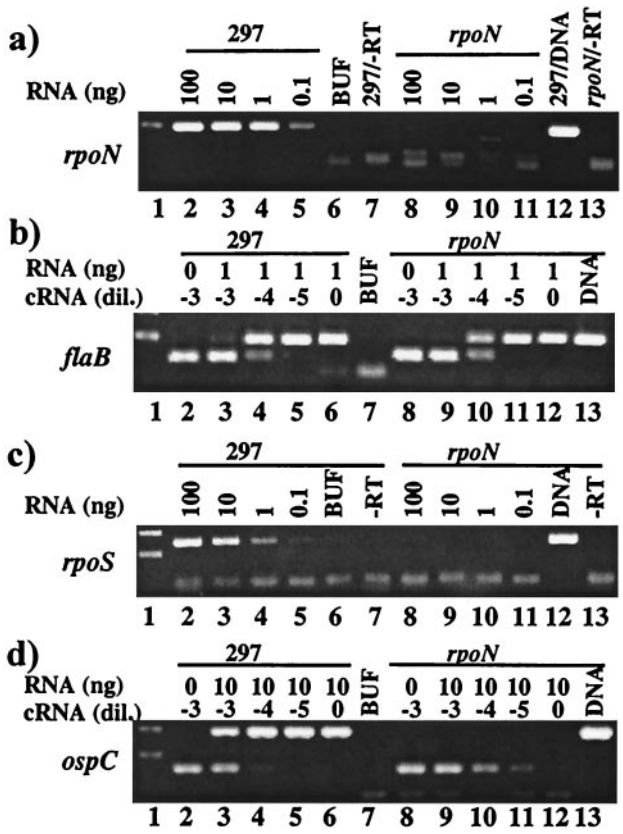

Fig. 4. RT-PCR analysis of a *rpoN* mutant. Borreliae were cultivated in BSK-H at pH 6.8. (*a* and *c*) Comparative RT-PCR; 10-fold dilutions of RNA from the wild type (strain 297) or the mutant were tested for *rpoN* (*a*) and *rpoS* (*c*) transcripts. Buf, buffer only; −RT, lacking RT. (*b* and *d*) Quantitative competitive RT-PCR; competitor RNA (cRNA) was used at exponential dilution, as indicated above the panels. The expression of constitutive *flaB* was unaffected by the *rpoN* mutation (*b*), whereas the expression of *ospC* was abolished (*d*).

gene and its appropriate flanking regions were PCR-amplified (based on B31 sequence information) and inserted into pGEM-T, followed by insertional inactivation with the Erm resistance gene, *ermC*. The constructs (*rpoN*: pALH394, Fig. 1*a*) (*rpoS*: pALH386, Fig. 1*b*) were electroporated into 297. Generally, after plating on selective medium, several Erm-resistant (Erm^r) colonies were obtained; typically, 12 were randomly chosen for PCR analysis. All clones yielded PCR amplification patterns consistent only with the desired genotypes (Fig. 3), with no evidence of a single crossover event and thus no evidence of plasmid (Amp resistance) incorporation. Also, no spontaneous Erm^r mutants were observed in any experiment. RT-PCR performed on RNA from one of the *rpoN*::*ermC* mutants confirmed that disrupted *rpoN* was not transcribed (Fig. 4*a*, lanes 8–11). The two mutants (*rpoN* and *rpoS*) displayed normal growth kinetics in BSK-H and appeared morphologically identical to 297 (not shown).

RpoN Controls the Expression of OspC, DbpA, and RpoS. Parental 297 displayed its conventional temperature dependence for the expression of OspC (23) (Fig. 5*a* arrow, lanes 1 and 2). In contrast, OspC was notably absent in the *rpoN* mutant cultivated at 37°C (Fig. 5*a*, lane 3); this negative result was corroborated by immunoblotting (Fig. 5*f*, lane 3) and RT-PCR (Fig. 4*d*, lanes 8–12). Because OspC, DbpA, and RpoS have been hypothesized to be coregulated as group I proteins (23, 40), expression of DbpA and RpoS also was examined in the *rpoN* mutant. Neither DbpA (Fig. 5*g*, lane 3) nor RpoS (Fig. 5*e*, lane 3) was detectable in the *rpoN* mutant; the absence of mRNA for *rpoS* was confirmed by RT-PCR (Fig. 4*c*, lanes 8–11). In contrast to these group I proteins, the expression of OspA and Lp6.6, proteins down-regulated during 297 mammalian infection (group II proteins) (23), was not influenced by the *rpoN* mutation (Fig. 5*d*, lane 3; not shown for Lp6.6).

To establish that the loss of expression of OspC, DbpA, and RpoS was the result of *rpoN* gene disruption, the mutant was complemented with a wild-type copy of *rpoN* (controlled by its native promoter) recombined into the chromosome by using pALH400 (Fig. 1*a*, line 3). A chromosomal location was selected to minimize gene dosage effects (i.e., copy number) and to maximize gene stability. As a result of complementation, not only was the expression of OspC, DbpA, and RpoS restored, but their expression patterns were pH-dependent (Fig. 5, lanes 4–6) as well as temperature-dependent (not shown), as has been reported (23).

RpoN Controls the Expression of OspC and DbpA Through RpoS. Because of its role as a transcriptional activator, it remained possible that RpoS actually regulated OspC and DbpA expression. To test this, we examined the expression of OspC and DbpA in an *rpoS* mutant. In the *rpoS* mutant, the expression of OspC and DbpA was abolished (Fig. 5, lane 8), suggesting that RpoS works directly to regulate the expression of these two lipoproteins. As further evidence of the regulatory role of RpoS, the *rpoN* mutant was complemented with a wild-type copy of the *rpoS* by using the shuttle plasmid pALH251 (Fig. 2*b*). As a means of rendering *rpoS* expression independent of *rpoN*, *rpoS* was engineered to be driven by the constitutive *B. burgdorferi flgB* promoter (P_{flgB}). In this *rpoS* diploid, OspC and DbpA were readily detectable (Fig. 5, lane 7), suggesting that constitutive production of RpoS could overcome the RpoN deficiency.

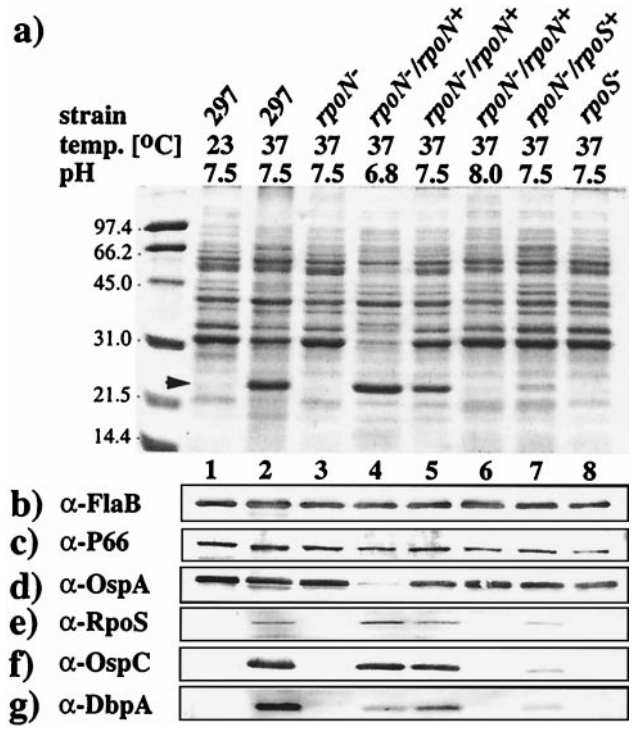

Fig. 5. SDS/PAGE (Coomassie blue stain) (*a*) and immunoblots (*b–g*) of whole cell lysates (39) from wild-type, mutant, and complemented strains of 297. Total protein from about 2×10^7 spirochetes (late-logarithmic phase of growth) was loaded per gel lane, except when probing for RpoS (10-fold more material). Monoclonal antibodies against OspA and FlaB and polyclonal antisera against all other proteins have been described (23, 51). Cultivation pH and temperature are indicated above each lane. Lanes 1 and 2, wild-type 297. Lane 3, *rpoN* mutant. Lanes 4–6, *rpoN* mutant complemented with wild-type *rpoN*. Lane 7, *rpoN* mutant complemented with pALH251 (*rpoS*). Lane 8, *rpoS* mutant.

Discussion

Significant advances have been made recently in the development of systems for the genetic manipulation of avirulent strains of *B. burgdorferi* (34, 36). Stewart *et al.* (37) in an initial attempt to transform virulent *B. burgdorferi* recently reported the construction of a shuttle plasmid that was stably maintained in strain N40. We chose to carry out our genetic studies in 297 because it is a human isolate (31) that is highly infectious (ID_{50} of about 50 organisms; unpublished data). Moreover, given that 297, unlike avirulent strain B31, expresses OspC very efficiently, it is particularly well suited to studies of the OspA/OspC regulatory paradigm. Although not a principal objective of this study, the use of 297 provided a chance for genetically manipulating virulent *B. burgdorferi*, a goal in borrelial genetics research that has been difficult to achieve. Nonetheless, herein we have extended the scope of *B. burgdorferi* genetics by performing genetic complementation in this highly intractable organism. Genetic complementation is an essential component of molecular Koch's postulates (41) and, as such, its application ultimately is vital for delineating borrelial gene function.

The use of Erm^r as a selectable marker for genetic studies with virulent *B. burgdorferi* should not be controversial. Although the precise mechanism is unknown, Erm^r occurs naturally among virulent *B. burgdorferi* (34, 42). Furthermore, antimicrobials other than Erm are preferred for the treatment of Lyme borreliosis (43). Finally, *B. burgdorferi* is not a free-living pathogen (requires a tick host), and thus inadvertent escape from the laboratory is virtually impossible. The presence of Amp resistance on pGEM-T, used as a convenient suicide plasmid for *B. burgdorferi*, also should be without measurable biohazardous risk because (*i*) pGEM-T is not stably maintained in *B. burgdorferi*, (*ii*) no attempt was made to select for Amp resistance in *B. burgdorferi*, and (*iii*) as stated earlier, it is highly implausible that a *B. burgdorferi* strain could escape from the laboratory.

The *rpoN* gene was selected for targeted disruption because it has been implicated in modulating the expression of bacterial virulence (27–30), and hence studies on *rpoN* could help to identify virulence-associated genes in *B. burgdorferi*. OspC was conspicuously absent in protein profiles of the *rpoN* mutant; this led to an initial hypothesis that RpoN directly regulated *ospC*, which was supported by the fact that *ospC* mRNA was undetectable by RT-PCR in the *rpoN* mutant. However, we also noted that under growth conditions that up-regulated OspC, DpbA, Mlp-8, OspF, and P35 (group I proteins) (23, 40) RpoS also was up-regulated (23). Moreover, analysis of the upstream promoter region of *ospC* predicted three putative $-35/-10$ promoters but did not highlight a $-24/-12$ consensus typical of an RpoN-dependent promoter (44). This prompted a revised hypothesis that perhaps RpoS, rather than RpoN, directly regulated OspC. Because the expression pattern for DbpA in all of the mutants mirrored that of OspC, it also was plausible that *dbpA*, and perhaps other group I genes, might be coordinately regulated along with *ospC* by RpoS.

RpoS is a stress-induced sigma factor of eubacteria (27), but its role in *B. burgdorferi* gene regulation and virulence expression has been obscure. Elias *et al.* (45) were the first to show that an *rpoS* mutant of avirulent B31 exhibited a growth phase-dependent sensitivity to 1 M NaCl. They also reported that a 454-bp intergenic region upstream of *rpoS* did not contain a sequence with more than 60% identity to a consensus σ^{70} promoter (45). Interestingly, computer analysis had predicted an excellent match for a RpoN-dependent $-24/-12$ promoter immediately (62 bp) upstream of the *rpoS* start codon in *B. burgdorferi* (46). We garnered experimental evidence for RpoN-dependent expression of *rpoS* in that both immunoblotting and RT-PCR failed to reveal any expression of *rpoS* in the *rpoN* mutant. Furthermore, a *rpoS* mutant did not express OspC or DbpA, and complementation of the *rpoN* mutant with constitutively expressed *rpoS* restored detectable levels of RpoS, OspC, and DbpA. Elias *et al.* (45) did not note an abrogation of *ospC* expression in a *rpoS* mutant of avirulent B31; however, expression of *ospC* in avirulent B31 tends to be low, thereby hampering studies of *ospC* expression. We thus conclude that RpoN controls the transcription of *rpoS*, which ultimately governs the expression of OspC, DbpA, and probably other group I-like proteins (23) of *B. burgdorferi*. Such a pathway of sigma factor interplay may represent an additional paradigm for bacterial gene regulation. It should be noted, however, that whereas our experiments imply that RpoN directly controls the expression of *rpoS* in *B. burgdorferi*, they do not entirely rule out the unlikely possibility that RpoN induces the expression of some other unknown regulator of *rpoS*; unfortunately, experiments to test this in *B. burgdorferi* currently are not feasible. We also have been unsuccessful thus far in isolating recombinant RpoN in a soluble form, a prerequisite for demonstrating in gel retardation assays RpoN binding specifically to the -12 region of P_{rpoS} (47). However, the prediction of an RpoN-dependent $-24/-12$ promoter upstream of *rpoS* genes in *B. burgdorferi* as well as in *Pseudomonas syringae* and *Enterobacter cloacae* (46) provides circumstantial evidence that RpoN directly controls *rpoS* expression, and that this type of regulatory network may be relatively common among prokaryotes.

A caveat of our studies is that we could not conclude whether RpoN or RpoS is essential for virulence expression by *B. burgdorferi*. Although the *rpoN* mutant and the *rpoN* mutant complemented with wild-type *rpoN* replicated normally in dialysis membrane chambers implanted into the peritoneal cavities

of rats (48) (implying that the *rpoN* mutant retained its ability to grow in a mammalian host-adapted state), thus far we have been unable to demonstrate that any of the 297 recombinants retained infectivity in the mouse model of Lyme borreliosis (49). No obvious differences in plasmid profiles between any of the recombinants and wild-type 297 have been observed. We thus have not discerned whether the inability of the *rpoN*- or *rpoS*-complemented strains to regain infectivity was caused by avirulent variants within the 297 population being preferentially transformed, or if repetitive *in vitro* manipulations somehow resulted in the loss of *B. burgdorferi* infectivity (50). Alternatively, if complete complementation was not achieved, the expression of other unknown proteins could have been adversely affected, resulting in loss of function(s) necessary for mouse infectivity. These uncertainties, however, do not detract from the demonstration that genetic complementation of the *rpoN* mutant with either *rpoN* or *rpoS* qualitatively restored the predicted protein expression of RpoS, OspC, and DbpA, which ultimately led to the discovery of a unique global regulatory pathway in *B. burgdorferi*. The genetic approaches described herein and the elucidation of the RpoN-RpoS regulatory pathway for key borrelial membrane lipoproteins have far-reaching implications for studying many aspects of *B. burgdorferi* infectivity, virulence, pathogenesis, and immune evasion.

The RpoN-RpoS regulatory pathway can be envisioned to serve the enzootic life cycle of *B. burgdorferi* in the following fashion. As a result of interdependent environmental signals (e.g., elevated temperature, reduced pH, and increased spirochete density) (23, 40) engendered during the tick's taking of a blood meal, activated RpoN activator protein likely binds to an enhancer region upstream of where RpoN is complexed with the RNA polymerase holoenzyme ($-24/-12$ region), leading to the synthesis of *rpoS* mRNA. RpoS then mediates the synthesis of OspC, DbpA, and ostensibly other group I-like proteins of *B. burgdorferi*. Other sensory information undoubtedly feeds into these processes, and thus details of this network likely will undergo refinements as new information emerges. The *rpoN* and *rpoS* mutants of 297 also provide systems in which to perform more global assessments of *B. burgdorferi* gene regulation.

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