Borrelia burgdorferi σ^{54} is required for mammalian infection and vector transmission but not for tick colonization

Mark A. Fisher, Dorothee Grimm, Amy K. Henion, Abdallah F. Elias, Philip E. Stewart, Patricia A. Rosa, and Frank C. Gherardini*

Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840

Edited by Sydney Kustu, University of California, Berkeley, CA, and approved February 1, 2005 (received for review November 17, 2004)

Previous studies have shown that a σ^{54}–σ^{S} cascade regulates the expression of a few key lipoproteins in *Borrelia burgdorferi*, the agent of Lyme disease. Here, we demonstrate that these sigma factors, both together and independently, regulate a much more extensive number of genes and cellular processes. Microarray analyses of σ^{54} and σ^{S} mutant strains identified 305 genes regulated by σ^{54} and 145 regulated by σ^{S}, whereas the σ^{54}–σ^{S} regulatory cascade appears to control 48 genes in *B. burgdorferi*. *In silico* analyses revealed that nearly 80% of genes with altered expression in the σ^{54} mutant were linked to potential σ^{54}-dependent promoters. Many σ^{54}-regulated genes are expressed *in vivo*, and through genetic complementation of the mutant, we demonstrated that σ^{54} was required by *B. burgdorferi* to infect mammals. Surprisingly, σ^{54} mutants were able to infect *Ixodes scapularis* ticks and be maintained for at least 24 wk after infection, suggesting the σ^{54}–σ^{S} regulatory network was not involved in long-term survival in ticks. However, σ^{54} mutants did not enter the salivary glands during tick feeding, indicating that σ^{54}-regulated genes were involved in the transmission process.

infectivity | microarray | Lyme | transcription

The agent of Lyme disease, *Borrelia burgdorferi*, colonizes both a mammalian reservoir host and an arthropod vector during its infective cycle. This transmission cycle requires: (*i*) migration of spirochetes from tick midgut to salivary glands during a blood meal, (*ii*) entry into and colonization of mammalian tissues, (*iii*) establishment of a chronic/systemic infection, and (*iv*) uptake and colonization of an uninfected feeding tick. This process undoubtedly involves recognition by the bacterium of multiple environmental cues that modulates the expression of key genes required for a successful adaptation process. A remarkable characteristic of this bacterium is its ability to adapt to and thrive in these very different host environments. It is perhaps more remarkable that *B. burgdorferi* is able to complete the complex events required for transmission even with its relatively small genome consisting of a 910-kbp linear chromosome and 21 circular and linear plasmids totaling 610 kbp (1, 2).

A number of *B. burgdorferi* genes are known to be regulated within its hosts (3, 4) or under conditions that mimic host infection (5–7). Unfortunately, the genetic regulation of adaptation to different hosts is not well understood. It has been shown that both alternative sigma factors in *B. burgdorferi* (σ^{54} and σ^{S}, encoded by *ntrA* and *rpoS*) act in a cascade where σ^{54} controls σ^{S} production to regulate the expression of two lipoproteins, OspC and DbpA (8), that potentially play roles in *B. burgdorferi's* survival in mammals (9, 10). In this study, microarrays were used to identify many other genes both independently and coregulated by σ^{54} and σ^{S}, and the *in vivo* role of σ^{54} was evaluated in the murine and arthropod hosts.

Methods

Detailed protocols are provided as *Supporting Methods*, which is published as supporting information on the PNAS web site.

Bacterial Strains and Growth Conditions. *B. burgdorferi* WT clone B31-A3 (11) and all derivatives were grown at 34°C in BSK-II medium supplemented with 6% rabbit serum under either normal atmosphere or microaerobic conditions (3–5% O_2, 5% CO_2, 95% N_2). Strain A3*rpoS* is a low-passage σ^{S} mutant that harbors all plasmids found in the parental strain B31-A3 (11). All plasmids were propagated in *Escherichia coli* DH5α or Top-10.

Construction of the σ^{54} Mutant and Complemented (Comp) Strains. The σ^{54} mutant strain was generated by allelic replacement of the WT copy of *ntrA* with a disrupted copy in the suicide vector pJSTJNK (12). This plasmid contains a 1.1-kb internal fragment of the *ntrA* gene (PCR-amplified with primers 1 and 2; all primers are shown in Table 2, which is published as supporting information on the PNAS web site) that is interrupted at the *Mun*I site with the P_{flaB}::*kan* cassette from pJLB12A (ref. 13; all plasmids are shown in Table 3, which is published as supporting information on the PNAS web site). Approximately 5×10^{9} cells (*B. burgdorferi* B31-A3 or A3 derivatives; all strains are shown in Table 3) were electroporated with 10–50 μg of plasmid DNA, and transformants were plated in solid BSK (200 μg/ml kanamycin, 40 μg/ml gentamicin, or 50 μg/ml streptomycin) (11). Screening for disruption of *ntrA* was done by PCR with primers 1 and 2 and confirmed with primers 5–6, 5–7, and 6–8. Before complementation and animal infections, A3*ntrA*, which harbors all plasmids but lp25, and B31-A3 were transformed with plasmid DNA from B31-A3 lp25-Gm containing a P_{flaB}::*aacC1* marker on lp25 (14), yielding clones A3-Gm and A3*ntrA*-Gm. Strain A3*ntrA*-Gm was transformed with vector pMFS+ or pMFSp54 generating strains *ntrA*-VC (vector control) and *ntrA*-comp. Both strains harbored all plasmids but lp56. Details of plasmid and strain construction are found in *Supporting Methods*. All plasmid constructs were confirmed by DNA sequencing.

Microarray, RT-PCR, and Quantitative RT-PCR Analyses. *B. burgdorferi* strains B31-A3, A3*ntrA*, and A3*rpoS* were grown in triplicate to 4×10^{7} cells per ml under microaerobic conditions, and growth rates were determined by dark-field microscopy. Bacteria were harvested by centrifugation at 25,000 × *g* for 1 min at 4°C, and RNA was extracted by TRI-reagent. RNA was treated with DNase-I, followed by DNA-Free DNase removal reagent, and purity was confirmed by PCR using primers 2 and 12.

RNA for microarray analysis was labeled by reverse transcription with Alexa Fluor 546 or Alexa Fluor 647 by using the ARES system (Molecular Probes). Labeled cDNAs were mixed (WT plus mutant) and hybridized to microarrays at 50°C for 16 h. Microarrays consisted of 1,742 custom 70-mer oligonucleotides

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: IFA, immunofluorescence assay; VC, vector control; comp, complemented.

See Commentary on page 4933.

*To whom correspondence should be addressed. E-mail: fgherardini@nih.gov.

(T_m-matched and screened to reduce cross-hybridization) covering essentially all ORFs in the B31 genome, printed in duplicate on Ultra-GAPS slides. Because of the large number of paralogs in the B31 genome (1), it was impossible to produce entirely unique probes for some genes. Those with identity >80% of the length of another probe are indicated in Tables 4–6, which are published as supporting information on the PNAS web site.

Fluorescence intensities from six hybridizations per strain (two hybridizations of three biological replicates) were quantified by a Scanarray 5000 with QUANTARRAY 3.0 software (PerkinElmer). Data were normalized by using the standard LOWESS algorithm in GENESPRING 5.1 (Silicon Genetics, Redwood City, CA). Genes significantly up- or down-regulated vs. WT were identified by using significance analysis of microarrays (SAM) version 1.21 (15). For a detailed description of the microarray analysis, see *Supporting Methods*.

For RT-PCR, total RNA (2 μg) from WT or mutant *B. burgdorferi* cells was converted to cDNA with 400 units of Superscript II reverse transcriptase with 40 units of RNase inhibitor and 3.8 μM arbitrary decamers (*Supporting Methods*) to prime cDNA synthesis. Reactions without reverse transcriptase confirmed the lack of DNA in RNA samples. The cDNA was used as template for PCR using primers 14 and 15 for *ntrA* expression and primers 17 and 18 for *rpoS* expression.

Quantitative RT-PCR (TaqMan) analysis (16) was used to confirm microarray data for 27 genes as described in detail in *Supporting Methods*. Log fold-change values from both microarray and TaqMan assays for selected genes were compared by linear regression with Microsoft EXCEL.

Bioinformatic Analyses. *B. burgdorferi* chromosomal and plasmid DNA sequences (1) were analyzed with SEQSCAN (www.bmb.psu.edu/seqscan) by using a scoring matrix derived from 186 known σ^{54}-dependent promoters (17). The resulting promoter candidates were screened for validity in EXCEL and SAS (SAS Institute, Cary, NC) by using criteria based on SEQSCAN score, orientation, and spacing of promoters relative to downstream genes, then compiled and merged with microarray data (*Supporting Methods*).

Experimental Infections. Naïve Swiss–Webster mice were anesthetized and bled before inoculation. Animals were infected with 4×10^3 spirochetes i.p. plus 1×10^3 s.c. or by tick bite as described (11). At 3 wk after inoculation, serum samples were collected, and preinoculation or postinoculation sera were screened by immunoblotting for P39 reactivity (*Supporting Methods*). Seroconversion, or development of antibodies, to P39 in mice is an indicator of active *B. burgdorferi* infection (18). At 5 wk after inoculation, spirochetes were cultured under aerobic conditions from tissues (ear, bladder, and joint) as described (11). Correct *ntrA* loci of tissue isolates were confirmed by PCR (primer pairs 1–2 and 13–16).

Tick Infection and Immunofluorescence Assays (IFAs). *Ixodes scapularis* larvae were infected by immersion in logarithmic growth phase cultures of *B. burgdorferi* strains A3-Gm, A3*ntrA*-Gm, *ntrA*-comp, and *ntrA*-VC as described (19). Infected larvae were allowed to feed to repletion on mice and molt to nymphs. The resulting nymphs were maintained for 18–24 wk at 98% humidity before being fed on mice and removed at 64 and 89 h after attachment. Midguts and salivary glands were isolated and examined by IFA (20) with rabbit anti-*B. burgdorferi* serum (1:100) as the primary antibody, and Alexa Fluor 488-labeled anti-rabbit IgG (1:100) as secondary antibody. Tissues were counterstained with DRAQ5 (1:1,000, Biostatus, Shepshed, U.K.). Midguts were analyzed by epifluorescent microscopy (Nikon Eclipse E800), and complete salivary glands were examined through their entire thickness by confocal microscopy (Zeiss LSM-510). 3D surface generating and surface cutaway projections of confocal image Z-stacks were generated by IMARIS SURPASS software (Bitplane, St. Paul) as described (21).

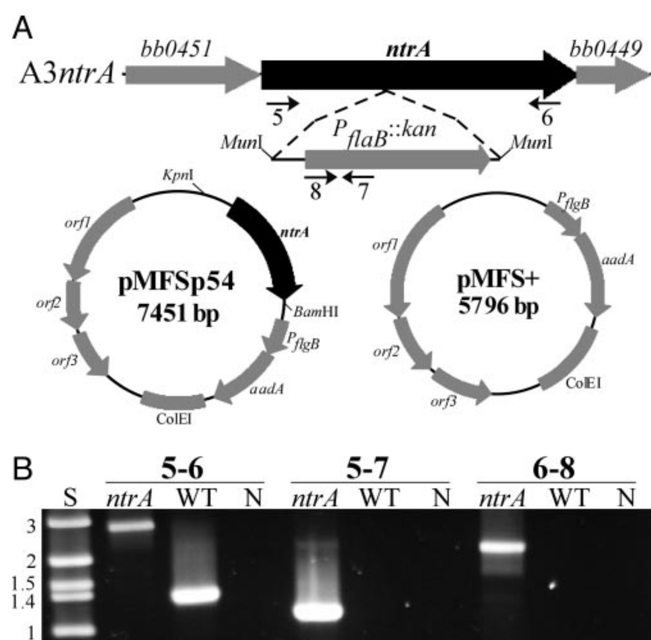

Fig. 1. Disruption of *ntrA* and complementation of the σ^{54} mutant. (*A*) Flanking ORFs and insertion point of the P_{flaB}::*kan* cassette in strain A3*ntrA* are shown above the complementation (pMFSp54) and VC (pMFS+) plasmids. Numbered arrows indicate the relative positions of primers used for PCR confirmation of the mutant. (*B*) PCR analyses to confirm *ntrA* disruption in A3*ntrA*. Size standards (S) in kbp and negative controls (N) are indicated.

Results

Inactivation of *ntrA*. Because the σ^{54}–σ^S cascade regulates the potential virulence factors *ospC* and *dbpA* (8–10), we used two genetic approaches to characterize mutants in this regulatory network. Microarray analysis was used to identify other genes regulated by these global regulators, and a σ^{54} mutant was evaluated for its role in the mouse-tick infectious cycle. A clone, A3*ntrA*, was confirmed to have *ntrA* insertionally inactivated (Fig. 1). RT-PCR was used to examine the expression of *ntrA* and

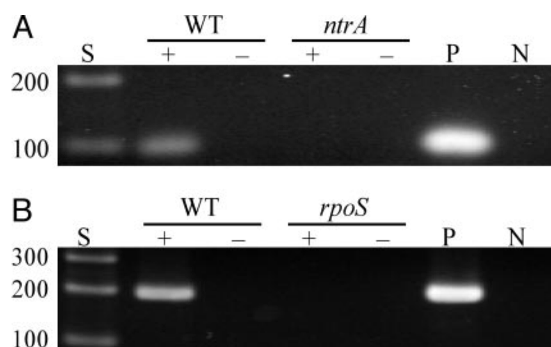

Fig. 2. RT-PCR analysis of *ntrA* and *rpoS* expression. RNA isolated from each strain was converted to cDNA and PCR-amplified with primers specific for *ntrA* or *rpoS*. (*A*) Expression of *ntrA* in B31-A3 (WT) and A3*ntrA* (*ntrA*, σ^{54} mutant). (*B*) Expression of *rpoS* in B31-A3 (WT) and A3*rpoS* (*rpoS*, σ^S mutant). Size standards (S) are in bp, and positive (P, B31-A3 DNA) and negative (N) controls are indicated. The presence or absence of reverse transcriptase is indicated by + or −.

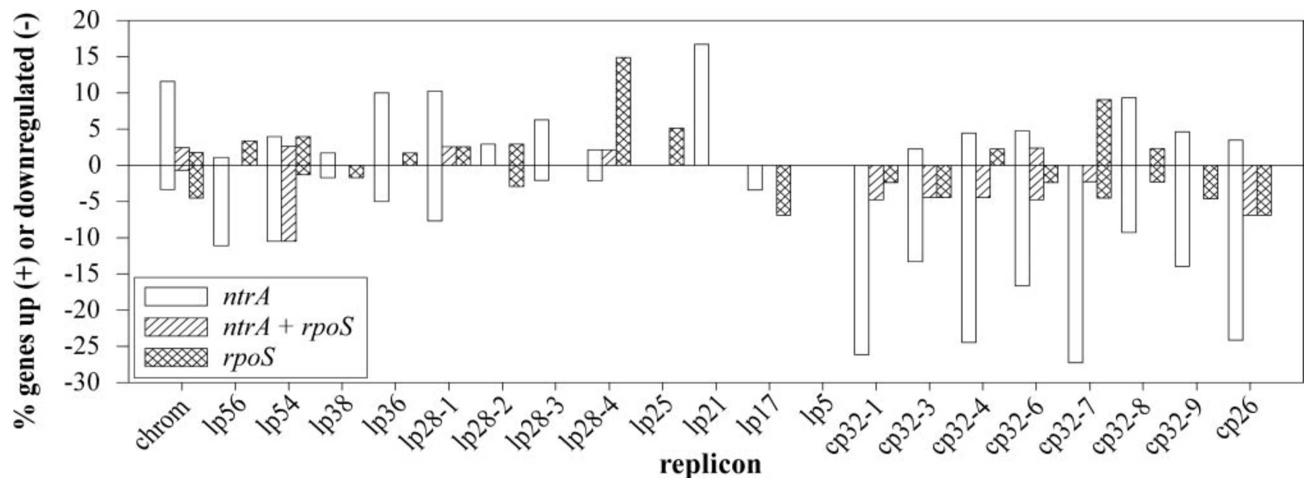

Fig. 3. Distribution of regulated genes by replicon. Microarray data were categorized by replicon and sigma factor regulatory group and are shown as percent of ORFs significantly changed on each replicon. *ntrA*, genes regulated only by σ^{54}; *ntrA* + *rpoS*, genes regulated by both σ^{54} and σ^{S}; *rpoS*, genes regulated only by σ^{S}.

rpoS in the respective mutants before microarray analysis. As expected, the σ^{54} mutant did not express *ntrA* (Fig. 2*A*), and the σ^{S} mutant failed to express *rpoS* (Fig. 2*B*), confirming inactivation of these genes. All strains exhibited growth characteristics very similar to WT when cultivated in BSK-II medium at 34°C.

Microarray-Based Identification of the σ^{54} and σ^{S} Regulons. A custom oligonucleotide array was used to identify genes differentially transcribed in the σ^{54} and σ^{S} mutants relative to WT. In the σ^{54} mutant, which lacked plasmid lp25, 305 genes were differentially expressed at statistically significant levels. Of these, 146 were underexpressed in A3*ntrA* compared with WT (Fig. 3 and Tables 4 and 6). In the σ^{S} mutant, 145 genes showed significantly altered expression. Of these, 81 were underexpressed in A3*rpoS* relative to WT (Fig. 3 and Tables 5 and 6). Both mutants shared a common set of 51 significantly changed genes. Of these, 48 were coordinately expressed in the mutants, thus they appeared to be regulated by the σ^{54}–σ^{S} cascade (Fig. 3 and Table 6). Therefore, three distinct regulatory groups were identified: group 1, 254 genes regulated by σ^{54} alone (Table 4); group 2, 94 genes regulated by σ^{S} alone (Table 5); and group 3, 51 genes regulated by both σ^{54} and σ^{S} (Table 6).

Cp26 and several cp32 plasmids had the largest percentage of their ORFs significantly regulated (25–30%) by σ^{54} (group 1), whereas lp28-4 had the largest percentage of genes (15%) differentially expressed by σ^{S} (group 2). As seen in other *B. burgdorferi* array experiments (5, 6), a large percentage of lp54 ORFs were also changed in both mutants (group 3, Fig. 3). The genes regulated by σ^{54} and σ^{S} fell into several major categories (e.g., cell envelope, cellular processes) as annotated in the *B. burgdorferi* genome (1). Role categories containing three or more regulated genes are shown in Fig. 6, which is published as supporting information on the PNAS web site. TaqMan assays (using primers and probes listed in Table 7, which is published as supporting information on the PNAS web site) were used to confirm differential expression of 27 genes identified in the microarray analyses (Tables 4–6). There was a strong, significant linear correlation between the log fold-change of the TaqMan data and that of the array data for both the σ^{54} and σ^{S} mutant comparisons ($r = 0.73$, $P = 3.8 \times 10^{-6}$), validating the microarray results.

Bioinformatic Analysis of Potential σ^{54}-Dependent Promoters. Because genes encoding potential regulatory proteins were differentially expressed in the σ^{54} mutant, a bioinformatic approach was used to identify putative σ^{54}-regulated promoters upstream of significantly regulated genes. This approach should identify genes that may be directly regulated by σ^{54}. When criteria were set to allow genes in potential operons to be recognized, 78% of the regulated ORFs could be linked to a potential σ^{54}-dependent promoter (data not shown). Even by limiting criteria to exclude all but a single gene within 500 bp of a promoter, 42% of the genes regulated in the σ^{54} mutant possessed potential σ^{54}-dependent promoters (*Supporting Methods* and Tables 4 and 6).

The σ^{54} Mutant Is Not Infectious in Mice. To determine whether σ^{54} is required by *B. burgdorferi* to infect mammals, we inoculated mice by i.p./s.c. injection (5×10^{3} cells) or tick bite with strains A3-Gm (WT), A3*ntrA*-Gm (σ^{54} mutant), *ntrA*-comp, or *ntrA*-VC. All strains harbored a selectable copy of lp25. Three of four mice injected with WT and 4/4 with *ntrA*-comp cells seroconverted to P39 (18), and spirochetes were cultured from tissue samples (Table 1). In contrast, no mice injected with A3*ntrA*-Gm or *ntrA*-VC seroconverted and no spirochetes were recovered from any tissues. Likewise, all mice challenged by ticks infected with WT or *ntrA*-comp seroconverted and yielded spirochetes from tissues, whereas none challenged with A3*ntrA*-Gm or *ntrA*-VC seroconverted and no bacteria were recovered from tissues (Table 1). PCR confirmed that all strains isolated from tissue contained the correct *ntrA* loci. These data show that σ^{54}

Table 1. Summary of experimental mouse infections with *B. burgdorferi* A3-Gm, A3*ntrA*-Gm, *ntrA*-comp, and *ntraA*-VC strains

Strain	Route*	Seroconversion†	Culture‡
A3-Gm	Needle	3/4	3/4
	Tick	3/3	3/3
A3*ntrA*-Gm	Needle	0/4	0/4
	Tick	0/3	0/3
ntrA-comp	Needle	4/4	4/4
	Tick	3/3	3/3
ntrA-VC	Needle	0/4	0/4
	Tick	0/3	0/3

*Needle inoculation with 5×10^{3} bacteria i.p./s.c. or by tick bite with larvae (one mouse) or nymphs (two mice).
†No. of P39 or culture positive/total no. of mice tested.
‡All tissues (skin, bladder, and joint) gave the same culture results.

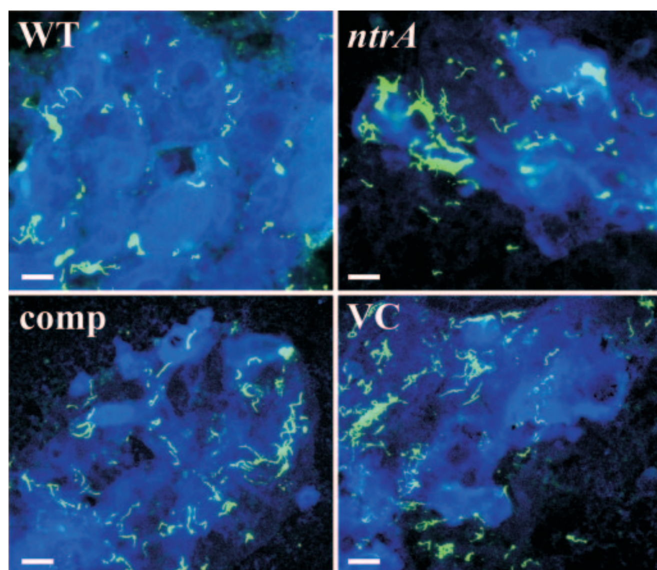

Fig. 4. IFA of midguts from fed ticks 24 wk after infection. Midguts isolated from ticks infected with A3-Gm (WT), A3*ntrA*-Gm (*ntrA*, σ^{54} mutant), *ntrA*-comp, and *ntrA*-VC strains were examined by IFA. Spirochetes (green) were detected by fluorescent microscopy with rabbit anti-*B. burgdorferi* primary and Alexa Fluor 488-labeled anti-rabbit secondary antisera. (Scale bars, 20 μm.)

is required for *B. burgdorferi* to infect mammals regardless of the infection route.

The σ^{54} Mutant Fails to Enter Tick Salivary Glands. *I. scapularis* ticks infected with A3-Gm, A3*ntrA*-Gm, *ntrA*-comp, and *ntrA*-VC strains were monitored by IFA for 24 wk. There was no apparent defect in the mutant's ability to survive in the midgut (Fig. 4). However, when salivary glands from feeding ticks were examined by confocal microscopy, a striking difference was observed. Both WT (13/19 ticks) and *ntrA*-comp (13/17) strains were able to enter the ascini of salivary glands, whereas neither the A3*ntrA*-Gm (0/19) nor the *ntrA*-VC (0/13) strain was observed inside salivary gland ascini (Fig. 5). These data suggest that σ^{54}-regulated genes are necessary for transmission of *B. burgdorferi*.

Discussion

Very little is known about gene regulatory systems in *B. burgdorferi*. We have begun to address this problem by identifying genes under the control of two global regulators, the alternative sigma factors σ^{54} and σ^S. In other bacteria, σ^{54} regulates such processes as nitrogen homeostasis and motility, and σ^S often regulates general stress responses (reviewed in refs. 22 and 23). Because these sigma factors are also required for virulence in other organisms (24, 25), we examined the ability of a *B. burgdorferi* σ^{54} mutant to infect a mammalian host. Through complementation analysis, we demonstrated that a functional *ntrA* gene is required for infectivity in mice (Table 1). Further, we showed that σ^{54} is required by *B. burgdorferi* to enter tick salivary glands, a critical step in vector transmission.

B. burgdorferi strain B31-A3 was chosen for *ntrA* mutagenesis because it is a clonal, infectious derivative of strain B31 used for genomic sequencing (1) and the parent strain of the σ^S mutant (11). Because both mutants were generated in the same background, differential gene expression in the mutants relative to WT could be directly compared. The σ^{54} and σ^S mutants were shown by RT-PCR to lack expression of *ntrA* and *rpoS*, respectively (Fig. 2), indicating that these mutants were appropriate for microarray analysis. Because we sought to identify all genes regulated by these alternative sigma factors, no arbitrary fold-change restriction was imposed on the microarray data. Rather, conservative statistical criteria were used to provide the most robust, yet complete data set possible. It is important to note that because sigma factors are primarily activators of transcription, genes up-regulated in the mutants are probably regulated indirectly (e.g., nonactivation of a repressor) or pleiotropically (e.g., nonactivation of an RNase slowing message turnover). However, σ^{54} can act directly as a negative regulator by a mechanism termed sigma factor antagonism (26). Therefore, some genes overexpressed in the σ^{54} mutant may in fact be directly repressed by σ^{54}. Down-regulated genes could be directly or indirectly (e.g., σ^{54}–σ^S regulation of *ospC*) regulated by the respective sigma factor.

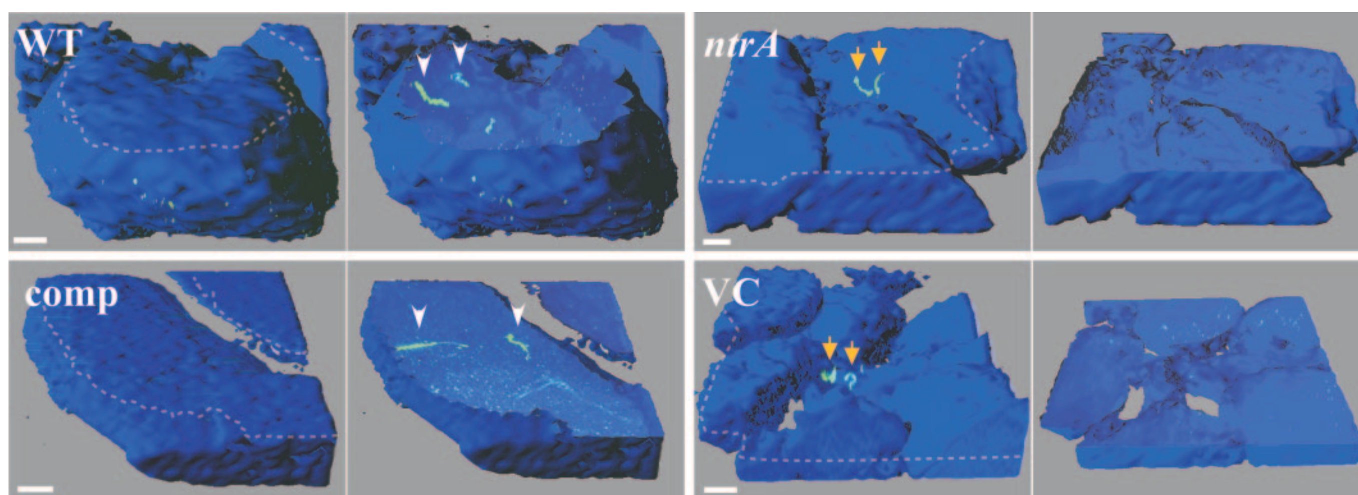

Fig. 5. 3D surface and cutaway projections of salivary gland ascini. Salivary glands were isolated from partially fed ticks infected with A3-Gm (WT), A3*ntrA*-Gm (*ntrA*, σ^{54} mutant), *ntrA*-comp, and *ntrA*-VC strains. The left image in each case shows the outer surface of intact ascini, and the right image shows a computer-generated internal section of the corresponding structure. The cutting plane used in the right image is denoted by the dotted line in the left image. Arrowheads indicate spirochetes (green) located within ascini, and arrows denote those on the exterior of the structure. Spirochetes were detected by confocal microscopy using rabbit anti-*B. burgdorferi* primary and Alexa Fluor 488-labeled anti-rabbit secondary antisera, and salivary glands were stained with DRAQ5. (Scale bars, 10 μm.)

By comparing both the σ^{54} and the σ^{S} mutants to WT, we identified sets of genes regulated by σ^{54} alone (group 1, Table 4), σ^{S} alone (group 2, Table 5), and both σ^{54} and σ^{S} (group 3, Table 6). Microarray comparison of the σ^{54} mutant with WT revealed 305 genes with significantly altered expression (Tables 4 and 6). Although the σ^{54} mutant analyzed by microarray lacked lp25, it is unlikely that this affected overall gene expression profiles because no transcriptional regulators or ribonucleases have been identified on this plasmid (1). In the σ^{S} mutant, only 145 genes showed significant changes (Tables 5 and 6). The expression of 254 genes was significantly altered in the σ^{54}, but not the σ^{S} mutant (Table 4), whereas 94 genes showed significant differential expression only in the σ^{S} mutant (Table 5).

Both mutants showed altered expression of 51 shared genes (Table 6). The vast majority (48/51) were expressed in the same direction in both mutants, suggesting they may be coregulated by the σ^{54}–σ^{S} cascade. Based on previous work (8), we would predict *ospC* (*bbb19*), *dbpA* (*bba24*), and *dbpB* (*bba25*, which is cotranscribed with *dbpA*) (27) to be in regulatory group 3. As expected, both *ospC* and *dbpB* were found in group 3 but *dbpA* was significantly changed only in the σ^{S} mutant (Tables 5 and 6). However, closer inspection revealed that *dbpA* was down-regulated in 5/6 microarray hybridizations (−2.1- to −1.3-fold) and up-regulated in one (1.3-fold) in the σ^{54} mutant. Without the latter data point, the average fold-change was −1.54, which was comparable to the −1.62 value observed in the σ^{S} mutant. This finding confirmed previous data (8) and suggested that our statistical criteria for identifying significantly regulated genes were too conservative, thus there are potentially more genes that are regulated by these sigma factors.

Previous work suggests *rpoS* (*bb0771*) should also be in regulatory group 3 (8) but the *rpoS* signal was below the detection threshold in all strains and all microarray hybridizations. This finding suggested that there was a problem with the *rpoS* oligo target or the levels of *rpoS* mRNA were below the detection limit. No *rpoS* mRNA was detected by RT-PCR in the σ^{S} mutant, but it was easily detectable in WT (Fig. 2*B*) and the σ^{54} mutant (TaqMan *rpoS* levels of −2.63-fold vs. WT), implicating a faulty array target. Together, these observations confirmed that *rpoS* was expressed under the conditions used for the arrays and was regulated by σ^{54}.

Because the σ^{54} mutant cannot infect mice, one would expect some genes regulated by σ^{54} to be involved in infectivity. Moreover, genes up-regulated in the host may be necessary for *in vivo* growth or survival. Thus, we hypothesized that a search for genes up-regulated in rat dialysis membrane chambers (5, 7) but down-regulated in the σ^{54} mutant should identify *B. burgdorferi* genes involved in infectivity. Not surprisingly, this comparison yielded 12 genes that may be required for infectivity and are likely under the control of σ^{54}. A similar analysis to identify genes expressed in mammalian tissues (3, 28) but down-regulated in the σ^{54} mutant yielded 25 additional genes, thus totaling 37 potentially involved in the virulence of *B. burgdorferi* (Table 8, which is published as supporting information on the PNAS web site). Several of these genes have been either shown (*ntrA* and *ospC*) or suggested (*dbpB*, *bba64*, *bbo39*, *bbs41*, and *vlsE*) to be required for the infectivity or pathogenesis of *B. burgdorferi* (this study and refs. 9 and 29–31).

Although we did not test the σ^{S} mutant in mice, 19 genes that are expressed in mammals were down-regulated in the σ^{S} mutant (Table 8), and thus may be another subset of genes involved in virulence (3, 5, 7, 28). In support of this idea, *bbl40* and another σ^{S}-regulated homolog (*bbo40*), both members of the *erp* gene family, have been implicated in survival in mammals by allowing evasion of the complement system (30). Another potential virulence gene down-regulated in the σ^{S} mutant is *bb0810*, which encodes a MviN homolog. *Salmonella typhimurium* mutants lacking MviN, a protein of unknown function, are attenuated in a mouse model of infection (32). Recently, Caimano *et al.* (33) demonstrated that a *B. burgdorferi* σ^{S} mutant was avirulent in mice. Our data could provide insight into which σ^{S}-regulated genes are required for virulence.

The genes regulated by both sigma factors fall into several major functional categories (1), including cell envelope, cellular processes, and transport/binding proteins (Fig. 6 and Tables 4–6). In the σ^{54} mutant, the expression of 18 cell envelope genes was affected, and 9 are potentially involved in virulence as described above (Table 8). Of the 22 σ^{S}-regulated genes involved in the cell envelope, 16 encode putative membrane proteins. This result is not surprising because *B. burgdorferi* devotes ≈8% of its genome to lipoproteins (1). Most of the envelope genes are down-regulated in the mutants, indicating direct regulation by σ^{54} and/or σ^{S}. Because it has been shown that *B. burgdorferi* lipoproteins are down-regulated during long-term colonization of mice (28), σ^{54} and/or σ^{S}-dependent regulation of these proteins may be important for chronic infections.

The largest group of σ^{54}- and σ^{S}-regulated genes in the cellular processes category is involved in chemotaxis and motility. In some bacteria (e.g., *Vibrio fischeri* and *Campylobacter jejuni*), motility genes are known to be regulated by σ^{54} and may play a role in colonization and pathogenesis (34, 35). The role of chemotaxis and motility systems in the pathogenesis of Lyme disease is not well characterized, but lack of flagella has been correlated with attenuation in cell culture and mouse infection models (36, 37). Although several chemotaxis and motility genes showed altered expression in both sigma factor mutants (17 genes, Tables 4–6), both remained motile. This finding is not surprising because most of the underexpressed chemotaxis genes are involved in regulation and *B. burgdorferi* has a redundant system for regulating motility (1). Additionally, most of the nonregulatory motility genes underexpressed in the σ^{S} mutant were related to flagellar protein secretion (e.g., *fliQ* and *fliR*) rather than flagellar structural proteins (e.g., FlaB). The down-regulation of these secretion genes in the σ^{S} mutant may have a more subtle effect on the function or number of flagella. Interestingly, Sellek *et al.* (37) demonstrated that a decrease in the number of flagella in *B. garinii* was correlated with reduced invasiveness. One could speculate, because of the different environments encountered during the complex infectious cycle, that chemotaxis would be very important at key stages of the process. For example, functional chemotaxis could be required for dissemination in the mammal and/or migration from the midgut to the salivary glands.

Microarray analysis of a *Listeria monocytogenes* σ^{54} mutant showed altered transcription of many genes involved in carbohydrate utilization and transport (38). Our data also show that 11 carbohydrate metabolism and transport genes are regulated by σ^{54} (Tables 4 and 6). Five of these (*bbb05-6 and bb002-4*) have putative roles in transport and metabolism of chitobiose (39). Because chitobiose is an amino sugar, this finding may indicate a role for nitrogen in σ^{54}-dependent regulation as is commonly seen in other bacteria (22). Alternatively, these gene products could simply be used, but not required (39), for acquiring precursors for peptidoglycan biosynthesis and energy because chitobiose is likely present in the arthropod vector.

Recently, Tokarz *et al.* (40) reported the transcriptional response of *B. burgdorferi* to blood. They demonstrated that *rpoS* was among 75 genes induced in blood. Presumably with increased transcription of *rpoS*, some σ^{S}-regulated genes would also be up-regulated. In fact, comparison of the blood-induced genes with our data shows that 17 genes underexpressed in the σ^{S} mutant (*bb0418*, *bb0548*, *bb0565*, *bb0567*, *bb0680-1*, *bb0728*, *bb0844*, *bba24*, *bba64-6*, *bba71-2*, *bbb19*, *bbj25*, and *bbn28*) were also induced in blood, further confirming our microarray data.

Neither K-means nor QT clustering analyses, methods to subset microarray data by expression patterns, provided insights

SEE COMMENTARY

into a unifying theme of sigma factor regulation in *B. burgdorferi*. Although many bacteria use alternative sigma factors to regulate distinct sets of genes for certain metabolic or physical processes, it appears that *B. burgdorferi*, perhaps because of its limited repertoire, may instead use alternative sigma factors as general regulators of many simultaneous biological processes.

Our *in vivo* analyses of the σ^{54} mutant demonstrated that *ntrA* is required by *B. burgdorferi* to infect mice and to enter tick salivary glands, an essential step in the transmission process. Because midgut contents (including spirochetes) often contaminate other tissues during dissection of fed ticks, it is unclear whether the mutant's defect in entry is caused by an inability to escape the midgut, penetrate the salivary glands, or both. Grimm *et al.* (9) reported that a B31-A3 *ospC* mutant was not infectious in mice but was able to enter tick salivary glands. Although the σ^{54} mutant did not express OspC *in vitro* and behaved like an *ospC* mutant in mice, it had a different phenotype in the tick; it could not enter the salivary glands. Recently, Pal *et al.* (41) showed that a different *ospC* mutant was unable to enter tick salivary glands. Their study used nymphs infected by microinjection, which differs considerably from the immersion-infection method used in this study and by Grimm *et al.* Microinjected *B. burgdorferi* cells were assayed for transmission 3 days after infection and were not tested after long-term survival or passage through the molting process. In contrast, immersion-infected larvae were assayed after molting to nymphs, which may account for the different salivary gland entry results seen in the two *ospC* mutants. Bacteria residing longer in the midgut may undergo OspC-independent adaptations, whereas short-term residence may not allow *B. burgdorferi* to fully adapt. Together, these observations suggest that OspC facilitates, but is not required for salivary gland entry.

An intriguing finding was that the σ^{54} mutant was able to survive long term in the tick. In an unfed tick, one might expect spirochetes to require stationary phase-regulated proteins. Because σ^{54} regulates the expression of σ^{S}, the stationary-phase sigma factor, we predicted the mutant would not persist in the tick. Its survival suggests a level of *rpoS* regulation independent of σ^{54}.

The search for sigma factor regulons by microarray analysis has provided a wealth of knowledge about the regulatory networks present in bacteria (42, 43). In this work, we used microarray technology to begin to identify the alternative sigma factor regulons of *B. burgdorferi* and demonstrated that they regulate distinct and overlapping sets of genes. Many of these are either required for infectivity or expressed *in vivo*, implicating their involvement in growth or survival in animals. In support of these observations, we have established, by means of targeted mutagenesis and genetic complementation, that the *ntrA* gene is a virulence determinant required by *B. burgdorferi* for transmission to, and infection of, its mammalian host.

We thank T. Schwan and P. Policastro for tick/IFA help, D. Sturdevant for array support, J. Treglown for pJSTJNK, D. Dorward and F. DeLeo for help with confocal microscopy, R. Larson for help with mice, G. Hettrick for graphics, and G. Sylva for sequencing.

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