

Function and Evolution of Plasmid-Borne Genes for Pyrimidine Biosynthesis in *Borrelia* spp.

Jianmin Zhong,¹ Stephane Skouloubris,² Qiyuan Dai,¹ Hannu Myllykallio,² and Alan G. Barbour^{1*}

Departments of Microbiology and of Molecular Genetics and Medicine, University of California Irvine, Irvine, California¹, and Université Paris-Sud, Institut de Génétique et Microbiologie, INSERM Avenir Group, CNRS UMR8621, Orsay, France²

Received 23 August 2005/Accepted 8 November 2005

The *thyX* gene for thymidylate synthase of the Lyme borreliosis (LB) agent *Borrelia burgdorferi* is located in a 54-kb linear plasmid. In the present study, we identified an orthologous thymidylate synthase gene in the relapsing fever (RF) agent *Borrelia hermsii*, located in a 180-kb linear plasmid, and demonstrated its expression. The functions of the *B. hermsii* and *B. burgdorferi* *thyX* gene products were evaluated both in vivo, by complementation of a thymidylate synthase-deficient *Escherichia coli* mutant, and in vitro, by testing their activities after purification. The *B. hermsii* *thyX* gene complemented the *thyA* mutation in *E. coli*, and purified *B. hermsii* ThyX protein catalyzed the conversion of dTMP from dUMP. In contrast, the *B. burgdorferi* ThyX protein had only weakly detectable activity in vitro, and the *B. burgdorferi* *thyX* gene did not provide complementation in vivo. The lack of activity of *B. burgdorferi*'s ThyX protein was associated with the substitution of a cysteine for a highly conserved arginine at position 91. The *B. hermsii* *thyX* locus was further distinguished by the downstream presence in the plasmid of orthologues of *nrdI*, *nrdE*, and *nrdF*, which encode the subunits of ribonucleoside diphosphate reductase and which are not present in the LB agents *B. burgdorferi* and *Borrelia garinii*. Phylogenetic analysis suggested that the *nrdIEF* cluster of *B. hermsii* was acquired by horizontal gene transfer. These findings indicate that *Borrelia* spp. causing RF have a greater capability for de novo pyrimidine synthesis than those causing LB, thus providing a basis for some of the biological differences between the two groups of pathogens.

Spirochetes are a coherent group of bacteria with a unique morphology and cell structure (38). The point of divergence of spirochetes from other bacterial groups was deep in time, and the topology of spirochetes' branching from other phyla, such as proteobacteria and cyanobacteria, remains unresolved. Phylogenetic inference has been advanced with the availability of genome sequences of pathogenic spirochete species from the following three genera: *Borrelia*, including *Borrelia burgdorferi* (13, 19) and *B. garinii* (23), two agents of Lyme borreliosis (LB); *Treponema*, including *Treponema pallidum* (20), the agent of syphilis, and *Treponema denticola* (51), a mouth organism associated with periodontal disease; and two serovars of *Leptospira interrogans* (36, 44). Leptospirens have free-living capacity and, not surprisingly, larger chromosomes, at ~4.5 Mb, than the ~1-Mb chromosomes of the obligately parasitic organisms *T. pallidum* (20) and *B. burgdorferi* (18). But the leptospirens' branching from the common ancestor of *Treponema* and *Borrelia* appears to be nearly as ancient as the spirochetes' origin itself, and for the most part, leptospire sequences have been of limited use for further defining evolution within the spirochete clade.

The several *Borrelia* species can be divided into two major groups on the basis of DNA sequence and biological differences (2). One group includes all the agents of LB, such as *B. burgdorferi* and *B. garinii*, as well as related species of uncertain pathogenicity for humans. The second monophyletic group comprises several species that cause relapsing fever (RF), such

as *B. hermsii* and *B. turicatae*, as well as some other species, such as *B. miyamotoi*. Lyme borreliosis is an illness with a more protracted course and a generally milder character than those experienced during relapsing fever (4). This clinical difference may be attributable in part to the much lower peak densities in the blood that are achieved by LB spirochetes than RF spirochetes (39, 47, 52). While RF spirochetes can be found in abundant numbers in blood smears from febrile patients or experimentally infected animals, LB agents in the blood are detectable only by culture or PCR assay (10). As suggested by Schwan et al. (49), these differences in the carrying capacities of the two types of *Borrelia* in the blood may be the consequence of differences in metabolic capacities of LB and RF agents.

The characteristics of the spirochetes in their vectors also may differentially correlate with the metabolic capabilities of RF and LB species (49). The tick vectors of RF are soft ticks whose feeding times on a host are measured in minutes rather than the days typical of the hard tick vectors of LB. As expected for this limited period for achieving transmission, RF spirochetes are already positioned in the tick's salivary glands at attachment, and they soon enter the host through the tick's saliva as it feeds. In unfed hard ticks, *B. burgdorferi* is mainly inside the midgut and does not migrate to the salivary glands until a day or two after being exposed to blood during feeding (7, 46). There is thus more time for *Borrelia* spp. causing LB to adjust to the vertebrate environment before they actually enter the host (50).

The genomes of *Borrelia* spp. are distinguished from those of other spirochetes and most other bacteria by having linear chromosomes and linear as well as circular plasmids (18, 41). Approximately 40% of the *B. burgdorferi* genome is carried on

* Corresponding author. Mailing address: Pacific-Southwest Center, 3046 Hewitt Hall, University of California Irvine, Irvine, CA 92697-4028. Phone: (949) 824-5626. Fax: (949) 824-6452. E-mail: abarbour@uci.edu.

plasmids (13, 19). The linear plasmids vary in size from 5 to 180 kb and have covalently closed hairpins for telomeres (5, 12). *Borrelia* organisms maintain on their plasmids the genes for most of the major lipoprotein surface antigens, but the plasmids also carry some metabolism genes, such as *guaA* and *guaB*, which are part of the purine biosynthetic pathway (32). The purine biosynthetic genes *purA* and *purB* are on the chromosomes of *B. hermsii* and other RF species, but their absence from LB species and their nucleotide sequences suggest that they were acquired through horizontal gene transfer (6).

What genes there are for de novo pyrimidine synthesis in *B. burgdorferi* and *B. garinii* are located on the chromosomes of these LB agents (19, 23). An exception is an orthologue of *thyX*, which is located near the telomere of a 54-kb linear plasmid of *B. burgdorferi* (19). For *B. garinii*, a *thyX* orthologue was not included in the annotation of the genome (23), but an examination of position 55200 to the end of the sequence at position 55560 of a 56-kb linear plasmid (accession number NC_006129) reveals an open reading frame encoding a 119-amino-acid polypeptide with 86% sequence identity with the N-terminal half of the *B. burgdorferi thyX* gene product. In other species of organisms, the *thyX* product, ThyX, is thymidylate synthase X (EC 2.1.1.148), which catalyzes the methylation of dUMP to yield the DNA precursor dTMP. Sequences homologous to *thyX* have been found in several groups of bacteria, including cyanobacteria, actinobacteria, and epsilon-proteobacteria such as *Helicobacter pylori*, but they are also present in some double-stranded DNA viruses of bacteria and at least one eukaryote, the slime mold *Dictyostelium discoides* (30, 35). Other groups of bacteria, including gamma-proteobacteria such as *Escherichia coli* and firmicutes such as *Bacillus subtilis*, usually have instead a *thyA* gene, which encodes thymidylate synthase A (ThyA; EC 2.1.1.45) and is not homologous to *thyX* (11, 30). ThyX proteins are homotetrameric and flavin adenine dinucleotide (FAD) dependent, while ThyA proteins are homodimeric and FAD independent in their catalysis (33, 35).

Using *B. hermsii* genomic DNA as a hybridization probe of a *B. burgdorferi* DNA array, we had previously found evidence that *B. hermsii* has a *thyX* gene (54). This finding prompted our further study of the RF species *B. hermsii*, first to confirm the presence of a *thyX* orthologue in this species and then, if present, to characterize it for further insight into *thyX* origins in spirochetes and to investigate the function of the *thyX* gene product. In the course of the study, we identified in *B. hermsii* other pyrimidine biosynthesis genes that were not present in LB species genomes.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* B31 (ATCC 35210) was grown at 34°C in tightly capped tubes of Barbour-Stoenner-Kelly broth medium (BSK) (3). *B. hermsii* HS1 (45) was grown in either BSK or Dulbecco's modified Eagle's medium (Invitrogen, San Diego, Calif.) supplemented with 4% fraction V of bovine serum albumin (US Biochemical Corp., Cleveland, Ohio), 1.1% gelatin, and 1.5 mM *N*-acetylglucosamine (SDMEM). Spirochetes were counted with a Petroff-Hauser chamber by phase-contrast microscopy, and cultivated cells were harvested by centrifugation at 2,000 × *g* for 20 min at 20°C. For some experiments, *B. hermsii* cells were recovered from citrated blood of infected CB17 *scid* mice by centrifugation of the citrated blood at 100 × *g* for 5 min as described previously (40). *E. coli* K-12 strain χ 2913 (Δ *thyA572 recA56 srlA::Tn10*) (14), which was provided by D. V. Santi of the University of California at San Francisco, was grown at 37°C in M9 minimal medium supplemented with 0.1 mM

CaCl₂, 2 mM MgSO₄, 0.2% Difco Casamino Acids, and 50 µg/ml thymidine. *E. coli* strains TOP10 (Invitrogen) and SURE (Stratagene, La Jolla, Calif.) were grown at 37°C in Luria-Bertani medium, with or without 100 µg/ml carbenicillin.

Extraction and labeling of DNA. Plasmid DNA was isolated from *E. coli* with a High Pure plasmid isolation kit (Roche Diagnostics, Mannheim, Germany), and total DNAs were isolated from *B. hermsii* and *B. burgdorferi* with a DNeasy tissue kit (QIAGEN Science, Valencia, Calif.). DNAs were labeled with [α -³²P]dATP (Perkin-Elmer, Boston, Mass.) by random priming using the Megaprime DNA labeling system (Amersham Biosciences, Piscataway, N.J.), as described previously (54).

Recombinant *B. hermsii* genomic library and screening. A genomic library in the plasmid pUC18 with inserts of 4 to 8 kb of *B. hermsii* strain HS1 DNA was described previously and was stored frozen in 50% glycerol in Luria-Bertani broth at -20°C in 384-well microtiter plates (42). Colony hybridization with labeled DNA probes was carried out as described previously (54).

PCRs. PCRs were carried out in Roche PCR buffer with 1.5 mM MgCl₂, a 0.2 mM concentration of each deoxynucleoside triphosphate, 3 units *Taq* polymerase (Roche Diagnostics), and 0.1 µM (each) of forward and reverse primers. The forward and reverse primers for amplification were as follows: 5'-GTACGGAT CCTATGCACGATCATGTAAGGC-3' and 5'-GCTGCTGCAGTTATAAA TTAAGTTTTCTTTTAACT-3' for *B. hermsii thyX*; 5'-GTACGGATCCTT TGAATAAAGAATATAAAATTTTG-3' and 5'-GCACCTGCAGTACTAAT ACACACTTACAAT-3' for *B. burgdorferi thyX* (accession number AE000790); 5'-GACGCATATGATGAAACAGTATTTAGAAT-3' and 5'-GACGCATA TGTTAGATAGCCACCGCGCTT-3' for *E. coli* K-12 *thyA* (U00096); 5'-GT TGATTCATCTGTAAGTTGCTCAAT-3' and 5'-ACTTGCTGTTCATC TGGTAATGG-3' for *B. hermsii flaB*; 5'-AGCTAAGAGTAATGATGGCAA T-3' and 5'-ATTTATCACCTTTAGCCATTCT-3' for the 28-kb linear plasmid lp7E of *B. hermsii* (Z11876); and 5'-CAGATGGTCTTACTGCTGAAGC-3' and 5'-CAGCAACAACCTTTTCTTTAG-3' for the 53-kb linear plasmid lp53 of *B. hermsii* (L24911). The PCR conditions were as follows: 1 cycle of 95°C for 4 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 1 cycle of 72°C for 7 min. For use as probes, the PCR products were labeled with [α -³²P]dATP by the random priming method (Amersham Biosciences). For cloning of the fragments, the PCR products were ligated into the TOPO TA cloning vector, and the products were transformed into *E. coli* TOP10 cells (Invitrogen).

Pulsed-field gel electrophoresis and Southern blot hybridization. Unsheared genomic DNAs were prepared in agarose plugs as described previously (18). In brief, 10⁹ *B. burgdorferi* cells from a culture or 10⁹ *B. hermsii* cells freshly harvested from mouse plasma were embedded in 80 µl 1% (wt/vol) SeaPlaque low-melting-temperature agarose (BioWhittaker Molecular Applications, Rockland, Maine). The agarose plugs were incubated for 24 h in 150 mM NaCl-50 mM Tris-HCl (pH 8.0) buffer with 1% sodium dodecyl sulfate and 1 mg/ml proteinase K at 50°C and then were washed with 10 mM Tris-HCl-1 mM EDTA (pH 8.0) buffer three times for 30 min each at 20°C. Electrophoresis in 1% GenePure LE agarose (ISC BioExpress, Kaysville, Utah) in a CHEF Mapper apparatus (Bio-Rad, Hercules, Calif.) was carried out in 45 mM Tris-45 mM borate-1 mM EDTA (pH 8.3) buffer at 4°C at 6 V/cm, with a pulsed-field angle of 120° and switch times that were linearly ramped between 0.35 and 89 s over 18 h; the gels were stained with ethidium bromide. Southern blot analysis was carried out as described previously (54).

DNA sequencing and analysis. Sequence analysis over both strands was carried out by using custom oligonucleotides and a CEQ 8000 automated DNA sequencer (Beckman Coulter, Fullerton, CA) as described previously (54). Nucleotide and protein databases were searched with the BLASTX, BLASTP, and TBLASTX algorithms (31). A sequence of 13,218 bp containing the *thyX*, *nrdI*, *nrdE*, and *nrdF* genes, as well as their flanking regions, of *B. hermsii* strain HS1 was obtained. For phylogenetic reconstruction, the homologous *thyX*, *nrdE*, and *nrdF* sequences of representative bacteria, viruses, or eukaryotes for which genome sequences were available, as well as selected organisms without full genome sequences, were obtained. The accession numbers of the sequences are given in the figure legends. Amino acid sequences were aligned using Clustal X, version 1.83, and this alignment (<http://spiro.mmg.uci.edu/data>) was the basis for a codon-based, gapped nucleotide alignment produced with CodonAlign, version 2.0 (<http://www.sinauer.com/hall/index.php>). Alignments of concatenated sequences were then edited manually with MacClade, version 4.06 (Sinauer Associates). To minimize effects of base composition bias across taxa, the third positions were excluded, and the evolutionary model of Galtier and Gouy was applied (21) using PHYLO_WIN phylogenetic analysis software (<http://pbil.univ-lyon1.fr/software/phylowin.html>). Positions with gaps were ignored. Phylograms showing nodes with bootstrap support values under distance (neighbor-joining) and maximum likelihood criteria were constructed.

Quantitative reverse transcriptase PCR. *B. hermsii* cells in logarithmic growth phase were harvested and resuspended at a density of 3×10^6 cells per ml of BSK or 1.0×10^7 cells per ml of SDMEM. In the case of SDMEM, the cells were washed twice in SDMEM to minimize carryover of BSK components. The cell suspensions were incubated at 37°C for 20 h and then harvested as described above. Total RNA was immediately extracted from the pellets, using TriReagent (Molecular Research Center, Cincinnati, OH). The RNAs were treated twice with 2 units of RNase-free DNase I (Ambion, Austin, TX) at 37°C for 30 min and then reverse transcribed with random hexamers and a Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics). Reactions with 200 ng substrate RNA were carried out in a 20- μ l volume containing 50 mM Tris (pH 8.5)–30 mM KCl–8 mM MgCl₂ with a 1 mM concentration of each deoxynucleotide, 20 units RNase inhibitor, 60 μ M random hexamers, and 10 units reverse transcriptase at 25°C for 10 min, followed by 50°C for 1 h. As a measure of DNA contamination in each sample, the reverse transcriptase was omitted in matched reactions. Real-time quantitative PCRs with the cDNA or plasmid DNA samples were carried out using a Rotor-Gene RG-3000 apparatus (Corbett Research, San Francisco, CA). TaqMan minor-groove binding probes were obtained from Applied Biosystems, Foster City, CA. The VIC-labeled probe, forward primer, and reverse primer for the *B. hermsii thyX* gene were 5'-TCATGTTAAAGCTCC-3', 5'-CTGGTCTTATTGACTATTTGATTCGAA-3', and 5'-CATCCATTGCCTTGCAACAA-3', respectively. The forward and reverse primers for the *flaB* gene of *B. hermsii* were those described above; the 6-carboxyfluorescein (FAM)-labeled probe for *flaB* was 5'-AACCTCTGTCTGCATC-3'. PCRs were carried out in triplicate in 25- μ l volumes with quantitative PCR Master Mix Plus containing HotGoldStar DNA polymerase (Eurogentec, San Diego, CA) and a 0.2 μ M concentration of each primer and probe. The PCR conditions were as follows: 94°C for 10 min and then 50 cycles of 15 s at 94°C followed by 1 min at 58°C. There were four replicates of each growth condition, and from each tube three cDNA reactions were carried out. The PCR results for the three cDNA samples were then averaged to yield a single value for each of the replicates. In a preliminary study, both PCR assays were negative with *B. burgdorferi* RNA.

Complementation assay. The full-length coding sequences of the *thyX* genes of *B. hermsii* and *B. burgdorferi* were amplified by PCR as described above and ligated between the PstI and BamHI sites of pUC18. The *thyA* gene of *E. coli* K-12 was similarly amplified and ligated into pUC18 at the NdeI site. A plasmid with the *Lactobacillus casei thyA* gene cloned into the PstI and BamHI sites of pUC18 was provided by D. V. Santi of the University of California, San Francisco (14). The different plasmid constructs were then transformed into the *thyA* mutant strain χ 2913 and plated on Luria-Bertani agar medium with 50 μ g/ml carbenicillin and 50 μ g/ml thymidine (Sigma, St. Louis, MO) (35). The different transformants were grown overnight in M9 minimal medium with 50 μ g/ml thymidine and 50 μ g/ml carbenicillin and then washed three times with unsupplemented M9 minimal medium before inoculation at a final concentration of 5×10^6 cells per ml into M9 minimal medium with carbenicillin alone. Cultures were incubated in duplicate at 37°C on a rotating shaker set at 250 rpm, and cell growth was measured spectrophotometrically by changes in transmittance, as measured by the optical density at 600 nm.

In vitro thymidylate synthase assay. Full-length *thyX* genes were amplified by PCR, using the conditions described above and the following forward and reverse primers: 5'-CGGGATCCCATGCACGATCATGTAAAG-3' and 5'-AACTGCAGTTATAAATTAAAGTTTTC-3' for *B. hermsii* and 5'-CGGGATCCCATGAATAAAGAATATAA-3' and 5'-GGGGTACCTCAATCAATTCCTAGCTT-3' for *B. burgdorferi*. The products were ligated between the BamHI and KpnI sites for *B. burgdorferi thyX* and the BamHI and PstI sites for *B. hermsii thyX* in the His tag-encoding plasmid vector pQE80L (QIAGEN Science) and transformed into *E. coli* SURE competent cells. The transformants were grown in Luria-Bertani medium at 37°C, and recombinant protein expression was induced when the culture reached an optical density at 600 nm of 0.4 by the addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 1 mM. After another 2 h of cultivation, the cells were harvested by centrifugation at $4,300 \times g$ for 20 min at 4°C. His-tagged proteins were purified by Ni-nitrilotriacetic acid affinity chromatography (QIAGEN Science) as described previously (35). The thymidylate synthase activity of the recombinant proteins was measured by using tritiated dUMP as the substrate, as described by Leduc et al. (29). The reaction was performed at 37°C for 30 min in a total volume of 50 μ l of 50 mM HEPES (pH 7.5)–10 mM MgCl₂–10% glycerol–0.5 mM methylenetetrahydrofolate (CH₂H₄ folate; Merck Eprova, Switzerland)–1.5 mM FAD–1.2 mM β -NADPH–0.6 mM β -NADH–20 μ M dUMP (Sigma). The amounts of His-tagged ThyX proteins in the reaction were 1.3 μ g for *B. hermsii* and 3 μ g for *B. burgdorferi*, and the specific activities of [³H]dUMP (Moravak Biochemicals, Brea, CA) were 0.15 Ci/mmol for *B. hermsii* ThyX and 0.3 Ci/mmol for *B. burgdorferi* ThyX. Reactions were terminated by the addition of activated charcoal at 10% (wt/vol)

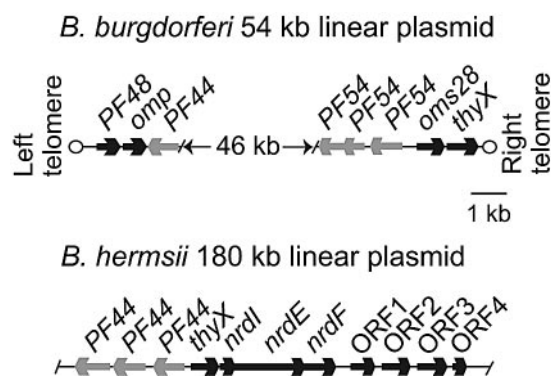


FIG. 1. Physical maps of the *thyX* locus and flanking regions of the 180-kb linear plasmid of *Borrelia hermsii* and the 54-kb linear plasmid of *Borrelia burgdorferi*. The solid arrows correspond to the lengths of the open reading frames and the presumed direction of transcription. PF numbers indicate the paralogous family designations for *B. burgdorferi* genes (13); the product of *omp* is a hypothetical outer membrane protein.

and 2% trichloroacetic acid. Released ³H ions in the supernatant were measured by scintillation counting as previously described (29).

Nucleotide sequence accession number. The sequence of 13,218 bp containing the *thyX*, *nrdI*, *nrdE*, and *nrdF* genes, as well as their flanking regions, of *B. hermsii* strain HS1 was assigned GenBank accession number AY623744.

RESULTS

***B. hermsii thyX* locus and adjacent genes.** We amplified by PCR the *B. burgdorferi thyX* gene from total DNA and used it in turn to probe a genomic library of *B. hermsii*. Three hybridizing clones were isolated, and their inserts were sequenced. The overlapping fragments comprised a single sequence of 13,218 bp with 11 open reading frames (ORFs) of at least 300 nucleotides on the two strands, as well as incomplete ORFs at each end of the 13.2-kb fragment. A physical map of the *B. hermsii* sequence along with selected regions of the 54-kb linear plasmid of *B. burgdorferi* (19) is shown in Fig. 1. The overall GC content of the combined sequence was 32%; there were no direct or inverted repeats of 10 or more nucleotides.

Possible functions were provisionally assigned to 4 of the 11 ORFs of *B. hermsii*, namely, *thyX*, *nrdI*, *nrdE*, and *nrdF*, on the basis of sequence identity and similarity. They are located on the same strand and in the middle of the fragment (Fig. 1). At the left end of the fragment (not shown) is a partial ORF that is similar to the portal protein genes of bacteriophages of firmicutes and *Fusobacterium nucleatum* (protein family 05133 [http://www.sanger.ac.uk/Software/Pfam/]). Upstream of *thyX*, on the opposite strand, are three ORFs with 36 to 41% pairwise amino acid identities with each other and with 31 to 33% identities with the “S2” proteins of LB *Borrelia* spp. (17). Genes for S2 in *B. burgdorferi* B31 are located on the lp54 plasmid, the same plasmid bearing *thyX* in the opposite orientation, but in *B. burgdorferi*, *thyX* and the S2 gene are ~50 kb apart. The S2-like genes of LB *Borrelia* spp. were assigned to paralogous family number 44 (13).

The *thyX* orthologue of *B. hermsii* is 75% identical in nucleotide sequence to that of *B. burgdorferi*. The GC contents of the two genes are 32 to 34%, which is similar to the overall GC content of *B. burgdorferi* (13, 19). In contrast to the *thyX* gene

of *B. burgdorferi*, which is a terminal ORF on the 54-kb linear plasmid (19; J. Aron and S. Casjens, personal communication), the *thyX* gene of *B. hermsii* is 30 bp upstream of three other closely spaced or overlapping ORFs on the same strand, with a GC content of ~33%. The putative gene products are components of both the pyrimidine biosynthesis and salvage pathways: they are the alpha (NrdE) and beta (NrdF) subunits of ribonucleoside diphosphate reductase and an associated ribonucleoprotein (NrdI) in the complex. The *nrdI*, *nrdE*, and *nrdF* genes were not reported for the genomes of *B. burgdorferi* and *B. garinii* (19, 23). This was confirmed for this study by TBLASTX searches of the deposited chromosome and plasmid sequences of *B. burgdorferi* and *B. garinii*, using the nucleotide sequences of the *nrd* genes. The gene order of *thyX-nrdI-nrdE-nrdF* was only identified for *B. hermsii*. The codon usages and GC skews of the *thyX* and *nrdIEF* genes were similar to the corresponding values calculated for the remaining sequence of the 13-kb fragment. There were consensus ribosomal binding sequences preceding the *thyX*, *nrdI*, *nrdE*, and *nrdF* genes, but a consensus σ^{70} -type, σ^{54} -type, or σ^{38} -type promoter was not discernible within 150 nucleotides upstream of the *thyX* start codon.

At the right end of the fragment and following the *nrdF* gene on the same strand are three complete ORFs and one partial ORF. The three complete ORFs have ~30% pairwise identity with each other over their aligned lengths, with BLASTP E values of $<10^{-7}$, but they are unlike any other protein in the database (E value, >0.1). The partial ORF at the end also appears to be homologous to this family of hypothetical proteins (E value, $<10^{-4}$).

Location of *B. hermsii thyX* gene. To identify the location of the *B. hermsii thyX* gene, we separated the linear chromosome and several linear and circular plasmids by pulsed-field gel electrophoresis and carried out Southern blot analysis. The chromosome and plasmids of *B. burgdorferi* were also included in the gel. The left panel of Fig. 2 shows the ethidium bromide-stained gel. There were major bands of approximately 920, 180, 53, and 30 kb, as expected, for *B. hermsii* and 920, 54, 32, and 28 kb for *B. burgdorferi* (18). The blot of this gel was sequentially probed first with the labeled, PCR-amplified *thyX* gene of *B. hermsii* and then with similarly prepared probes for a 28-kb linear plasmid and the 53-kb linear plasmid of *B. hermsii*. The *thyX* probe bound to the 180-kb plasmid of *B. hermsii* and, by cross-hybridization, to the 54-kb plasmid of *B. burgdorferi*. The two other probes that were subsequently hybridized with the blot bound as expected to the 28-kb and 53-kb plasmids in the gel.

Phylogenetic analyses. We compared the *thyX*, *nrdE*, and *nrdF* sequences of *B. hermsii* with a selection of corresponding orthologues from other organisms that represented the diversity of these genes. The nucleotide sequences were aligned on a codon basis with the corresponding *B. hermsii* sequences. The *nrdE* and *nrdF* sequences are tandemly arrayed in all the selected organisms used for the comparison, and accordingly, the aligned sequences of these genes were concatenated for the analysis. To adjust for differences in base composition across the taxa, we excluded the third position of each aligned codon and used the algorithm of Galtier and Gouy (21).

Figure 3 shows a phylogram of the *thyX* genes. Although the *thyA* gene, much studied in the gamma-proteobacterium *E. coli*

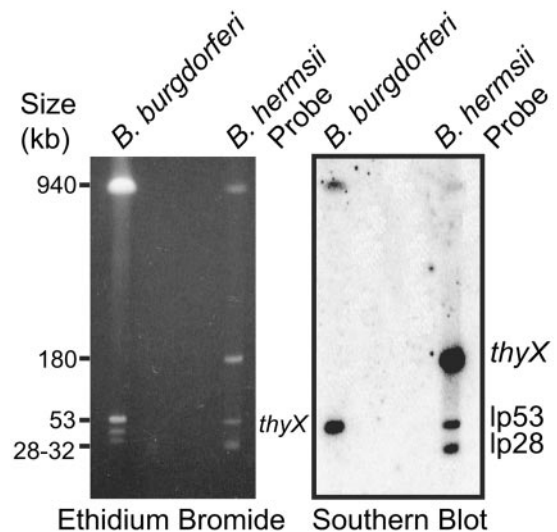


FIG. 2. Southern blot analysis of replicons of *B. hermsii* and *B. burgdorferi* separated by pulsed-field gel electrophoresis and sequentially hybridized with radiolabeled probes for *thyX*, the lp53 linear plasmid, and the lp28 linear plasmid of *B. hermsii*. An ethidium bromide-stained gel is shown on the left, and an autoradiograph is shown on the right. Sizes (in kb) of linear plasmids of *B. hermsii* and of size standards are indicated.

and the firmicute *B. subtilis*, is considered the usual thymidylate synthase gene among bacteria, the cumulative bacterial genomes sequenced to date indicate that the *thyX* gene is at least as widely distributed as *thyA* among bacterial phyla. The figure shows that the *B. hermsii* and *B. burgdorferi* genes are monophyletic. There is moderate support for a node with two other spirochetes, *T. pallidum* and *T. denticola*, besides the two *Borrelia* species, and for a deeper node encompassing the spirochetes, some alpha-proteobacteria, *Dictyostelium discoideum*, and the thermophilic bacterium *Thermus thermophilus*. The borrelial genes are distant from the *thyX* genes of the epsilon-proteobacteria *H. pylori* and *Campylobacter jejuni* as well as those of some clostridia and the actinobacterium *Corynebacterium glutamicum*.

In contrast to the *thyX* genes of *B. hermsii* and *B. burgdorferi*, the *nrdEF* genes of *B. hermsii* clustered with *nrdEF* genes of the firmicutes *Staphylococcus* and *Bacillus* and some of their viruses (Fig. 4). The same clustering was noted in phylograms of *nrdE* and *nrdF* genes examined separately (data not shown). The arthropod-borne *Ehrlichia*, *Rickettsia*, and *Wolbachia* species whose genomes have been sequenced to date do not have *nrdEF* orthologues. *Agrobacterium tumefaciens* and *Bartonella henselae*, the alpha-proteobacteria known to have *nrdEF* genes, are represented in a more distant lineage which is shared with gamma-proteobacteria and actinobacteria.

Expression of *B. hermsii thyX* gene. Expression of the *B. hermsii thyX* gene was examined under the following two in vitro conditions: complete BSK medium, which includes supplemental pyrimidines and purines as well as rabbit serum (3), and SDMEM, which contained bovine serum albumin, gelatin, and *N*-acetylglucosamine but not serum and was based on Dulbecco's modified Eagle's medium, which lacks purines or pyrimidines. The lack of purines in SDMEM was confirmed by

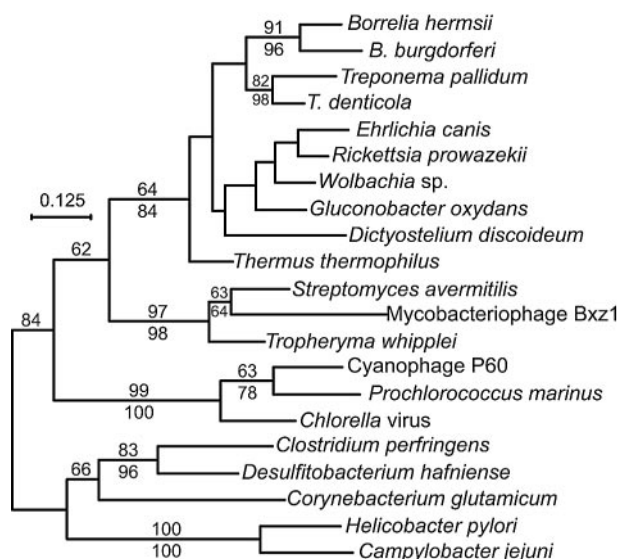


FIG. 3. Phylogram of codon-aligned nucleotide sequences of *thyX* genes from the following organisms (with accession numbers): *Borrelia burgdorferi* (NC_001857), *B. hermsii* (AY623744), *Campylobacter jejuni* (NC_003912), *Chlorella* virus (NC_000852), *Clostridium perfringens* (BA000016), *Corynebacterium glutamicum* (BX927153), cyanophage P60 (AF338467), *Desulfotobacterium hafniense* (NZ_AAAW03000212), *Dictyostelium discoideum* (M27713), *Ehrlichia canis* (NZ_AAEJ01000001), *Gluconobacter oxydans* (NC_006677), *Helicobacter pylori* (NC_000921), mycobacteriophage Bxz1 (AY129337), *Prochlorococcus marinus* (AE017161), *Rickettsia prowazekii* (NC_000963), *Streptomyces avermitilis* (BA000030), *Thermus thermophilus* (NC_005835), *Treponema denticola* (NC_002967), *T. pallidum* (AE001268), *Tropheryma whipplei* (AE016852), and a *Wolbachia* endosymbiont of *Drosophila melanogaster* (NC_002978). The third position of each codon and positions with gaps were excluded from the analysis. Nodes with bootstrap values with >61% support by maximum likelihood (100 replicates; shown above the line) or neighbor-joining (1,000 replicates; shown below the line) distance criteria are indicated. Bar, nucleotide distance.

the inability of an *E. coli purA* mutant strain, H1238 (*purA54 thr fluA argF relA spoT argI*), to grow in the medium (data not shown). Transcription of *thyX* and the constitutively expressed *flaB* gene was measured with probe-based real-time PCR of cDNAs produced with random hexamers; copy numbers were estimated from a standard curve produced with cloned *thyX* and *flaB* genes of *B. hermsii*.

BSK medium supported a threefold increase in cell density over 20 h. There was no detectable growth of the spirochetes in SDMEM, and both suspensions were harvested at cell densities of 10^7 per ml. In the absence of reverse transcriptase, there was no detectable PCR product when *thyX* and *flaB* primers and probes were used, but there were products with both reactions when reverse transcriptase was added. By quantitative PCR, the mean (95% confidence intervals) ratio of *thyX* to *flaB* cDNA was 0.020 (0.017 to 0.024) in SDMEM and 0.022 (0.019 to 0.025) in BSK medium ($P = 0.4$ by a two-tailed *t* test). The experiment showed that *thyX* was transcribed under two different in vitro conditions, but in smaller amounts than the constitutively expressed *flaB* gene (9, 37).

Complementation of *thyA* mutation. A *thyA* mutant of *E. coli* was transformed with expression plasmids encoding the *thyX* gene of *B. hermsii* or *B. burgdorferi* and, as positive controls, the

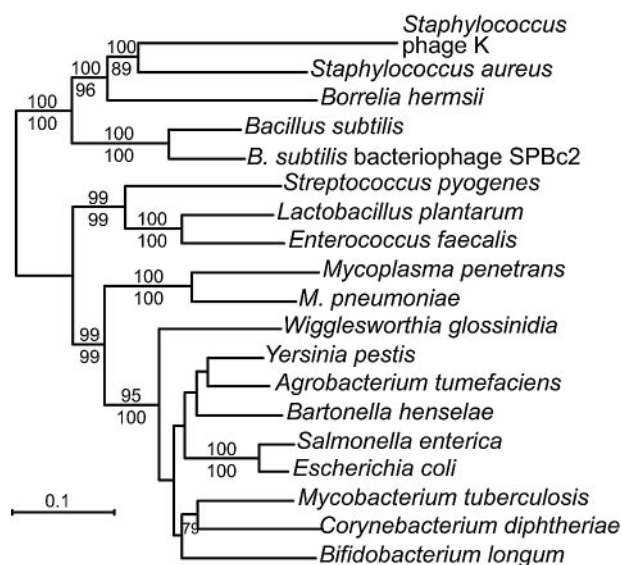


FIG. 4. Phylogram of codon-aligned concatenated nucleotide sequences of *nrdE* and *nrdF* genes from the following bacteria and viruses (with accession numbers): *Agrobacterium tumefaciens* (AE008981), *Bacillus subtilis* (NC_000964), *B. subtilis* bacteriophage SPBc2 (AF020713), *Bartonella henselae* (BX897699), *Bifidobacterium longum* (AE014295), *Borrelia hermsii* (AY623744), *Corynebacterium diphtheriae* (NC_002935), *Enterococcus faecalis* (NC_004668), *Escherichia coli* (U00096), *Lactobacillus plantarum* (NC_004567), *Mycobacterium tuberculosis* (NC_000962), *Mycoplasma penetrans* (BA000026), *Mycoplasma pneumoniae* (AE000050), *Salmonella enterica* (NC_006511), *Staphylococcus aureus* (NC_002758), *Staphylococcus phage K* (NC_005880), *Streptococcus pyogenes* (AE010059), *Wigglesworthia glossinidia* (BA000021), and *Yersinia pestis* (AE017136). Nucleotide positions with gaps and the third base of each codon in the aligned sequences were excluded from the analysis. Bootstrap values of nodes with >71% support by maximum likelihood (100 replicates; shown above the line) or neighbor-joining (1,000 replicates; shown below the line) distance criteria are indicated. Bar, nucleotide distance.

thyA genes of *E. coli* and *L. casei*. *E. coli* transformed with the plasmid vector without an insert served as the negative control. The *thyA* mutant host had previously been used to demonstrate complementation by the *thyX* gene of *H. pylori* (35). In a preliminary study, expression of the recombinant ThyX protein was detectable and measurable by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels and was similar in quantity for *E. coli* transformants with either the *B. hermsii* or *B. burgdorferi* *thyX* gene (data not shown).

All transformants were grown in minimal medium with or without thymidine, and then their growth was monitored by a spectrophotometer. Figure 5 shows the growth of the different isolates under nonselective and selective conditions. In the presence of supplemental thymidine, all isolates grew at approximately the same rate. In the absence of thymidine, *E. coli* cells with the *B. burgdorferi* *thyX* gene, like *E. coli* with the plasmid vector alone, did not detectably grow under either aerobic (Fig. 5) or anaerobic conditions (data not shown). In contrast, *E. coli* cells with the *B. hermsii* *thyX* gene grew in the absence of thymidine, albeit with a longer generation time (~10 h) than cells bearing the *thyA* gene of either *E. coli* or *L. casei*, which had generation times of ~2 h. By 50 h of incubation of the broth cultures, the average cell density of *B. hermsii*

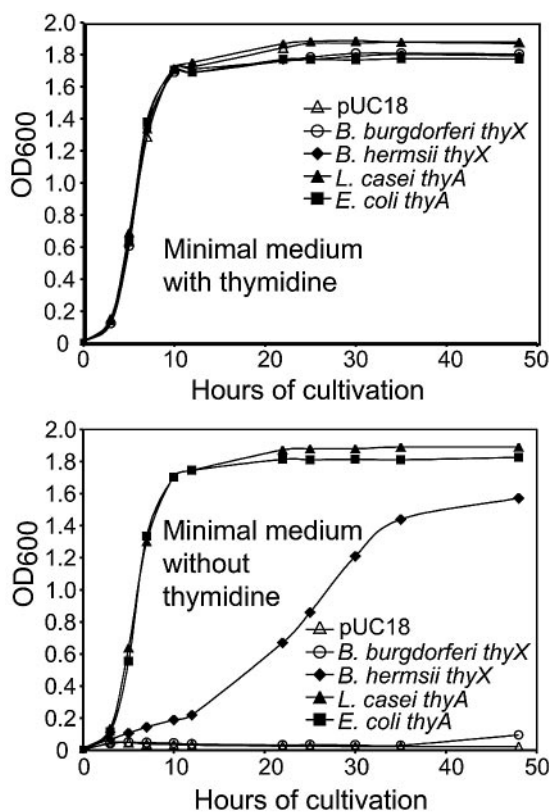


FIG. 5. Growth in the presence and absence of thymidine of a *thyA* mutant of *Escherichia coli* transformed with the plasmid vector pUC18 without an insert (open triangles) or with an insert of *thyA* of *E. coli* (solid squares), *thyA* of *Lactobacillus casei* (solid triangles), *thyX* of *Borrelia burgdorferi* (open circles), or *thyX* of *B. hermsii* (solid diamonds). Growth was determined by measurement of the optical density at 600 nm by a spectrophotometer.

thyX-expressing cells was 86% as high as the densities of *L. casei thyA*- and *E. coli thyA*-expressing cells (Fig. 5).

Thymidylate synthase activity. The failure of the *B. burgdorferi thyX* gene to complement the *thyA* mutation in *E. coli* was further investigated by purifying the recombinant gene products and subjecting the proteins to an assay for thymidylate synthase activity (35). The amount of protein expression in *E. coli* SURE cells and the purification yields of the ThyX proteins of *B. hermsii* and *B. burgdorferi* were comparable, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The assay's end point was the amount of free ^3H ions in the supernatant after precipitation of dUMP and dTMP with trichloroacetic acid. The results are shown in Fig. 6. The *B. hermsii thyX* gene product had thymidylate synthase activity as high as 2.51 pmol $^3\text{H}^+/\mu\text{g}/\text{min}$, and its activity remained linear for at least 20 min. This specific activity was approximately twofold higher than that achieved with purified ThyX of *H. pylori* in the same assay (35). In contrast, the in vitro activity of the *B. burgdorferi* protein was barely detectable above the background (Fig. 6).

Alignment of deduced proteins. Partial amino acid sequences of the gene products of the *thyX* orthologues of *B. burgdorferi*, *B. garinii*, and *B. hermsii* as well as of the ThyX proteins of other selected organisms are shown in Fig. 7. All

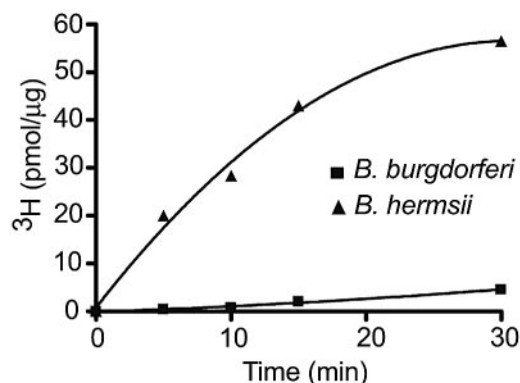


FIG. 6. In vitro thymidylate synthase activities of purified recombinant ThyX proteins of *B. hermsii* and *B. burgdorferi*. Activity was measured as released ^3H ions, in pmol/ μg (y axis), from the deprotonation of C-5 of the dUMP ring over time (x axis).

the *Borrelia* sp. proteins have the ThyX motif of RHRX₇S (positions 90 to 100 of *B. hermsii* ThyX) that is common to these proteins at their conserved amino-terminal domain (30, 35). The gene products of *B. hermsii* and *B. burgdorferi* share the following residues in common with other ThyX orthologues (*B. hermsii* ThyX numbering): H65, E70, F76, R90, H91, R92, S100, Y103, E168, R171, L174, P175, L197, R198, E206, R208, A211, and P223 (29). However, in the *B. burgdorferi* and *B. garinii* ThyX proteins, a cysteine residue substitutes for the conserved arginine at position 102 in *B. hermsii* ThyX, at position 86 in *H. pylori* ThyX (29), and at comparable positions in the ThyX proteins of other organisms, including *Treponema* spp.

DISCUSSION

The *thyX* gene was first discovered in the eukaryote *D. discoideum* (15), but the great majority of subsequent examples of *thyX* have been described for bacterial species or their viruses (30, 34). For some of these species, the *thyX* gene product's function as a thymidylate synthase was demonstrated by genetic complementation and/or a biochemical assay (16, 22, 24, 25, 33, 35), but in most cases the *thyX* orthologues in bacterial genomes were identified on the basis of sequence identity alone. The latter situation was the case for a *thyX*-like sequence in *B. burgdorferi*'s genome (19) as well as for similar sequences identified in the genomes of *T. pallidum* (20) and *T. denticola* (51). Using a *B. burgdorferi* genome array hybridized with DNA from *B. hermsii*, we obtained evidence that the RF agent has a *thyX* gene as well (54). Further microarray experiments indicated that the *thyX* gene of *B. burgdorferi* and the putative *thyX* gene of *B. hermsii* were transcribed both in vitro and in vivo (37, 54).

For the present study, we confirmed that *B. hermsii* has a *thyX* orthologue by identifying it in a genomic library and then sequencing the gene and ~ 13 kb of its flanking regions. As was the case for *B. burgdorferi* and *B. garinii*, the *thyX* gene of *B. hermsii* was carried on a plasmid, but in the RF species it was carried on the 180-kb linear plasmid instead of the 54- to 56-kb plasmids. The *thyX* gene is the first gene located so far on these large linear plasmids of *B. hermsii* and other RF species (18).

<i>Borrelia burgdorferi</i>	44	ELIDYLIRNGHTSPLEQVVFTHVK-APIFVARQWMRHRRTAR-INEVSGCYSLAREE	98
<i>B. garinii</i>	44	ELIDYLIRNGHTSPLEQVVFTHVK-APIFVARQWMRHRRTAR-INEASGCYSLARKE	98
<i>B. hermsii</i>	55	GLIDYLIRNEHTSPLEQVVLTFHVK-APIFVARQWMRHRRTSR-INEVSGRYSMLMREE	109
<i>Treponema pallidum</i>	84	ALIDFLLRNKHTSPFEQVVLTFHVR-APIFVARQWMRHRRTAR-ISEVSSRYSLLSHD	138
<i>T. denticola</i>	45	ALIDYLLRNKHTSPFEQVVFTHVK-LPIFVARQWIRHRRTAR-LNEISGRYSILKAE	99
<i>Thermus thermophilus</i>	47	ALIDYLMRHRHTSPFEMVVKFHVK-APIFVARQWFRHRRTAS-VNEISGRYSILKEE	101
<i>Thermotoga maritima</i>	43	HLIEYLMKHGHETPFHEIVVFTHVK-APIFVARQWFRHRRTAS-VNELSGRYSKLSYE	97
<i>Streptomyces avermitilis</i>	57	GLINYLMDRDRHGSPFEHNSMTFFIS-APIFVREFMRHRVGVSYNEESGRYRELEPV	112
Mycobacteriophage Bx21	44	GLINALMRDRHGTPFEHMNATFRVT-APIFVWREHHRHRSVSYNEESGRYKQLDPV	99
<i>Tropheryma whipplei</i>	56	GLITFLMRERHGSPFEHSHMTFRIS-APIFVREFMRHRRTAS-VNEESGRYKQLDPV	110
<i>Helicobacter pylori</i>	38	LIHRVGNIFRHSSTLEHLYNFEIKGLSRGALQELSRHRRTAS-LSVKSRYTTLRELK	93
<i>Ehrlichia canis</i>	70	ALINYLMDRHSHTPFEMCEIKFHIK-LPIFVARQWVRHRRTAN-VNEYSARYSVLDRE	124
<i>Wolbachia</i> sp.	61	ALIKYLMRHHHTPFEMCEIKFHVK-LPIFVARQWIRHRRTAN-VNEYSARYSILDNE	115
<i>Rickettsia prowazekii</i>	61	GLINYLRLRHHTPFEMCDIKFHIK-LPIFVARQWIRHRRTAS-VNEYSARYSILGNE	115
<i>Dictyostelium discoideum</i>	83	GLIRYLRYRHQHTSPFEMVEFKFHCV-MPVFIARQWIRHRRTAN-VNEYSARYSVLPDK	137
Cyanophage P60	61	RLIRYLKIKHGHWSPFEMAHMVVEIE-TTRAIAPQILRHRHSFS-FQEFSSQRYA-VADL	114
<i>Prochlorococcus marinus</i>	36	KLIKYCINNEHWSVFEQAYMTLQIE-TNRAIAAQILRHRHSFT-FQEFSSQRYADSTQL	90
<i>Chlorella</i> virus	43	GLLYKCIKHKHWSIFETAFMTLELK-TSRGIAAQVLRHRHSFH-FQEFSSQRYASVME	97
		.L.....H..P.E.....RHR.....E.S.RY.....	

FIG. 7. Amino acid alignment of partial ThyX proteins of the following organisms (with accession numbers): *Borrelia burgdorferi* (NP_045749), *B. garinii* (CP000015), *B. hermsii* (AAT68707), *Chlorella* virus (NP_049030), cyanophage P60 (NP_570363), *Dictyostelium discoideum* (XP_641335), *Ehrlichia canis* (ZP_00211002), *Helicobacter pylori* (NP_224139), *Magnetococcus* sp. (ZP_00605242), mycobacteriophage Bx21 (NP_818164), *Prochlorococcus marinus* (NP_874667), *Rickettsia prowazekii* (NP_220685), *Streptomyces avermitilis* (NP_823693), *Thermotoga maritima* (NP_228259), *Thermus thermophilus* (YP_004706), *Treponema denticola* (NP_970925), *T. pallidum* (AAC65955), *Tropheryma whipplei* (NP_787744), and a *Wolbachia* endosymbiont of *Drosophila melanogaster* (NP_966910). Numbers indicate the positions of the amino acids aligned according to the *B. burgdorferi* sequence. The consensus amino acids are shown at the bottom. The cysteine-for-arginine substitution in *B. burgdorferi* and *B. garinii* at position 91 is highlighted in gray.

Upstream of the *B. hermsii* *thyX* gene are three ORFs that are homologous to the PF44 genes, which are also located on the lp54 plasmid of *B. burgdorferi*. This is an indication that at least part of the 180-kb linear plasmid of RF species has a common lineage with the lp54-type plasmids of LB species.

Unlike the *B. burgdorferi* *thyX* gene, which is the rightmost ORF on the linear plasmid, the *B. hermsii* *thyX* gene was flanked on its right by several other ORFs, none of which were similar to sequences in the *B. burgdorferi* or *B. garinii* genome (Fig. 1). These additional genes included what may be an operon comprising the *nrdI*, *nrdE*, and *nrdF* genes. The *nrdE* and *nrdF* genes encode the large and small subunits, respectively, of ribonucleoside diphosphate reductase, an enzyme that catalyzes the de novo synthesis of deoxyribonucleotides required for DNA replication and repair (43). The *nrdI* gene encodes a small protein that is also part of the enzyme complex (53). On the basis of their deduced amino acid sequences, the NrdI, NrdE, and NrdF proteins of *B. hermsii* most closely resemble components of the class Ib ribonucleoside diphosphate reductase complex of bacteria (26–28). To investigate the function of the *nrdEF* genes of *B. hermsii*, they were cloned in tandem and expressed in *E. coli*, but the transformant was not able to detectably complement a *nrdAB* mutation in the host strain (unpublished findings). The gene products of *nrdAB* and *nrdEF* are in two different classes of ribonucleoside diphosphate reductase enzymes with limited sequence similarity (28), and it is possible that the *B. hermsii* proteins were not functional in the *E. coli* enzyme complex.

Using quantitative reverse transcriptase PCR assays with specific primers and probes for *thyX* and *flaB*, we confirmed our previous microarray result that the *thyX* gene of *B. hermsii* was expressed (54). In the present study, this was shown during in vitro cultivation in both complete medium, which provides a source of purines and pyrimidines, and a more defined medium lacking purines and pyrimidines. The expression of *thyX* of *B. hermsii* may be constitutive: under both conditions, the relative level of expression of the *B. hermsii* *thyX* gene was about 1/50

that of *flaB*, which is a highly expressed gene that encodes a major structural protein of spirochetal flagella (54).

Unlike the ribonucleoside diphosphate reductase complex, which may be dependent on proper interactions of the different subunits for full or even partial enzyme activity, the single polypeptide of ThyX appears to be sufficient for thymidylate synthase activity in complementation and in vitro assays in studies of other organisms (28). Accordingly, we examined the activities of the recombinant ThyX proteins of *B. burgdorferi* and *B. hermsii* in providing complementation of a *thyA* mutant of *E. coli* and then in an in vitro assay of thymidylate synthase activity. Although the ThyA thymidylate synthase is not discernibly homologous to ThyX, recombinant ThyX proteins of *H. pylori* and *C. jejuni* complemented *thyA* mutations in *E. coli* in other studies (22, 35). Here we showed that recombinant ThyX of *B. hermsii* had thymidylate synthase activity in both types of experiments. In contrast, recombinant ThyX of *B. burgdorferi* had very weak activity in the in vitro assay and was not capable of substituting for ThyA in an *E. coli* mutant.

Although *B. burgdorferi* ThyX appeared to be well expressed in *E. coli*, it is possible that the recombinant protein's lack of activity was attributable to misfolding or other posttranslational events that inexplicably did not affect the recombinant protein of *B. hermsii* under similar conditions. A more plausible explanation for the outcomes of the function experiments was found in the deduced amino acid sequence of the *B. burgdorferi* polypeptide. While the ThyX protein of *B. burgdorferi*, as well as that of *B. garinii*, has the motif RHRX₇S that serves to define ThyX proteins (35), there is a substitution of a cysteine for the highly conserved arginine at position 91 in the *B. burgdorferi* and *B. garinii* proteins (Fig. 7). This arginine corresponds to an arginine at position 90 of *Thermotoga maritima* ThyX (33) and position 86 of *H. pylori* ThyX (29). Crystallographic studies of the *T. maritima* ThyX protein suggest that hydrogen bonding occurs between this arginine and the dUMP substrate (33). We propose that replacement of a charged side chain-containing amino acid with an uncharged

one might abolish the interaction between the ThyX protein and the dUMP substrate. It is possible that the LB ThyX proteins have functional thymidylate synthase activity in spirochetes, but they also may have other roles in cells or be examples of plasmid genes that are undergoing degradation in *Borrelia* spp. (13). Whatever its role, if any, the *thyX* gene of *B. burgdorferi* is not essential for in vitro growth, at least under usual laboratory conditions: an isolate of *B. burgdorferi* strain B31 without the lp54 plasmid or its genes grew at the same rate as wild-type B31 in broth medium (48).

The *nrdIEF* genes were adjacent to the *thyX* gene on the 180-kb linear plasmid of *B. hermsii*, which suggests a common origin for these genes involved in pyrimidine metabolism. But we propose that the *nrdIEF* cluster, and perhaps other genes on the 180-kb plasmid, were acquired by a *Borrelia* ancestor at a time other than that of the acquisition of the *thyX* gene. The *thyX* genes of *Borrelia* spp. are in what may be a monophyletic cluster containing treponemes as well as some alpha-proteobacteria, notably some arthropod-borne species of *Ehrlichia*, *Rickettsia*, and *Wolbachia* (Fig. 3). None of these other species, including *T. pallidum* and *T. denticola*, have discernible orthologues of the *nrdE* or *nrdF* gene in their genomes. On the other hand, the *nrdEF* genes of *B. hermsii* are in a monophyletic cluster with species and their bacteriophages of the *Bacillales* group of *Firmicutes*, most or all of which have *thyA* genes and not *thyX* genes providing thymidylate synthase function to the cells (Fig. 4). Although the phylogenetic evidence of separate events for the acquisition of *thyX* and *nrdIEF* genes is compelling, we cannot at this time distinguish between selective acquisition by RF *Borrelia* spp. of the *nrdIEF* genes and selective loss of this operon by an ancestor of LB *Borrelia* spp. after the divergence of the RF group.

The serial propagation of *Borrelia* species outside their arthropod and vertebrate hosts requires a complex growth medium such as BSK, which is based on a tissue culture medium containing deoxyribonucleotides as well as amino acids and vitamins (3, 52). Besides possessing a *thyX* gene, *B. burgdorferi* is known to have some genes that are capable of interconversion of pyrimidine and purine intermediates, such as uridine kinase (*udk*) (8), cytidylate kinase (*cmk*), nucleoside diphosphate kinase (*ndk*), thymidylate kinase (*tmk*), and thymidine kinase (*tdk*) (1, 19, 20). On the basis of the cross-species hybridization study of *B. hermsii* with a *B. burgdorferi* array, we concluded that *B. hermsii* has *cmk*, *ndk*, *tmk*, and *tdk* genes. From the results of the hybridization study, we could not detect the presence of a *udk* gene in *B. hermsii* using genomic DNA or cDNAs as probes, but evidence of a *udk* gene in the *B. hermsii* chromosome was found in its sequence (T. Schwan et al., unpublished data).

Figure 8 summarizes partial pathways for dTTP and dCTP biosynthesis, as well as our inferences about the presence of the genes for these pathways in *B. burgdorferi* and *B. hermsii*. *B. hermsii* apparently has all the genes for these pathways, while *B. burgdorferi*, lacking *nrdEF* genes and a functional *thyX* gene, does not. The LB agents *B. burgdorferi* and *B. garinii* would be unable to convert UDP to dUDP and then dUMP to dTMP. While both groups of *Borrelia* pathogens presumably could use their thymidine kinases in a salvage pathway to convert thymidine provided by their arthropod and vertebrate hosts to dTMP, only *B. hermsii*, and possibly other RF species, appears

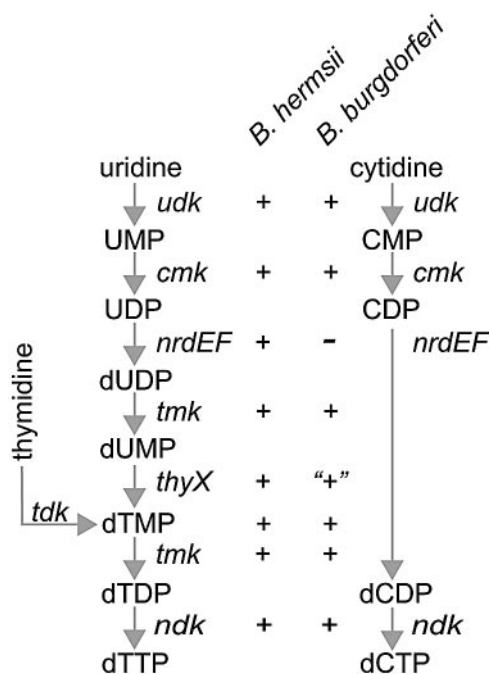


FIG. 8. Genes for pyrimidine salvage pathways for synthesis of dTTP and dCTP and their inferred presence in (+) or absence from (-) *Borrelia burgdorferi* and *B. hermsii*. The genes are uridine kinase (*udk*; EC 2.7.1.48), cytidylate kinase (*cmk*; EC 2.7.4.14), ribonucleoside diphosphate reductase (*nrdEF*; EC 1.17.4.1), thymidylate kinase (*tmk*; EC 2.7.4.9), thymidine kinase (*tdk*; EC 2.7.1.21), nucleoside diphosphate kinase (*ndk*; EC 2.7.4.6), and thymidylate synthase (*thyX*; EC 2.1.1.148). The lack of function of the *B. burgdorferi* *thyX* gene product in complementation and in vitro assays is indicated by quotation marks.

to be fully capable of synthesizing pyrimidines de novo. These supplemental capabilities in *B. hermsii* may permit greater deoxynucleoside triphosphate synthesis when only low concentrations of precursors are present in a host and, consequently, higher peak densities in the blood. Although there may be additional or alternative explanations for the differences between RF and LB spirochetes in the burdens in the blood of their hosts, the greater capability of *B. hermsii* for pyrimidine and purine biosynthesis may be sufficient.

ACKNOWLEDGMENTS

We thank Daniel Santi for providing the *E. coli* strain; Sherwood Casjens, John Aron, and Tom Schwan for sharing their results before publication; and Hung Le, April Phillips, and Hany Mattaous for assistance with experiments.

This work was supported by NIH grant AI24424 to A.G.B. H.M. is supported by research funds from INSERM (BioAvenir program), CNRS (Fundamental Microbiology Program), and the Bettencourt-Schueller Foundation. S.S. is supported by an INSERM Young Researcher grant.

REFERENCES

- Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland. 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**:133-140.
- Barbour, A. G. 2001. *Borrelia*: a diverse and ubiquitous genus of tick-borne pathogens, p. 153-173. In W. M. Scheld, W. A. Craig, and J. M. Hughes (ed.), *Emerging infection*, vol. 5. ASM Press, Washington, D.C.
- Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57**:521-525.

4. Barbour, A. G. 1999. Relapsing fever and other Borrelia infections, p. 535–546. In R. L. Guerrant, D. H. Walker, and P. F. Weller (ed.), Tropical infectious diseases—principles, pathogens, and practice, vol. 1. Churchill Livingstone, Philadelphia, Pa.
5. Barbour, A. G., and C. F. Garon. 1987. Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. *Science* **237**:409–411.
6. Barbour, A. G., A. D. Putteet-Driver, and J. Bunikis. 2005. Horizontally acquired genes for purine salvage in *Borrelia* spp. causing relapsing fever. *Infect. Immun.* **73**:6165–6168.
7. Benach, J. L., J. L. Coleman, R. A. Skinner, and E. M. Bosler. 1987. Adult Ixodes dammini on rabbits: a hypothesis for the development and transmission of *Borrelia burgdorferi*. *J. Infect. Dis.* **155**:1300–1306.
8. Boursaux-Eude, C., D. Margarita, A. M. Gilles, O. Barzu, and I. Saint Girons. 1997. *Borrelia burgdorferi* uridine kinase: an enzyme of the pyrimidine salvage pathway for endogenous use of nucleotides. *FEMS Microbiol. Lett.* **151**:257–261.
9. Brooks, C. S., P. S. Hefty, S. E. Joffill, and D. R. Akins. 2003. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. *Infect. Immun.* **71**:3371–3383.
10. Bunikis, J., and A. G. Barbour. 2002. Laboratory testing for suspected Lyme disease. *Med. Clin. N. Am.* **86**:311–340.
11. Carreras, C. W., and D. V. Santi. 1995. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64**:721–762.
12. Casjens, S. 1999. Evolution of the linear DNA replicons of the *Borrelia* spirochetes. *Curr. Opin. Microbiol.* **2**:529–534.
13. Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser. 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol. Microbiol.* **35**:490–516.
14. Climie, S. C., C. W. Carreras, and D. V. Santi. 1992. Complete replacement set of amino acids at the C-terminus of thymidylate synthase: quantitative structure-activity relationship of mutants of an enzyme. *Biochemistry* **31**:6032–6038.
15. Dynes, J. L., and R. A. Firtel. 1989. Molecular complementation of a genetic marker in *Dictyostelium* using a genomic DNA library. *Proc. Natl. Acad. Sci. USA* **86**:7966–7970.
16. Fan, H. Z., G. McClarty, and R. C. Brunham. 1991. Biochemical evidence for the existence of thymidylate synthase in the obligate intracellular parasite *Chlamydia trachomatis*. *J. Bacteriol.* **173**:6670–6677.
17. Feng, S., S. Das, T. Lam, R. A. Flavell, and E. Fikrig. 1995. A 55-kilodalton antigen encoded by a gene on a *Borrelia burgdorferi* 49-kilobase plasmid is recognized by antibodies in sera from patients with Lyme disease. *Infect. Immun.* **63**:3459–3466.
18. Ferdows, M. S., P. Serwer, G. A. Griess, S. J. Norris, and A. G. Barbour. 1996. Conversion of a linear to a circular plasmid in the relapsing fever agent *Borrelia hermsii*. *J. Bacteriol.* **178**:793–800.
19. Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, et al. 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390**:580–586.
20. Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. G. Sutton, R. Dodson, M. Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, J. C. Venter, et al. 1998. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* **281**:375–388.
21. Galtier, N., and M. Gouy. 1995. Inferring phylogenies from DNA sequences of unequal base compositions. *Proc. Natl. Acad. Sci. USA* **92**:11317–11321.
22. Giladi, M., G. Bitan-Banin, M. Mevarech, and R. Ortenberg. 2002. Genetic evidence for a novel thymidylate synthase in the halophilic archaeon *Halobacterium salinarum* and in *Campylobacter jejuni*. *FEMS Microbiol. Lett.* **216**:105–109.
23. Glockner, G., R. Lehmann, A. Romualdi, S. Pradella, U. Schulte-Spechtel, M. Schilhabel, B. Wilske, J. Suhnel, and M. Platzer. 2004. Comparative analysis of the *Borrelia garinii* genome. *Nucleic Acids Res.* **32**:6038–6046.
24. Graziani, S., Y. Xia, J. R. Gurnon, J. L. Van Etten, D. Leduc, S. Skouloubris, H. Myllykallio, and U. Liebl. 2004. Functional analysis of FAD-dependent thymidylate synthase ThyX from *Paramecium bursaria* Chlorella virus-1. *J. Biol. Chem.* **279**:54340–54347.
25. Griffin, J., C. Roshick, E. Iliffe-Lee, and G. McClarty. 2005. Catalytic mechanism of *Chlamydia trachomatis* flavin-dependent thymidylate synthase. *J. Biol. Chem.* **280**:5456–5467.
26. Jordan, A., E. Aragall, I. Gibert, and J. Barbe. 1996. Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli* *nrdEF* operons encoding one of two class I ribonucleotide reductases present in both bacteria. *Mol. Microbiol.* **19**:777–790.
27. Jordan, A., I. Gibert, and J. Barbe. 1994. Cloning and sequencing of the genes from *Salmonella typhimurium* encoding a new bacterial ribonucleotide reductase. *J. Bacteriol.* **176**:3420–3427.
28. Jordan, A., E. Pontis, M. Atta, M. Krook, I. Gibert, J. Barbe, and P. Reichard. 1994. A second class I ribonucleotide reductase in Enterobacteriaceae: characterization of the *Salmonella typhimurium* enzyme. *Proc. Natl. Acad. Sci. USA* **91**:12892–12896.
29. Leduc, D., S. Graziani, G. Lipowski, C. Marchand, P. Le Marechal, U. Liebl, and H. Myllykallio. 2004. Functional evidence for active site location of tetrameric thymidylate synthase X at the interphase of three monomers. *Proc. Natl. Acad. Sci. USA* **101**:7252–7257.
30. Leduc, D., S. Graziani, L. Meslet-Cladiere, A. Sodolescu, U. Liebl, and H. Myllykallio. 2004. Two distinct pathways for thymidylate (dTMP) synthesis in (hyper)thermophilic Bacteria and Archaea. *Biochem. Soc. Trans.* **32**:231–235.
31. Madden, T. L., R. L. Tatusov, and J. Zhang. 1996. Applications of network BLAST server. *Methods Enzymol.* **266**:131–141.
32. Margolis, N., D. Hogan, K. Tilly, and P. A. Rosa. 1994. Plasmid location of *Borrelia* purine biosynthesis gene homologs. *J. Bacteriol.* **176**:6427–6432.
33. Mathews, I. L., A. M. Deacon, J. M. Canaves, D. McMullan, S. A. Lesley, S. Agarwalla, and P. Kuhn. 2003. Functional analysis of substrate and cofactor complex structures of a thymidylate synthase-complementing protein. *Structure (Cambridge)* **11**:677–690.
34. Myllykallio, H., D. Leduc, J. Filee, and U. Liebl. 2003. Life without dihydrofolate reductase FoaA. *Trends Microbiol.* **11**:220–223.
35. Myllykallio, H., G. Lipowski, D. Leduc, J. Filee, P. Forterre, and U. Liebl. 2002. An alternative flavin-dependent mechanism for thymidylate synthesis. *Science* **297**:105–107.
36. Nascimento, A. L., A. I. Ko, E. A. Martins, C. B. Monteiro-Vitorello, P. L. Ho, D. A. Haake, S. Verjovski-Almeida, R. A. Hartskeerl, M. V. Marques, M. C. Oliveira, C. F. Menck, L. C. Leite, H. Carrer, L. L. Coutinho, W. M. Degraive, O. A. Dellagostin, H. El-Dorry, E. S. Ferro, M. I. Ferro, L. R. Furlan, M. Gamberini, E. A. Gigliotti, A. Goes-Neto, G. H. Goldman, M. H. Goldman, R. Harakava, S. M. Jeronimo, I. L. Junqueira-de-Azevedo, E. T. Kimura, E. E. Kuramae, E. G. Lemos, M. V. Lemos, C. L. Marino, L. R. Nunes, R. C. de Oliveira, G. G. Pereira, M. S. Reis, A. Schriefer, W. J. Siqueira, P. Sommer, S. M. Tsai, A. J. Simpson, J. A. Ferro, L. E. Camargo, J. P. Kitajima, J. C. Setubal, and M. A. Van Sluys. 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* **186**:2164–2172.
37. Ojaimi, C., C. Brooks, S. Casjens, P. Rosa, A. Elias, A. Barbour, A. Jasin-skas, J. Benach, L. Katona, J. Radolf, M. Caimano, J. Skare, K. Swingle, D. Akins, and I. Schwartz. 2003. Profiling of temperature-induced changes in *Borrelia burgdorferi* gene expression by using whole genome arrays. *Infect. Immun.* **71**:1689–1705.
38. Paster, B. J., and F. E. Dewhirst. 2000. Phylogenetic foundation of spirochetes. *J. Mol. Microbiol. Biotechnol.* **2**:341–344.
39. Pennington, P. M., C. D. Allred, C. S. West, R. Alvarez, and A. G. Barbour. 1997. Arthritis severity and spirochete burden are determined by serotype in the *Borrelia turicatae*-mouse model of Lyme disease. *Infect. Immun.* **65**:285–292.
40. Pennington, P. M., D. Cadavid, and A. G. Barbour. 1999. Characterization of VspB of *Borrelia turicatae*, a major outer membrane protein expressed in blood and tissues of mice. *Infect. Immun.* **67**:4637–4645.
41. Plasterk, R. H., M. I. Simon, and A. G. Barbour. 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. *Nature* **318**:257–263.
42. Putteet-Driver, A. D., J. Zhong, and A. G. Barbour. 2004. Transgenic expression of RecA of the spirochetes *Borrelia burgdorferi* and *Borrelia hermsii* in *Escherichia coli* revealed differences in DNA repair and recombination phenotypes. *J. Bacteriol.* **186**:2266–2274.
43. Reichard, P. 1993. From RNA to DNA, why so many ribonucleotide reductases? *Science* **260**:1773–1777.
44. Ren, S. X., G. Fu, X. G. Jiang, R. Zeng, Y. G. Miao, H. Xu, Y. X. Zhang, H. Xiong, G. Lu, L. F. Lu, H. Q. Jiang, J. Jia, Y. F. Tu, J. X. Jiang, W. Y. Gu, Y. Q. Zhang, Z. Cai, H. H. Sheng, H. F. Yin, Y. Zhang, G. F. Zhu, M. Wan, H. L. Huang, Z. Qian, S. Y. Wang, W. Ma, Z. J. Yao, Y. Shen, B. Q. Qiang, Q. C. Xia, X. K. Guo, A. Danchin, I. Saint Girons, R. L. Somerville, Y. M. Wen, M. H. Shi, Z. Chen, J. G. Xu, and G. P. Zhao. 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* **422**:888–893.
45. Restrepo, B. I., T. Kitten, C. J. Carter, D. Infante, and A. G. Barbour. 1992. Subtlety of expression regions of *Borrelia hermsii* linear plasmids are highly polymorphic. *Mol. Microbiol.* **6**:3299–3311.
46. Ribeiro, J. M., T. N. Mather, J. Piesman, and A. Spielman. 1987. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae). *J. Med. Entomol.* **24**:201–205.
47. Sadziene, A., A. G. Barbour, P. A. Rosa, and D. D. Thomas. 1993. An OspB mutant of *Borrelia burgdorferi* has reduced invasiveness in vitro and reduced infectivity in vivo. *Infect. Immun.* **61**:3590–3596.
48. Sadziene, A., D. D. Thomas, and A. G. Barbour. 1995. *Borrelia burgdorferi*

- mutant lacking Osp: biological and immunological characterization. *Infect. Immun.* **63**:1573–1580.
49. **Schwan, T. G., J. M. Battisti, S. F. Porcella, S. J. Raffel, M. E. Schrupf, E. R. Fischer, J. A. Carroll, P. E. Stewart, P. Rosa, and G. A. Somerville.** 2003. Glycerol-3-phosphate acquisition in spirochetes: distribution and biological activity of glycerophosphodiester phosphodiesterase (GlpQ) among *Borrelia* species. *J. Bacteriol.* **185**:1346–1356.
 50. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. USA* **92**:2909–2913.
 51. **Seshadri, R., G. S. Myers, H. Tettelin, J. A. Eisen, J. F. Heidelberg, R. J. Dodson, T. M. Davidsen, R. T. DeBoy, D. E. Fouts, D. H. Haft, J. Selengut, Q. Ren, L. M. Brinkac, R. Madupu, J. Kolonay, S. A. Durkin, S. C. Daugherty, J. Shetty, A. Shvartsbeyn, E. Gebregeorgis, K. Geer, G. Tsegaye, J. Malek, B. Ayodeji, S. Shatsman, M. P. McLeod, D. Smajs, J. K. Howell, S. Pal, A. Amin, P. Vashisth, T. Z. McNeill, Q. Xiang, E. Sodergren, E. Baca, G. M. Weinstock, S. J. Norris, C. M. Fraser, and I. T. Paulsen.** 2004. Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. *Proc. Natl. Acad. Sci. USA* **101**:5646–5651.
 52. **Stoenner, H. G., T. Dodd, and C. Larsen.** 1982. Antigenic variation of *Borrelia hermsii*. *J. Exp. Med.* **156**:1297–1311.
 53. **Torrents, E., I. Roca, and I. Gibert.** 2003. *Corynebacterium ammoniagenes* class Ib ribonucleotide reductase: transcriptional regulation of an atypical genomic organization in the nrd cluster. *Microbiology* **149**:1011–1020.
 54. **Zhong, J., and A. G. Barbour.** 2004. Cross-species hybridization of a *Borrelia burgdorferi* DNA array reveals infection- and culture-associated genes of the unsequenced genome of the relapsing fever agent *Borrelia hermsii*. *Mol. Microbiol.* **51**:729–748.