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Regulation of Expression of the Paralogous Mlp Family in *Borrelia burgdorferi*

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The Mlp (multicopy lipoproteins) family is one of many paralogous protein families in *Borrelia burgdorferi*. To examine the extent to which the 10 members of the Mlp family in *B. burgdorferi* strain 297 might be differentially regulated, antibodies specific for each of the Mlps were developed and used to analyze the protein expression profiles of individual Mlps when *B. burgdorferi* replicated under various cultivation conditions. All of the Mlps were upregulated coordinately when *B. burgdorferi* was cultivated at either elevated temperature, reduced culture pH, or increased spirochete cell density. Inasmuch as the expression of OspC is influenced by a novel RpoN-RpoS regulatory pathway, similar induction patterns for OspC and the Mlp paralogs prompted an assessment of whether the RpoN-RpoS pathway also was involved in Mlp expression. In contrast to wild-type *B. burgdorferi*, both RpoN- and RpoS-deficient mutants were unable to upregulate OspC or the Mlp paralogs when grown at lower pH (6.8), indicating that pH-mediated regulation of OspC and Mlp paralogs is dependent on the RpoN-RpoS pathway. However, when RpoN- or RpoS-deficient mutants were shifted from 23°C to 37°C or were cultivated to higher spirochete densities, some induction of the Mlps still occurred, whereas OspC expression was abolished. The combined findings suggest that the Mlp paralogs are coordinately regulated as a family and have an expression profile similar, but not identical, to that of OspC. Although Mlp expression as a family is influenced by the RpoN-RpoS regulatory pathway, there exists at least one additional layer of gene regulation, yet to be elucidated, contributing to Mlp expression in *B. burgdorferi*.

It is now widely accepted that *Borrelia burgdorferi* differentially regulates a number of its genes as the spirochete adapts to its diverse environmental niches in *Ixodes* ticks and mammalian hosts (1, 17, 18, 27). A paradigm of this differential gene expression process is the reciprocal downregulation of outer surface (lipo)protein A (OspA) and upregulation of OspC by spirochetes within tick midguts during tick engorgement (7, 19, 21, 31, 32). Additional studies have examined the upregulation or downregulation of OspA and OspC as well as other genes of *B. burgdorferi* when the spirochete is grown under various in vitro culture conditions; in particular, three parameters (temperature, pH, and increased spirochete cell density) have emerged as important environmental cues that likely influence gene expression profiles in various stages of the enzootic life cycle of *B. burgdorferi* (4, 5, 15, 25, 34).

Among those genes or families of genes that appear to be differentially regulated are the Mlps (multicopy lipoproteins), one of the many paralogous families (Gbb Family_113) in *B. burgdorferi* (24, 35) (www.tigr.org/tigr-scripts/CMR2/ParalogousList.spl?db = gbb&alignid = 20325). In *B. burgdorferi* strains 297 and B31, there are at least 10 copies of the *mlp* genes, distributed among each of the cp32/cp18 circular plasmids (3, 6, 24, 35). Based on their inferred molecular weights, sequence similarities, and reactivity with polyclonal antisera, the Mlps have been divided into two antigenic classes (24). Antigenic class I Mlps have masses of 18 to 23 kDa, whereas antigenic class II Mlps possess shorter C termini and range from 13 to 15 kDa. Polyclonal antiserum raised against an Mlp from one antigenic class is cross-reactive with other Mlps within the same class, but not with Mlps of the other antigenic class. This is a reflection of the fact that Mlps within the same class are 60 to 80% identical, whereas there is only about 30% identity between the two classes (3, 24). In strain 297, five Mlps fall within antigenic class I (Mlp1, Mlp4, Mlp5, Mlp7B, and Mlp9), and the remaining five are members of antigenic class II (Mlp2, Mlp3, Mlp7A, Mlp8, and Mlp10). In contrast, virtually all of the Mlps identified in *B. burgdorferi* strain B31 are of the antigenic class II type, with the exceptions being BBQ35 and the newly identified MlpB (3, 6, 23).

Limited studies have attempted to infer a role for the Mlps in the pathogenesis of Lyme borreliosis. Theisen (33) reported that expression of an *mlp* homolog conferred Congo Red as well as hemin binding to a recombinant *Escherichia coli* strain. Gilmore et al. (12) reported that *mlpA* was transcriptionally upregulated in *B. burgdorferi* strain B31 during tick engorgement. It also has been reported that the cp32 plasmids (and their derivative cp18 counterparts) encoding the *mlp* genes actually are bacteriophage genomes or remnants of such genomes (9, 10), but the biological ramifications of this contention remain poorly understood. Despite these provocative findings, the physiological function(s) of the Mlps remains largely unknown.

Work in recent years is beginning to clarify the molecular mechanisms of differential gene expression in *B. burgdorferi*. Yang et al. (34) showed that the global regulator RpoS (σ^S) is coexpressed with OspC and other proteins (e.g., DbpA, OspF,

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TABLE 1. Primers used in this study

Primer name	Sequence	Purpose
*mlp1-Bgl*II-Ep-5′	GGAAGATCTGACTGCAAAAATAATAATAATAATAAA	For epitope cloning of *mlp1*
*mlp1-Hin*dIII-Ep-3′	CACAAGCTTTTGCTCTATTAAGTTGCCAC	For epitope cloning of *mlp1*
mlp2 Bgl II-Ep-5′	GGAAGATCTGCGGGTTTCTTTAGCGGC	For epitope cloning of *mlp2*
*mlp2*HindIII-Ep-3′	CACAAGCTTGGGCCCGTTGCAGGTACT	For epitope cloning of *mlp2*
*mlp3-Bgl*II-Ep-5′	GGAAGATCTCAAGGAGCTCTTAGTGGG	For epitope cloning of *mlp3*
*mlp3-Hin*dIII-Ep-3′	CACAAGCTTGGAACCACCATTGCCGCAGGT	For epitope cloning of *mlp3*
*mlp5-Bgl*II-Ep-5′	GGAAGATCTGATTGTAAAGCTAATACTCCT	For epitope cloning of *mlp5*
*mlp5-Hin*dIII-Ep-3′	CACAAGCTTTTCGTTAGTACCTTTTCCATA	For epitope cloning of *mlp5*
*mlp7A-Bgl*II-Ep-5′	GGAAGATCTCAAGTAGTTAAAGGGGCCC	For epitope cloning of *mlp7A*
*mlp7A-Hin*dIII-Ep-3′	CACAAGCTTATTATTGCATGTAGTAGTTGC	For epitope cloning of *mlp7A*
mlp-ATG-G-5′	ATGAAAATYATCAAYATATTATTTTG	RT-PCR, 5′ primers for all *mlp*s
mlp1-SO-SHORT	TGCTCTATTAAGTTGCCACTACC	RT-PCR, 3′ primers for *mlp1*
mlp2-SO-SHORT	GCTAAAGAAACCCGCAACTGTG	RT-PCR, 3′ primers for *mlp2*
mlp3-SO-2-SHORT	GGAACCACCATTGCCGCAGG	RT-PCR, 3′ primers for *mlp3*
mlp4-SO-SHORT	CATTTTGTGCTTTTGATTTTAAGAAG	RT-PCR, 3′ primers for *mlp4*
mlp5-SO-SHORT	TTTTTACAATCTTGTGGAGTATTAGC	RT-PCR, 3′ primers for *mlp5*
mlp7A-SO-SHORT	GCCGTGAAAGTATCTATACCGC	RT-PCR, 3′ primers for *mlp7A*
mlp7B-SO-SHORT	CTGCTTTCTGCTTGCTGGACAC	RT-PCR, 3′ primers for *mlp7B*
mlp8-SO-410	CTGTAAATTTATCTATGTCCCCTTC	RT-PCR, 3′ primers for *mlp8*
mlp9-SO-SHORT	CATGTTTATTATCATTACATTTGTTTG	RT-PCR, 3′ primers for *mlp9*
mlp10-SO-2	TTAAGAGATTCTTTAACTAAAGTTTTGTATGTGG	RT-PCR, 3′ primers for *mlp10*

and Mlp8) comprising an expression group denoted as group I proteins. The group I proteins tend to be upregulated by elevated temperature, lower culture pH (6.8), and increased spirochete cell density (34). That RpoS is coexpressed with the other group I proteins inferred that RpoS is a potential regulator governing the differential expression of one or more group I proteins. Subsequently, Hübner et al. (14) demonstrated that inactivation of the *rpoS* gene in *B. burgdorferi* abolished the expression of both *ospC* and *dbpA*, providing the first direct genetic evidence that RpoS controls production of at least some of the group I proteins. Moreover, the expression of RpoS, in turn, is controlled by another alternative sigma factor, RpoN (σ^N or σ^{54}) (14). These combined findings have prompted the hypothesis that a novel RpoN-RpoS regulatory pathway exists which governs not only the expression of *ospC* and *dbpA* but also likely other group I lipoprotein genes in *B. burgdorferi* (14).

Previous studies in our laboratory indicated that Mlp8 also had an expression profile akin to the group I proteins; that is, *mlp8* expression was upregulated by elevated temperature, lower culture pH (6.8), and increased cell density (34). However, thus far a comprehensive analysis of the expression pattern for all 10 of the Mlps (at the individual protein level) has been hampered by the antigenic relatedness (antibody cross-reactivity) of the Mlps (24, 35). In this report, antibodies specific for each of the Mlps were developed and used to analyze the protein expression profiles of individual Mlps when wild-type *B. burgdorferi* or selected mutants were cultivated under various environmental conditions. The combined efforts have allowed a more complete elucidation of the overall pattern of Mlp expression in *B. burgdorferi* and its complex relationship to the novel RpoN-RpoS regulatory pathway (14).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Low-passage, virulent *B. burgdorferi* strain 297 was described previously (20). According to PCR analysis (8), strain 297 contains 21 plasmids, 9 of which are the cp32/cp18 derivatives encoding the various *mlp* genes. Borreliae were cultivated in vitro in BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) (22) under various environmental conditions. Spirochetes stored at −70°C from not more than three prior serial passages were resurrected into BSK-H medium. The resurrected cultures were incubated at 34°C under an atmosphere of 1% CO_2 and were grown to a final concentration of less than 10^7 cells per ml. To adapt *B. burgdorferi* to culture conditions at 23°C, spirochetes first were diluted to about 10^6 cells per ml and were incubated at 23°C for 1 week. These 23°C-adapted spirochetes then were used for subsequent inoculation into fresh BSK-H cultures adjusted to various pH conditions. HCl or NaOH was used to adjust the pH of BSK-H medium, and the medium was then filter sterilized before inoculation. Cultures were inoculated at a final concentration of 10^3 spirochetes per ml and then incubated at either 23 or 37°C. Cultures were allowed to grow to various cell densities, as determined by counting spirochetes via dark-field microscopy. *E. coli* strains XL1-Blue (Stratagene, La Jolla, Calif.) and TOP 10 (Invitrogen, Carlsbad, Calif.) were used as a cloning host, and strain BL21 was used as the host for protein purification.

Antibodies and antisera. Rat polyclonal antisera directed against fusion proteins were prepared according to a previously published protocol (16). Polyclonal antisera against OspC, Mlp8, and Mlp10 and monoclonal antibodies 14D2-27, 8H3-33, and 17C3-73, directed against OspA, FlaB, and Mlp9, respectively, were described previously (35). Monoclonal antibodies 1H5-65 and 10F11-76, directed against Mlp4 and Mlp7B, respectively, were generated by immunizing BALB/c mice with full-length fusion proteins according to previously published protocols (1, 30).

Epitope-specific (monospecific) polyclonal antisera directed against either Mlp1, Mlp2, Mlp3, Mlp5, or Mlp7A were generated as described previously (35). First, for each *mlp* gene, a DNA sequence was identified that theoretically encoded an epitopic region (about 20 amino acids long) unique to each Mlp. The selected epitope for each Mlp was chosen such that it contained less than five consecutive amino acids in common with any other Mlp. The selected DNA sequence was first amplified by PCR using oligonucleotide primers listed in Table 1. Each amplicon, flanked by a restriction enzyme site, was then cloned into the appropriate polylinker region of pQE40 (Qiagen, Inc.). The resulting construct encoded a six-His tag and the peptide specific for each Mlp fused to the murine dihydrofolate reductase protein. Dihydrofolate reductase has been shown to be poorly immunogenic in mice and rats and acts to protect the attached oligopeptide from proteolysis (The QIAexpressionist; Qiagen, Inc.). Fusion proteins were then purified by affinity chromatography on a nickel-nitrilotriacetic acid matrix according to protocols from the manufacturer (Qiagen, Inc.). Purified recombinant fusion proteins were subsequently used for generating rat polyclonal antisera as previously described (16).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as previously

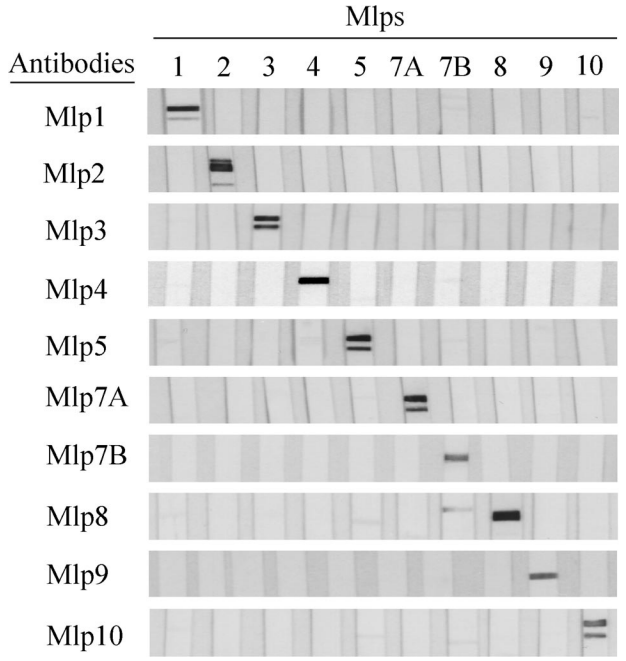

FIG. 1. Specificities of antibody reagents directed against each of the 10 Mlps. Approximately 1 μg of each recombinant Mlp protein (denoted at top of figure) was subjected to SDS-PAGE and immunoblotting. Immunoblot analyses were performed using either monoclonal antibodies (for Mlp4, Mlp7B, and Mlp9; hybridoma supernatants diluted 1:10; labeled at left of figure) or epitope-specific rat antisera (other seven Mlps; 1:500 dilutions).

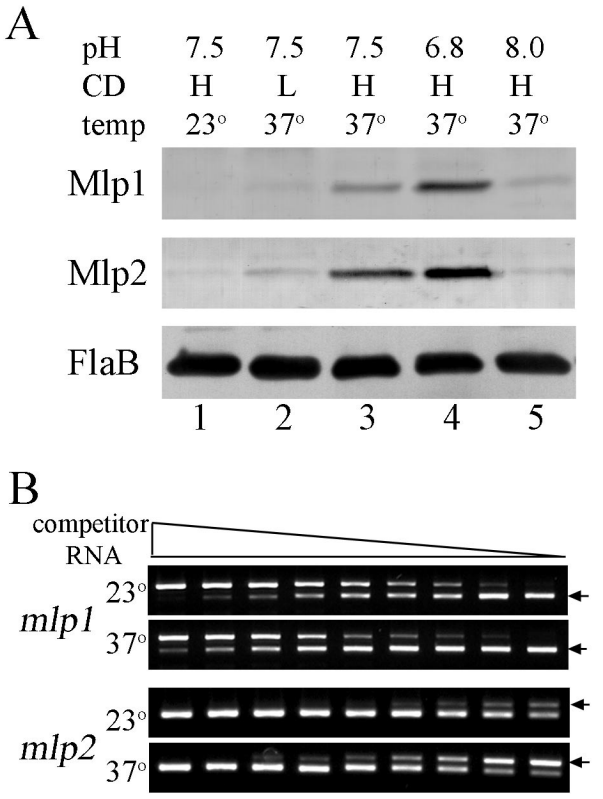

FIG. 2. Environmental factors influencing Mlp1 and Mlp2 expression in *B. burgdorferi*. Low-passage spirochetes were cultivated in BSK-H medium under various temperatures (23°C or 37°C), pH levels (6.8, 7.5, or 8.0), or cell densities (CD) (3×10^6 per ml [L] or 3×10^7 per ml [H]). (A) Whole-cell lysates of 5×10^7 spirochetes were subjected to immunoblot assays using the antibody reagents shown in Fig. 1; immunoblotting for FlaB served as an internal loading control. (B) qcRT-PCR analysis of *mlp1* and *mlp2* gene expression. Total RNA was isolated from spirochetes grown at either temperature and used in qcRT-PCRs. Arrows denote amplicons emanating from borrelial *mlp* mRNA. Twofold serial dilutions of in vitro-synthesized competitor RNA (from left to right on figure) were added in each experiment for comparative analysis. PCR products were resolved by agarose gel electrophoresis.

described (35). To achieve higher sensitivity for the detection of σ^s, immunoblotting was carried out using the Western Lightning Chemiluminescence Reagent Plus system (Perkin-Elmer Life Sciences, Boston, Mass.) according to instructions supplied by the manufacturer. For quantitation of individual protein bands detected by immunoblotting, densitometry was performed using a Kodak Digital Science Image Station with ID Image Analysis software.

Quantitative competitive reverse transcription-PCR (qcRT-PCR). Total RNA was isolated from spirochetes using a total RNA isolation kit (Biotecx Laboratory, Houston, Tex.). RNA (10 μg) was then incubated with 10 U of RQ1 DNase I (Promega Corp., Madison, Wis.) at 37°C for 3 h, followed by extraction once with phenol-chloroform and precipitation with cold ethanol. Precipitated RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated water. Occasionally, DNase treatment was repeated once if DNA contamination was detected by PCR. RNA was subjected to agarose gel electrophoresis in formamide-containing gels to assess RNA quality, and quantitation was performed by UV spectrophotometry.

To acquire in vitro-synthesized RNA to be used as competitor in individual qcRT-PCRs (for each of the native *mlp* transcripts), a series of plasmids first were constructed that contained altered *mlp* genes; alterations constituted either insertions or deletions relative to the native *mlp* genes. First, PCRs were performed to generate DNA fragments that contained highly conserved 5′ regions for all of the *mlp* genes. The primers used for PCR were TCCCCGCGGATG AAAATCATCAATATATTA and CGC**GGATCC**CGCAGCTGTTTAATACGAG (bold indicates a *Bam*HI site), and the template was plasmid DNA encoding the *mlp7B* gene. The resultant 75-bp PCR fragment was then cloned into pCR-Script (Stratagene) at the *Srf*I site, downstream of the T3 promoter. Then, a nonrelated (irrelevant) 220-bp *Sal*3A1 fragment derived from *E. coli* DNA was inserted into the *Bam*HI site located at the 3′ end of the conserved *mlp* fragment. The resulting plasmid DNA was digested with *Pvu*II and *Bam*HI; the 330-bp *Pvu*II-*Bam*HI fragment was then purified and cloned into the *Pvu*II and *Bam*HI sites of each of the pGEX-4T-2-Mlp plasmids as previously described (24, 35). pGEX-4T-2-Mlp plasmids are a series of expression vectors for Mlp recombinant proteins that encode glutathione *S*-transferase fused with individual mature or C-terminal (truncated) Mlp peptides (24, 35). The final plasmids contained each respective DNA fragment with the T3 promoter, followed by altered *mlp* genes having either a 141- to 153-bp deletion (for *mlp2* and *mlp4*) or a 47- to 149-bp insertion (for all other *mlp* genes) relative to their native genes. The size differences between resultant mutant transcripts and corresponding native transcripts were readily discernible on agarose gels. Each plasmid DNA was then linearized by digestion with *Eco*RI and subjected to in vitro synthesis of RNA using a MEGAscript kit (Ambion, Austin, Tex.). The quality of in vitro-transcribed RNA was evaluated by gel electrophoresis, and the concentration was determined by UV spectrometry.

qcRT-PCR assays (28, 36) were performed using the one-step RT-PCR kit (Titan One Tube RT-PCR system; Boehringer, Mannheim, Germany). Conditions for the RT-PCRs were as recommended by the manufacturer; in a 20-μl buffered reaction mixture were 40 ng of bacterial RNA, a 0.4 μM concentration of each of the oligonucleotide primers, 5 mM dithiothreitol, a 0.2 mM concentration of each deoxynucleoside triphosphate, 5 U of RNase inhibitor, and 1 μl of enzyme mixture. In each experiment, a series of tubes contained twofold dilutions of each respective competitor RNA (the starting concentration of each dilution series ranged from 2 to 225 pg). The amount of native *mlp* RNA present in each reaction tube was estimated by comparing the UV intensities of PCR bands amplified from the native mRNA species and that of its competitor. Results were expressed as the fold change of RNAs obtained from *B. burgdorferi* cells cultivated under different environmental conditions.

TABLE 2. Environmental factors influencing Mlp expression in *B. burgdorferi*

Class and name	Location	Protein level[a] induced by condition							RNA level[a]	
		Temp[b]		pH[c]			Cell density[d]			
		23°C	37°C	6.8	7.5	8.0	3×10^6	3×10^7	23°C	37°C
Class I										
Mlp1	cp32-4	1.0	2.5	4.7	2.1	1.7	1.0	2.5	1.0	3.5
Mlp4	cp32-?[e]	1.0	1.8	2.1	1.8	1.2	0.8	1.8	1.0	2.5
Mlp5	cp32-5	1.0	2.2	2.7	2.2	1.8	1.3	2.2	1.0	3.0
Mlp7B	cp32-1	1.0	1.1	1.1	1.1	1.0	1.1	1.1	1.0	3.0
Mlp9	cp18-2	1.0	1.3	1.2	1.3	1.3	1.0	1.6	1.0	2.0
Class II										
Mlp2	cp32-?[e]	1.0	2.4	5.1	2.4	1.5	1.1	2.4	1.0	5.5
Mlp3	cp18-1	1.0	3.7	3.2	3.7	1.0	1.8	3.7	1.0	6.0
Mlp7A	cp32-1	1.0	8.0	10.8	11.0	2.3	2.3	11.0	1.0	6.0
Mlp8	cp32-3	1.0	16.5	19.9	16.5	1.0	2.8	16.5	1.0	8.0
Mlp10	cp32-2	1.0	8.1	13.2	9.1	1.0	2.2	9.1	1.0	4.0

[a] Values are presented as fold increase indexed to levels (1.0) considered to be baseline.
[b] Cultures were at pH 7.5, harvested at 3×10^7 cells/ml.
[c] Cultures were incubated at 37°C, harvested at 3×10^7 cells/ml.
[d] Spirochetes per milliliter under culture conditions of pH 7.5, 37°C.
[e] "?" denotes uncertainty regarding plasmids encoding Mlp2 and Mlp4. See reference 3 for strain 297 plasmid assignments.

RESULTS

Influence of in vitro growth parameters on Mlp expression in *B. burgdorferi*. Previously, our laboratory demonstrated that the expression of Mlp8 was influenced by pH, spirochete cell density, and temperature during the growth of *B. burgdorferi* in vitro (34). However, a comprehensive analysis of the expression of the other Mlps under similar *B. burgdorferi* growth parameters has been hampered by the lack of specific antibody reagents for each Mlp. Two strategies were used to address this obstacle. First, monoclonal antibodies were developed in this study against Mlp4 and Mlp7B, for use in conjunction with a monoclonal antibody previously generated for Mlp9 (35). Second, along with "epitope-specific" antisera generated previously for Mlp8 and Mlp10 (35), epitope-specific antisera were raised against each of the remaining Mlps (Mlp1, Mlp2, Mlp3, Mlp5, and Mlp7A) for which specific antibody probes had been lacking. As shown by immunoblotting, each of the nine monoclonal antibody preparations or monospecific polyclonal antisera reacted only with its cognate recombinant protein (Fig. 1). In the case of antiserum directed against Mlp8, only very minor cross-reactivity with Mlp7B sometimes was detectable (Fig. 1), but not to an extent that hampered interpretations from subsequent immunoblotting experiments. Some of the recombinant Mlps migrated on SDS-PAGE as doublet bands, with the faster-migrating band (lower mass than the predicted mass of the full-length fusion protein) most likely the result of endogenous protease cleavage occurring within *E. coli* cell lysates (Fig. 1).

The various antibody reagents were used to examine the expression of all 10 of the Mlps individually when *B. burgdorferi* was cultivated under various in vitro growth conditions. First, *B. burgdorferi* cells were inoculated in BSK-H medium adjusted to various pH levels (pH 6.8, 7.6, or 8.0) and then was cultivated at either 23 or 37°C. Spirochetes were allowed to achieve cell densities of either 3×10^6 per ml or 3×10^7 per ml. As shown in Fig. 2A, Mlp1 and Mlp2 were upregulated when *B. burgdorferi* was cultivated at elevated temperature (compare lanes 1 and 3), reduced pH (compare lanes 3, 4, and 5), or higher cell density (compare lanes 2 and 3). These results were representative of what occurred for all 10 of the Mlps (data summarized in Table 2). In general, induction of class II Mlps was higher than for class I Mlps (Table 2). Although increased protein levels ranged from 2- to 20-fold during induction, the highest levels of Mlps were expressed by spirochetes grown at pH 6.8 (37°C) to a higher cell density (Table 2).

To examine whether elevated protein levels correlated with increased mRNA levels, qcRT-PCR was performed (28, 36). There were at least two- to eightfold-higher levels of mRNA from each *mlp* gene detected by qcRT-PCR when spirochetes were cultivated at 37°C as opposed to 23°C (Fig. 2B for *mlp1* and *mlp2*; Table 2 for all *mlp* genes). In general, the highest levels of mRNA correlated with the greatest degrees of protein induction (e.g., Mlp7A and Mlp8) (Table 2).

The RpoN-RpoS pathway in *B. burgdorferi* influences temperature-dependent regulation of the Mlps. Recently, our investigators reported that two alternative sigma factors, RpoN and RpoS, act in concert and are essential for the temperature-dependent regulation of at least two major *B. burgdorferi* lipoproteins, OspC and DbpA (14). Given that the Mlps also are regulated by temperature, we examined whether their expression also is controlled by the novel RpoN-RpoS regulatory pathway in *B. burgdorferi* (14). As previously reported (14), inactivation of either *rpoN* or *rpoS* abolished the expression of OspC (Fig. 3A, lanes 4 and 8). Complementing the *rpoN* mutant with either wild-type *rpoN* or a constitutively expressed *rpoS* gene restored the expression of both OspC and RpoS (Fig. 3A, lanes 6 and 7), as expected given that the expression of OspC is dependent on RpoS which, in turn, is governed by RpoN. Regarding an examination of the potential influence of the RpoN-RpoS pathway on Mlp expression, monospecific polyclonal antisera directed against each individual Mlp for this purpose

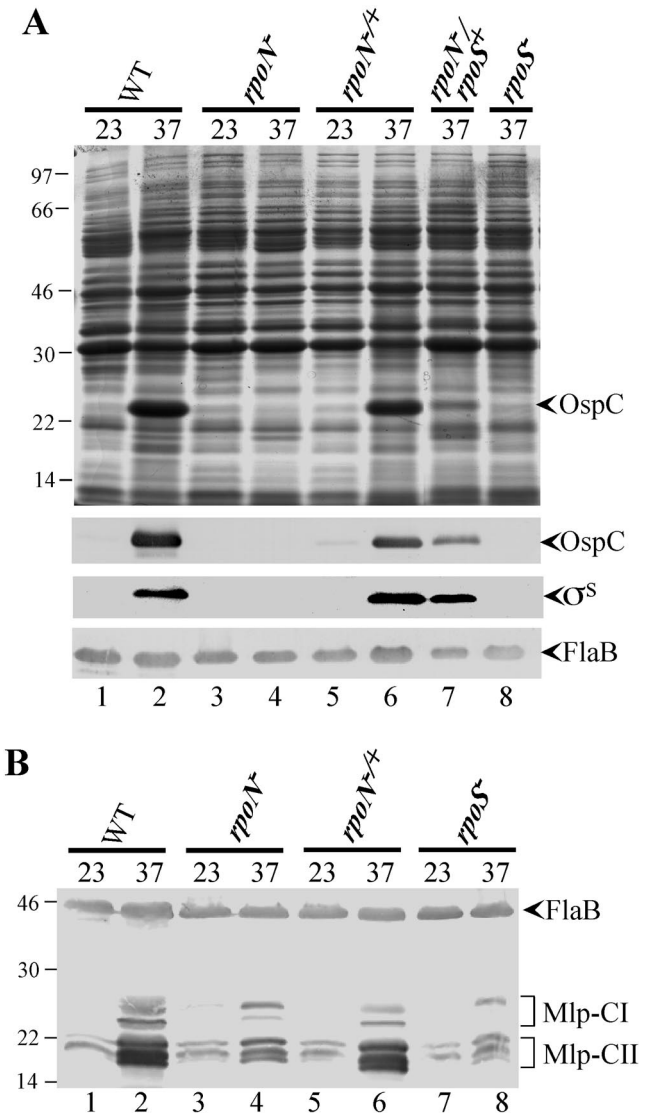

FIG. 3. Influence of RpoN or RpoS on temperature-dependent regulation of Mlp and OspC expression in *B. burgdorferi*. Wild-type and mutant strains of low-passage *B. burgdorferi* were cultivated at either at 23°C or 37°C in BSK-H medium (pH 7.5). Spirochetes were harvested at a cell density of 3×10^7 per ml and then were subjected to SDS-PAGE and immunoblotting. Each gel lane for SDS-PAGE was loaded with approximately 5×10^7 spirochetes. WT, wild-type *B. burgdorferi* strain 297; *rpoN*$^-$, RpoN-deficient mutant; *rpoN*$^{-/+}$, RpoN-deficient mutant complemented with a wild-type copy of *rpoN*; *rpoN*$^-$/*rpoS*$^+$, RpoN-deficient mutant complemented with a constitutively expressed wild-type copy of *rpoS*; *rpoS*$^-$, RpoS-deficient mutant. Numbers at the left of the figure denote molecular mass markers in kilodaltons. (A) Coomassie-stained SDS-PAGE (top) and immunoblot analyses (bottom three panels) performed by using antisera directed against OspC, σ^s (RpoS), or FlaB (loading control) (positions denoted by arrowheads). For the detection of low-abundance σ^s, 2×10^8 spirochetes per gel lane were used, and immunoblotting was performed using chemiluminescence. (B) Immunoblot analysis performed by using a mixture of antibody reagents (see Fig. 1) directed against each of the Mlps. Mlp-CI, gel position where antigenic class I Mlps migrate; Mlp-CII, gel position where antigenic class II Mlps migrate.

were pooled and subsequently used in immunoblotting analyses to track expression of the Mlps as a family. Differentiation of the two Mlp antigenic classes (class I and class II) on immunoblots was readily discerned by antigenic class-based differences in molecular masses (e.g., 19 to 22 kDa for antigenic class I and 13 to 15 kDa for class II). Consistent with results shown in Table 2, expression of both antigenic classes of Mlps in the wild-type strain was elevated when the culture temperature was shifted from 23 to 37°C (Fig. 3B, lanes 1 and 2). However, when RpoN- or RpoS-deficient mutants were shifted from 23 to 37°C, although greatly reduced, in contrast to OspC, some induction of the Mlps still occurred (Fig. 3B, lanes 4 and 8). Genetic complementation of the *rpoN*-disrupted mutant with a wild-type copy of *rpoN* restored expression of the Mlps in temperature-shifted spirochetes to nearly wild-type levels (Fig. 3B, lane 6).

The RpoN-RpoS pathway influences pH-dependent regulation of the Mlps. Because pH also is a major parameter influencing the expression of Mlps (Table 2) and other genes in *B. burgdorferi* (4, 5, 26, 34), we further sought to determine whether pH-dependent regulation of the Mlps was influenced by inactivation of either *rpoN* or *rpoS*. pH also has been found to influence the expression of OspC in that lower pH tends to upregulate its expression (4, 5, 34). Of note, OspC expression is abolished in *rpoN*- or *rpoS*-deficient mutants when they are cultivated at 37°C in BSK-H medium at routine pH (7.5) (14). However, there remained the possibility that cultivation of *B. burgdorferi* at lower pH (which further enhances OspC expression) may overcome such an expression defect. To first examine this possibility, wild-type *B. burgdorferi* and the *rpoN* and *rpoS* mutants were cultivated at 37°C at a low (6.8) or high (8.0) pH. As expected, in wild-type 297, the lower culture pH resulted in an increase in OspC expression (Fig. 4A, lanes 1 and 2). However, inactivation of either *rpoN* or *rpoS* completely abolished OspC expression among spirochetes cultivated at either pH (Fig. 4A, lanes 4 and 8). Complementation of the *rpoN* mutant with wild-type *rpoN* restored the expression of OspC (Fig. 4A, lane 5), as expected, and a shuttle vector containing a constitutively expressed *rpoS* gene also restored the expression of OspC to near wild-type levels when spirochetes were cultivated at pH 6.8 (lane 7). The combined results suggest that a lower pH, which enhances the expression of OspC in wild-type cultures of *B. burgdorferi* (4, 5, 34), was not able to overcome the loss of RpoN or RpoS function in the respective mutants.

With respect to Mlp expression under similar circumstances, a significant increase in the expression of the Mlps was observed among wild-type spirochetes cultivated at pH 6.8 (Fig. 4B, lanes 1 and 2), consistent with data presented in Table 2. In contrast, increased expression of the Mlps above some basal level was not observed among the *rpoN* or *rpoS* mutants cultivated at pH 6.8 (lanes 3 and 7). When the *rpoN* mutant complemented with a wild-type *rpoN* gene was cultivated at pH 6.8, the Mlps again were upregulated (lane 5), as in wild-type 297 (lane 1).

The RpoN-RpoS pathway affects cell density-dependent regulation of the Mlps. Given the results shown in Table 2, it also was of interest to examine the relationship between the RpoN-RpoS pathway and cell density-dependent regulation of the

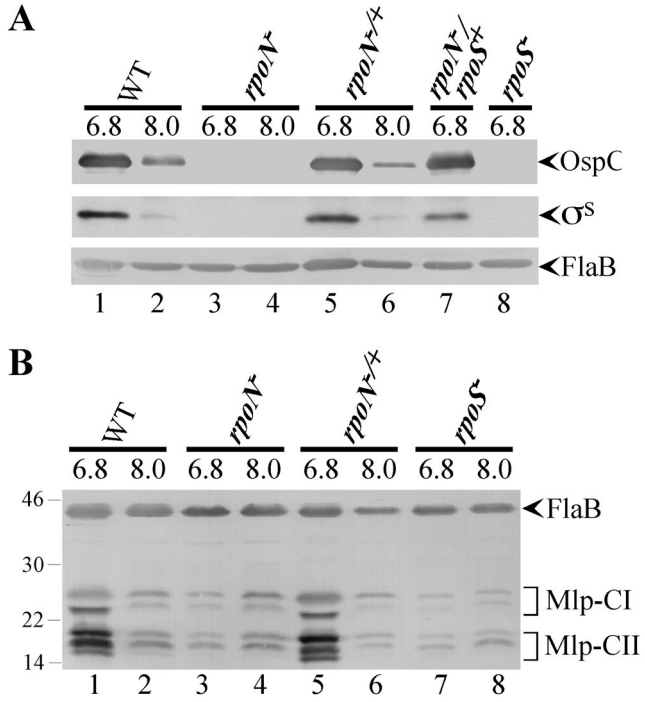

FIG. 4. Influence of RpoN or RpoS on pH-dependent regulation of Mlp and OspC expression in *B. burgdorferi*. Wild-type and mutant strains of low-passage *B. burgdorferi* were cultivated at 37°C in BSK-H medium adjusted to pH 6.8 or 8.0. Spirochetes were harvested at a cell density of 3×10^7 per ml and then were subjected to SDS-PAGE and immunoblotting. Each gel lane for SDS-PAGE was loaded with approximately 5×10^7 spirochetes. Strains used (denoted at tops of panels) were designated as for Fig. 3. Numbers at the left of the figure denote molecular mass markers in kilodaltons. (A) Immunoblot analyses performed by using antisera directed against OspC, σ^s (RpoS), or FlaB (loading control) (positions denoted by arrowheads). For the detection of low-abundance σ^s, 2×10^8 spirochetes per gel lane were used, and immunoblotting was performed using chemiluminescence. (B) Immunoblot analysis performed by using a mixture of antibody reagents (see Fig. 1) directed against each of the Mlps. Mlp-CI, gel position where antigenic class I Mlps migrate; Mlp-CII, gel position where antigenic class II Mlps migrate.

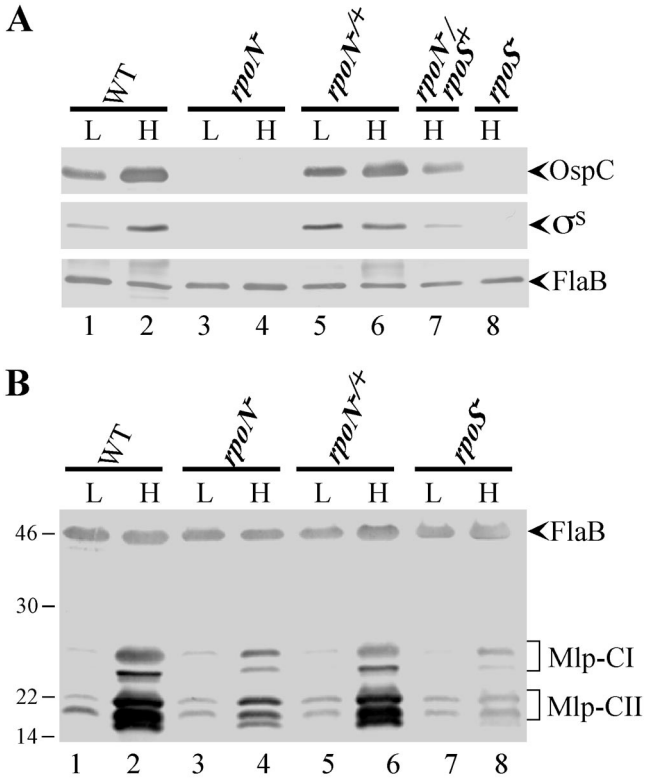

FIG. 5. Influence of RpoN or RpoS on cell density-dependent regulation of Mlp and OspC expression in *B. burgdorferi*. Wild-type and mutant strains of low-passage *B. burgdorferi* were cultivated at 37°C in BSK-H medium. Spirochetes were harvested when the cell density reached either 3×10^6 per ml (L) or 3×10^7 per ml (H) and then were subjected to SDS-PAGE. Each gel lane for SDS-PAGE was loaded with approximately 5×10^7 spirochetes. Strains used (denoted at tops of panels) were designated as for Fig. 3. Numbers at the left of the figure denote molecular mass markers in kilodaltons. (A) Immunoblot analyses performed by using antisera directed against OspC, σ^s (RpoS), or FlaB (loading control) (positions denoted by arrowheads). For the detection of low-abundance σ^s, 2×10^8 spirochetes per gel lane were used. (B) Immunoblot analysis performed by using a mixture of antibody reagents (see Fig. 1) directed against each of the Mlps. Mlp-CI, gel position where antigenic class I Mlps migrate; Mlp-CII, gel position where antigenic class II Mlps migrate.

Mlps and OspC. As has been shown by others (25), the level of OspC expression increased significantly when wild-type 297 was allowed to grow to an elevated cell density (Fig. 5A, lane 2). However, as observed in earlier experiments, OspC expression was completely abolished in either the *rpoN* or *rpoS* mutants regardless of cell growth phase (Fig. 5A, lanes 3, 4, and 8). Although inactivation of *rpoN* or *rpoS* reduced overall the expression of the Mlps in spirochetes harvested at either an earlier or later stage of growth (Fig. 5B, lanes 3, 4, 7, and 8), some increase in Mlp expression still was apparent at elevated cell densities in both the *rpoN* and *rpoS* mutants (Fig. 5B, lanes 4 and 8). This was in contrast to the finding that pH-dependent induction of Mlp expression did not occur in either of these mutants (Fig. 4B). In comparable experiments, the *rpoN* mutant complemented with wild-type *rpoN* (Fig. 5B, lanes 5 and 6) behaved essentially like wild-type 297 (Fig. 5B, lanes 1 and 2).

DISCUSSION

The importance of membrane lipoproteins in *B. burgdorferi* (2) is underscored by the fact that more than 150 putative lipoprotein genes are encoded within the borrelial genome, accounting for 10% of the spirochete's genomic capacity (6, 11). In a microarray study, 29% of genes differentially regulated by *B. burgdorferi* were putative lipoprotein genes (27). Although the physiological functions of the lipoproteins remain largely unknown, understanding the regulation of lipoprotein gene expression likely will be essential for elucidating many aspects of the complex enzootic life cycle of *B. burgdorferi* and the organism's ability to cause human disease (13). However, further complicating an analysis of lipoprotein gene expression in *B. burgdorferi* is the fact that many of the lipoproteins are among the more than 175 paralogous protein families in *B. burgdorferi* (www.tigr.org/tigr-scripts/CMR2

/ParalogousList.spl?db = gbb). While the biological ramifications of this rather remarkable redundancy remain to be fully appreciated, one of the most challenging questions concerns whether some genes within a family are differentially regulated at the expense of others, or whether all genes comprising a paralogous family are coordinately regulated. In the case of the paralogous Mlp family, which is comprised of two antigenic classes, initially we were attracted to the hypothesis that the two antigenic classes might subserve different physiological functions in *B. burgdorferi*, and thus they might be reciprocally regulated under different growth conditions. The generation of antibody reagents specific for each Mlp paralog was instrumental in making possible the tracking of the expression of individual Mlp paralogs to address this hypothesis. One of the principal findings of this study was that, despite different expression levels for each of the Mlp paralogs, the Mlps appeared not to be differentially regulated at the expense of one another, but rather were upregulated or downregulated as a family when *B. burgdorferi* replicated under three different culture conditions (i.e., temperature, pH, and cell density). This coordinate regulation for the Mlp family seems to contrast the more complex pattern of gene regulation noted for Bdr proteins, another large paralogous protein family in *B. burgdorferi* strain B31 (29).

The coordinate regulation of the *mlp* genes is consistent with previous Northern blotting data showing that mRNA levels for all *mlp* genes of *B. burgdorferi* strain B31 were increased by elevated temperature (23). Although there is relatedness but as yet no distinct identities between the *mlp* genes of strains 297 and B31 (23), microarray data for strain B31 have revealed that mRNA levels for *mlpG* (BB028), *mlpC* (BBS30), and *mlpF* (BBM28) all increased 2.2- to 10.6-fold when spirochetes were cultivated at 37°C (culture pH of 6.8) as opposed to 23°C (culture pH of 7.5) (27). Despite this coordinate regulation, there were differences in the levels of each individual Mlp at both the protein and mRNA levels after induction. Sequence alignments have not uncovered precise differences in the upstream regulatory regions of the *mlp* genes that can account for these differences in expression levels. However, in an analysis of the promoter regions of the *mlp* genes in *B. burgdorferi* strain B31, Porcella et al. (23) noted minor nucleotide differences that may account for the differences in the degrees of *mlp* expression.

Inasmuch as previous work has categorized *B. burgdorferi* lipoproteins that are upregulated by temperature, pH, and cell density as members of "group I" lipoproteins (34), our results suggest that the Mlps as a family also belong to this expression group. We previously showed that the expression of other group I lipoproteins, such as OspC and DbpA, is controlled by a novel RpoN-RpoS pathway (14). In this pathway, the alternative sigma factor RpoN controls the expression of another alternative sigma factor (RpoS) which, in turn, governs the expression of the group I lipoproteins (14). We showed previously that an upregulation of *ospC* and *dbpA* in response to temperature was markedly affected by this RpoN-RpoS pathway (14). Although OspC expression also is influenced by the pH of the culture medium as well as spirochete cell density (34), previously it was not known whether these induction effects also were influenced by RpoN and/or RpoS. Our results herein showed that inactivation of RpoN or RpoS also abolished both the pH- and cell density-dependent regulation of *ospC*. The combined results suggest that *ospC* transcription is under the absolute control of RpoN and RpoS, at least under the culture conditions tested. Moreover, we have observed that OspC was not expressed by RpoN- or RpoS-deficient mutants cultivated in dialysis membrane chambers implanted into rat peritoneal cavities (i.e., in a mammalian host-adapted state), supporting a broader hypothesis that RpoN and, hence, RpoS may be essential for the expression of OspC under any environmental circumstance.

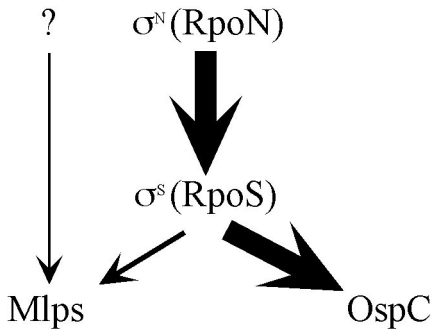

FIG. 6. A model for Mlp regulation and its relationship to OspC regulation in *B. burgdorferi*. Central to the model is the RpoN-RpoS regulatory pathway, in which RpoN governs the expression of RpoS which, in turn, influences the expression of OspC and Mlp paralogs. Under conditions of either elevated culture temperature, lower pH, or increased cell density, the RpoN-RpoS regulatory pathway is absolutely required for OspC expression (large arrows). However, in the absence of the RpoN-RpoS regulatory pathway (thin arrow at left), Mlp expression can be partially activated by some other yet-unknown (?) regulatory pathway.

That the Mlps have a group I-like expression profile (akin to OspC and DbpA) prompted an examination of whether Mlp expression also is governed by the RpoN-RpoS regulatory pathway. The results presented suggest that although the regulation of Mlps is influenced by the RpoN-RpoS pathway, unlike OspC, their dependency on RpoN and RpoS differed depending on the environmental conditions tested. In the case of culture pH, as with OspC expression, inactivation of *rpoN* or *rpoS* abolished pH-dependent regulation of the Mlps. However, unlike OspC expression, temperature- or cell density-induced Mlp expression, although reduced, still was readily detectable in both *rpoN* and *rpoS* mutants. Thus, given that the temperature- or cell density-dependent induction of the Mlps is only partially influenced by the RpoN-RpoS pathway, there appears to be an additional layer of gene regulation, yet undefined, that contributes to the regulation of Mlp expression. This layer of regulation also may contribute to the basal level of Mlp expression observed when *B. burgdorferi* is cultivated under more unfavorable Mlp expression conditions (i.e., elevated pH, lower ambient temperature, or lower cell density).

We and others have contended that changes in the pH of the borrelial culture medium (from 7.5 to 6.8) can markedly alter lipoprotein expression by *B. burgdorferi* (4, 5, 34). Inasmuch as the pH of a borrelial culture can drop as spirochetes replicate to increased cell densities (34), the possibility remained that cell density effects modulating gene expression in *B. burgdorferi* (15, 25) may actually be due to a concomitant drop in pH of the

culture. In this regard, cultures were allowed to reach cell densities of only 3×10^7 per ml, spirochete levels that should minimize pH changes (less than 0.2 pH units) to the BSK-H medium. Under these culture conditions of no or minimal pH change, the cell density effect on both Mlp and OspC expression was marked. Most importantly, whereas pH-dependent upregulation of Mlp expression was abolished in the *rpoN* or *rpoS* mutants, cell density-dependent upregulation of Mlp expression still occurred. Clearly, the increase in Mlp expression in these mutants upon elevated cell density therefore could not be attributed to any potential pH change in the BSK-H medium. The combined results thus suggest that increased spirochete density, independent of culture pH, can act to upregulate Mlp expression in *B. burgdorferi*.

On the basis of previous work (14) and data presented herein, a simplified working model for the regulation of expression of OspC and the Mlps is proposed (Fig. 6). In this model, RpoN and RpoS both influence the expression of OspC and the Mlps. The RpoN-RpoS regulatory circuit appears to be required at any level for OspC expression, at least under the culture conditions examined. In the case of the Mlps, although their expression is influenced by RpoN and RpoS, there exists at least one other unknown pathway, independent of RpoN or RpoS, contributing to Mlp expression in response to temperature and spirochete cell density (Fig. 6). This working model also likely applies to the regulation of other group I lipoproteins, but additional data will be needed to assess the broader significance of the RpoN-RpoS pathway and other regulatory pathways potentially converging with this network.

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