Molecular Microbiology (2001) **39**(2), 370–378

Two independent transcriptional units control the complex and simultaneous expression of the *bmp* paralogous chromosomal gene family in *Borrelia burgdorferi*

Elena Yu Dobrikova, Julia Bugrysheva and Felipe C. Cabello*
Department of Microbiology and Immunology, New York Medical College, Basic Science Building, Valhalla, NY 10595-1690, USA.

Summary

The chromosomal paralogous gene family 36 encodes for four lipoproteins with high amino acid homology that are expressed *in vivo* in humans and animals and are immunogenic. Transcriptional analysis of the *bmp* gene cluster indicated that all four genes of this cluster are expressed *in vitro* and constitute two transcriptional units with a complex pattern of transcription, including alternative monocistronic and polycistronic messages. One unit consists of *bmpD*, whose transcription is coupled to the transcription of the ribosomal protein genes, *rpsG* and *rpsL*. The second unit includes *bmpC*, *bmpA* and *bmpB*. The simultaneous expression of the four *bmp* genes in *Borrelia burgdorferi* suggests that their gene products may have either different or complementary functions. Primer extension experiments identified promoters for *bmpD*, *bmpC* and *bmpA*, but not for *bmpB*. The concentration of gene-specific mRNA paralleled its promoter homology to the *Escherichia coli* σ^{70} promoter. The linkage of *bmpD* expression to *rpsL and rpsG* suggests that the expression of this gene may be controlled by growth-related global regulation mechanisms in *B. burgdorferi*. These results indicate that the *bmp* family constitutes a good model for the investigation of complex regulation of chromosomal gene expression in this bacteria.

Introduction

Borrelia burgdorferi is a culturable extracellular bacteria that has a rather small genome of approximately 1000 chromosomal and 400 plasmid genes (Fraser *et al*., 1997). The multiplication and persistence of *B. burgdorferi* in different microenvironments in its hosts in the face of limited genetic material suggests that it may modulate its gene expression while in these niches in a very tight and discriminating fashion in answer to external stimuli (Schwan *et al*., 1995; Suk *et al*., 1995). Analysis of the DNA sequence of *B. burgdorferi* indicated that it contains only a few homologues to regulatory genes of other eubacteria and only three sigma factors and one *rho* terminator factor (Fraser *et al*., 1997). Previous studies have shown that the basic units of transcription in *B. burgdorferi* appear to be single genes and operons with few genes (*ospA–ospB*, *oppA*) and others with many genes (*fla* operon) that are transcribed as unique polycistronic messages (Howe *et al*., 1986; Ge and Charon, 1997; Bono *et al*., 1998). Preliminary characterization of several *B. burgdorferi* promoters indicates that, in general, they are homologous to the σ^{70} promoter of *Escherichia coli* (Jonsson *et al*., 1992; Margolis *et al*., 1994; Porcella *et al*., 1996; Cloud *et al*., 1997; Ge *et al*., 1997; Indest *et al*., 1997). However, preliminary experiments with gene fusions suggest that DNA sequence upstream of the −35 region can influence the level of gene expression (Sohaskey *et al*., 1999). Although environmental stimuli are able to modulate *in vitro* and *in vivo* gene expression in *B. burgdorferi*, there is no evidence yet for the presence of regulons, global regulators and hierarchical networks of regulatory genes in this pathogen (Fraser *et al*., 1997). Only response regulators of two different two-component systems have genes in the genome of this organism (Fraser *et al*., 1997; Subramanian *et al*., 2000). Although the *B. burgdorferi* genome contains genes encoding for DNA-binding proteins with HU/IHF-like function that could be involved in gene regulation (Tilly *et al*., 1996), it lacks homologues of eubacterial alternative regulatory proteins, such as activators and repressors of transcription, and modulators of gene expression such as CAP (Fraser *et al*., 1997). On the other hand, *Borrelia* has genes and gene families (especially ones harboured by plasmids) that do not share homology with genes of other bacteria (Fraser *et al*., 1997). This information suggests that *B. burgdorferi* may have evolved mechanisms to control gene expression that are different from those of other eubacteria, and that studies of gene expression in *B. burgdorferi* may reveal

Accepted 28 September, 2000. *For correspondence. E-mail Cabello@nymc.edu; Tel. (+1) 914 594 4182; Fax (+1) 914 594 4176.

novel insights into bacterial gene modulation in answer to host microniche stimuli.

Analysis of the genome of *B. burgdorferi* indicates that about 5% of the chromosomal genes and 14.5% of the plasmid genes encode putative lipoproteins, many of them encoded by redundant paralogous gene families (Casjens *et al*., 2000) that are probably located on the cell surface of *B. burgdorferi* (Cox *et al*., 1996). The chromosomal *bmp* gene cluster encodes the paralogous gene family 36 of lipoproteins with high amino acid homology, which are expressed *in vivo* and are immunogenic (Aron *et al*., 1994; Simpson *et al*., 1994; Ramamoorthy *et al*., 1996). Antibodies against one of the members of this family, BmpA (formerly p39), appear early in the development of infection with *B. burgdorferi* in humans and animals (Simpson *et al*., 1994). These antibodies are bactericidal and protective (Barthold *et al*., 1997). These findings, the conservation of the *bmp* genes, their sequential genetic structure on the chromosome in all *B. burgdorferi sensu lato* strains (but not in the genome of the relapsing fever spirochaetes; Gorbacheva *et al*., 2000) and the presence of homologues of these genes in *Treponema pallidum* (Aron *et al*., 1994; Simpson *et al*., 1994), suggest that the *bmp* genes may be related to virulence properties specific to *B. burgdorferi*. Analysis of the DNA sequence of the *bmp* genes for putative promoters revealed an *E. coli* σ^{70}-like promoter for *bmpD*, two promoters for *bmpC*, one for *bmpA* and no promoter for *bmpB* (Aron *et al*., 1994; Simpson *et al*., 1994; Ramamoorthy *et al*., 1996). Preliminary and incomplete evidence indicates that *bmpD* and *bmpA* are transcribed *in vitro* (Ramamoorthy and Philipp, 1998), but there is no information regarding the relative levels of expression of these genes. The clustered and sequential location of the *bmp* genes in the chromosome, the apparent overlapping of their transcriptional signals and the expression of some of them *in vivo* suggest that they may constitute a regulatory unit (McClure, 1985) modulated by *in vivo* conditions encountered by *B. burgdorferi* in its hosts and able to generate polycistronic messages encompassing different members of the family. In an effort to answer some of these questions, we decided to investigate promoter usage, levels of transcription, message size and potential modulation resulting from external stimuli in the *bmp* cluster of *B. burgdorferi*.

Results

Expression of the bmp *genes in* B. burgdorferi *growing* in vitro

Published work by other investigators has indicated that *B. burgdorferi* B31 growing in culture expressed *bmpD* as a monocistronic mRNA modulated by the phase of growth (Ramamoorthy and Philipp, 1998) and that a *bmpA* mRNA was expressed constitutively, but there has been no

Table 1. Primers used and size of the amplicons obtained in studies of the expression of genes of the *bmp* cluster.

Gene	Primer	Sequence (5′−3′)	Size of amplified fragment		Position on *bmp* region (see Fig. 1)
			Wild type	Competitor	
RT−PCR of the partial coding regions					
bmpA	21(+)	CCAAGGTTGCGGCTCTTC	307	219	2922−2939
	22(−)	CTTCTACCAGCTTCAAGGTCAG			3207−3228
bmpB	23(+)	TGGTGATGATGTTCAGATTCC	339	241	4098−4118
	24(−)	TTTGCTGCCTCAATAATAACACC			4417−4436
bmpC	1(+)	GATGAGGCAATGACTGAGGATGC	486	337	1770−1792
	2(−)	GCAGCGTCATAAACTCCAAGACC			2232−2254
bmpD	19(+)	CTGATGATGGCAAGTCGGAG	613	509	238−257
	20(−)	ACGCCTATACCAGAAAGCCC			831−850
flaB	49(+)	CTAGTGGGTACAGAATTAATCGAGC	880	691	
	50(−)	TAACATAAAAATATCCTCCTTGC			
RT−PCR of the intergenic regions					
*bmp*A−	13(+)	GGCCTTAAAGAAGGAGTTGTGGG	529	–	3484−3506
bmpB	14(−)	GCCAAATCAAGTCTGAGCC			3993−4011
*bmp*C−	5(+)	TGATCGGGGGTTAAAGGAAGG	809	721	2420−2440
bmpA	22(−)	CTTCTACCAGCTTCAAGGTCAG			3207−3228
*bmp*D−	4(+)	AGGCCGCAAAAGAGTTGGG	786	–	856−874
bmpC	3(−)	GCTACCATGAGCCAAAACACC			1620−1640
rpsL−	55(+)	GGAACAAAAAAGCCTAAAGC	694	564	600 upstream *bmpD*
bmpD	52(−)	CGACTTGCCATCATCAGAGC			235−254
Primer extension					
bmpA	53(−)	CGCTCCCAAGACTACCTTTACC			2683−2704
bmpB	54(−)	GTCGTCAAGAACACCATCTACC			3831−3852
bmpC	3(−)	AGGCCGCAAAAGAGTTGGG			1620−1640
bmpD	52(−)	CGACTTGCCATCATCAGAGC			235−254
bmpD	56(−)	TAACATAAAAATATCCTCCTTGC			163−185

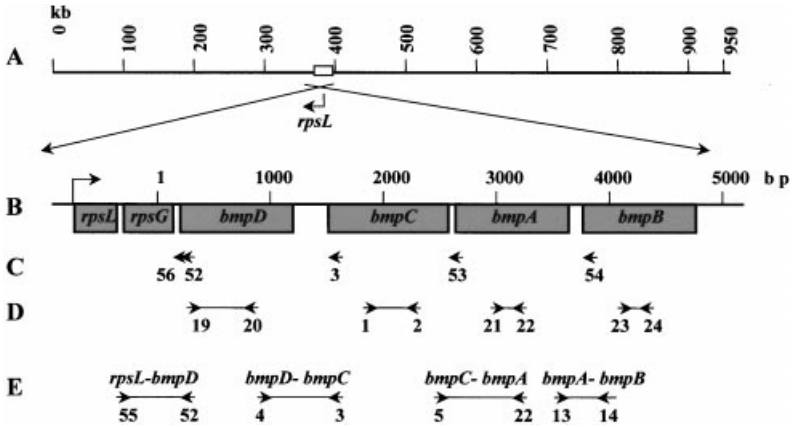

Fig. 1. Organization of the *B. burgdorferi bmp* gene cluster and localization of primer binding sites in the *bmp* region.
A. Diagram of the linear chromosome of *B. burgdorferi*.
B. Schematic map of the *bmp* region. Position 1 corresponds to nucleotide 396706, and 5000 to 391707 on the *B. burgdorferi* B31 chromosomal map. The arrow indicates the direction of transcription.
C. Localization of the primers used in the primer extension experiments. Primers used in RT–PCR and in competitive RT–PCR of *bmp* coding (D) and intergenic (E) regions.

information regarding the transcription of the other genes of the *bmp* family. We have used reverse transcriptase–polymerase chain reaction (RT–PCR) to investigate whether all four *bmp* genes are expressed in *B. burgdorferi* growing *in vitro* and also to discern the transcriptional organization of this gene cluster. The primers used for RT–PCR are listed in Table 1, and the organization of *bmp* gene cluster is shown in Fig. 1. We were able to detect amplicons for *bmpA*, *bmpB*, *bmpC* and *bmpD* (Fig. 2). The presence of RT–PCR-generated amplicons spanning the *bmpA–bmpB*, *bmpC–bmpA* and *rpsL–bmpD* regions but not *bmpD–bmpC* indicated that these genes were also transcribed as polycistronic messages. Thus, all four genes of the *bmp* cluster are transcribed *in vitro*, and their transcription can produce polycistronic messages encompassing neighbouring members of the cluster. *bmpD* does not generate a polycistronic message with other members of the *bmp* cluster, but it is transcribed as part of another transcriptional unit along with the *rpsG* and *rpsL* gene homologues located upstream of its 5′ DNA sequence and encoding the ribosomal proteins S7 and S12 (Fig. 1).

Identification of the promoters of the bmp *genes*

As mentioned above, previous comparative analysis of the DNA sequence of the *bmp* gene region resulted in the identification of two putative *E. coli* σ^{70}-like promoters for *bmpC*, one for each *bmpD* and *bmpA* and none for *bmpB* (Aron *et al.*, 1994; Simpson *et al.*, 1994; Ramamoorthy *et al.*, 1996). To determine the promoters and transcription starting sites of the *bmp* genes while *B. burgdorferi* was growing *in vitro*, primer extension experiments for all four *bmp* genes were performed with the primers complementary to corresponding mRNA sequence downstream of the translation initiation codon (Table 1 and Fig. 1C). As predicted by comparative analysis, *bmpA*, *bmpC* and *bmpD* genes, but not *bmpB*, have their own transcriptional start points (Fig. 3A). The transcriptional start site for *bmpC* mRNA is located at position −66, upstream of the translational initiation codon, *bmpD* mRNA has two start points at positions −74 and −76 upstream of its translation initiation codon, and the transcriptional start site of the *bmpA* gene is situated at the −105 position relative to its translational initiation codon. Although the *bmp* gene promoters deduced from the sequence upstream of the transcriptional start points have reasonable homology with the *E. coli* σ^{70} consensus (Harley and Reynolds, 1987) (Fig. 3B), they do not correspond to the putative promoters identified by DNA sequence comparisons (Aron *et al.*, 1994; Simpson *et al.*, 1994; Ramamoorthy *et al.*, 1996). However, the transcription start point for *bmpA*, as determined experimentally, was still located within the coding sequence of the *bmpC* gene (60 bases from the *bmpC* stop codon), whereas the promoter region of the *bmpD* gene overlapped with the last 11 nucleotides of the *rpsG* coding region.

Quantitative analysis of the expression of the genes of the bmp *cluster*

The experiments described above indicated that some genes of the *bmp* cluster could be transcribed from their

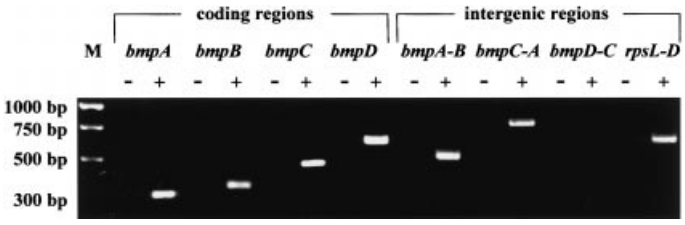

Fig. 2. Detection by RT–PCR of *bmp* gene-specific RNAs in *B. burgdorferi* growing *in vitro*. Amplifications were performed in the absence (–) or presence (+) of reverse transcriptase using the primers shown in Fig. 1D and E. M, molecular weight marker.

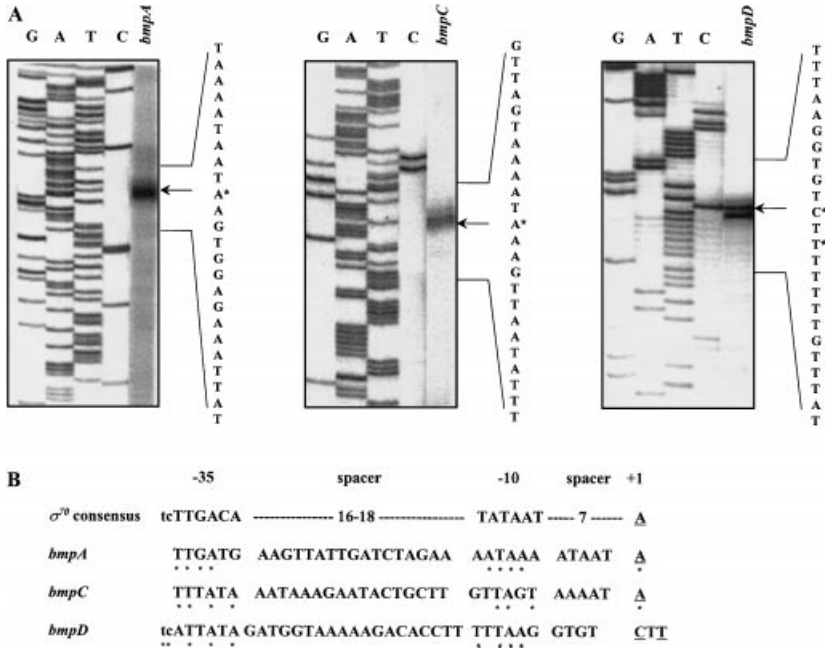

Fig. 3. Determination of the transcription start sites of *bmp* genes.
A. Primer extension reactions (lanes marked *bmpA*, *bmpC and bmpD*) were performed with primers complementary to mRNA sequences downstream from the translational start sites (Fig. 1C). The same primers were used to generate sequencing ladders (lanes G, A, T and C) for the corresponding primer extension reactions. The sequences of the regions around transcriptional start sites are shown on the right; start points are indicated by arrows and asterisks.
B. Nucleotide sequence upstream of transcriptional start points of *bmp* genes. The top line presents the *E. coli* σ^{70} promoter (Harley and Reynolds, 1987). The homology of deduced *bmp* promoters with σ^{70} consensus is indicated by asterisks; transcriptional start sites are underlined.

own promoters and as part of polycistronic messages, suggesting that the transcriptional level of each gene may be affected by the expression of the other members of the cluster. To determine the level of *bmp* gene expression *in vitro*, we performed competitive RT–PCR, using as competitors RNAs generated by *in vitro* transcription from plasmids containing cloned DNA fragments of the corresponding genes with internal deletions in the region to be amplified (the construction of competitors is described in *Experimental procedures*). Figure 4 illustrates the results of the competitive RT–PCR assays directed at measuring the transcriptional levels of individual *bmp* genes, and it can be seen that the reaction displayed in lane 3 of all the experiments gave bands of similar intensities for the competitor and target RNA, indicating that they are present in equivalent amounts. The competitive RT–PCR assays for each *bmp* gene were repeated at least three times, and the means of these independent determinations were normalized to 10 ng of total *B. burgdorferi* RNA. *bmp* genes are expressed in significantly different amounts during *B. burgdorferi* growth *in vitro*; *bmpA* is transcribed at the highest level (about 65 fg of specific mRNA per 10 ng of total RNA), *bmpD* and *bmpB* at intermediate levels (about 20 fg) and *bmpC* at the lowest level (1.1 fg). *bmpA* levels of mRNA transcription are significantly higher than those of *bmpC*, *bmpD* and *bmpB* ($P < 0.01$, Tukey–Kramer multiple comparison test). Because *bmpB* does not have its own promoter, it is assumed to be transcribed exclusively with *bmpA* mRNA (Fig. 5). Northern analysis with *bmpA* specific probe performed by other investigators (Ramamoorthy and Philipp, 1998) and ourselves (data not shown) detected several transcripts of different size, one of which corresponds to bicistronic *bmpA–bmpB* mRNA. Moreover, competitive RT–PCR, using primers 5 and 22 to amplify the *bmpC–bmpA* intergenic region (see Fig. 1 and Table 1) resulted in the same amount of product as when primers 1 and 2 were used to amplify individual *bmpC* mRNA. This last finding suggests that *bmpC* is always expressed as a polycistronic message with *bmpA* (Fig. 5). The comparison of expression levels of *bmpC* (1.1 fg), *bmpA* (65 fg) and *bmpB* (20 fg) permits the conclusion that *bmpA* can transcribe as individual mRNA

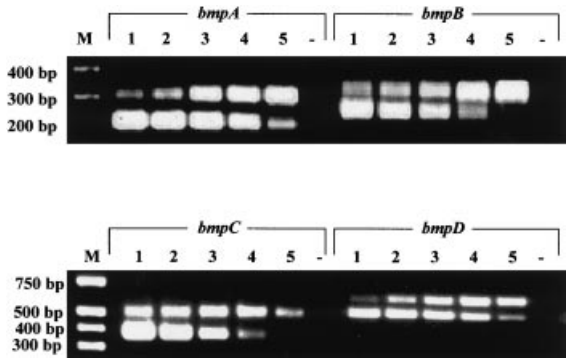

Fig. 4. Measurement of the concentration of *bmp* gene-specific mRNAs by competitive RT–PCR. All reactions in *bmpA*, *bmpB and bmpD* (lanes 1–5) contained 10 ng of total *B. burgdorferi* RNA and, in *bmpC*, there was 150 ng. The amount of competitor RNA in *bmpB*, *bmpC and bmpD* was 80 fg in lane 1, 40 fg in lane 2, 20 fg in lane 3, 10 fg in lane 4 and 5 fg in lane 5; in *bmpA*, there was 320 fg in lane 1, 160 fg in lane 2, 80 fg in lane 3, 40 fg in lane 4 and 20 fg in lane 5. Lane (–) is a negative control; RT–PCR without RNA. Lane 3 shows equal signal intensities from target mRNA and competitor, indicating that, in this reaction, they are present in equal concentrations. M, molecular weight marker.

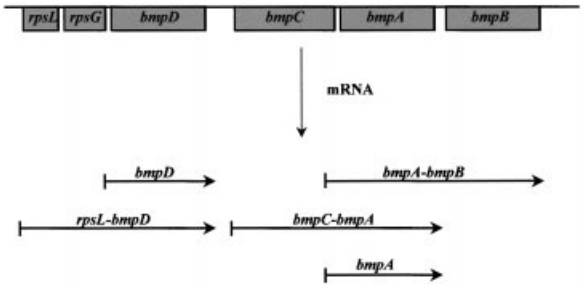

Fig. 5. Model for transcription of the *bmp* gene cluster in *Borrelia burgdorferi* growing *in vitro*.

as well. In support of this assumption, the smallest transcript identified by Northern hybridization (data not shown) corresponds to individual *bmpA* mRNA. *bmpD* can be expressed mainly as monocistronic mRNA (Fig. 5), as the concentration of *rpsL–bmpD* message is approximately 20% of the total amount of *bmpD* mRNA.

Expression of the genes of the bmp *cluster in* Borrelia *growing under different conditions*

As preliminary evidence suggested that expression of some *bmp* genes can be modulated by growth conditions (Ramamoorthy and Philipp, 1998), we have analysed the influence of temperature and stage of growth on the transcription of these genes. In these experiments, the constitutively expressed *flaB* gene (de Silva *et al.*, 1999) was used as a control to compare the different RNA isolates. We could not detect any marked changes in the transcriptional level of the *bmp* genes in *B. burgdorferi* growing *in vitro* at three temperatures (23°C, 32°C and 37°C) and taken at two different cell concentrations ($\approx 10^7$ – exponential phase of growth; and $\approx 10^8$ – early stationary phase of growth; data not shown).

We also compared the expression of *bmp* genes in two *B. burgdorferi* strains: high-passage non-infectious strain B31 and low-passage infectious strain N40. No significant variations were found in *bmpA and bmpD* expression levels, whereas the amount of *bmpC* mRNA in the N40 strain was four or five times higher than in B31. However, DNA sequencing of the entire *bmp* region of the N40 strain demonstrated the presence of only a few nucleotide substitutions in the *bmpC* 5′ region outside the promoter sequence (data not shown). Similar levels of *bmpC* mRNA to the N40 strain were found in another recent clinical isolate of *B. burgdorferi*, BL206, suggesting that the transcription of this gene can be regulated by as yet uncharacterized transcriptional factors.

Discussion

These results extend previous observations by other investigators who had detected the expression of only *bmpD* and *bmpA* using Northern hybridization (Ramamoorthy and Philipp, 1998). Specifically, we have determined that the four members of the *bmp* gene cluster are transcribed in *B. burgdorferi* growing *in vitro*. This unexpected result suggests that perhaps the four gene products of this paralogous gene family are needed for *B. burgdorferi* growing *in vitro* and that, in spite of their homology, the gene products of this family may have different or complementary *in vitro* and *in vivo* functions (Cotter and Miller, 1998; Subramanian *et al.*, 2000). Our ability to detect *bmpC* mRNA is probably the result of increased sensitivity of RT–PCR compared with the Northern procedure to detect mRNA (Ramamoorthy and Philipp, 1998). The simultaneous expression of the four genes of the cluster could be explained by the synthesis of polycistronic RNA message whose transcription starts from a putative dominant member of the *bmp* cluster, either *bmpD* or *bmpC*, and extends distally (Ge and Charon, 1997), as suggested by the presence of mRNAs corresponding to *bmpC–bmpA* and *bmpA–bmpB* regions

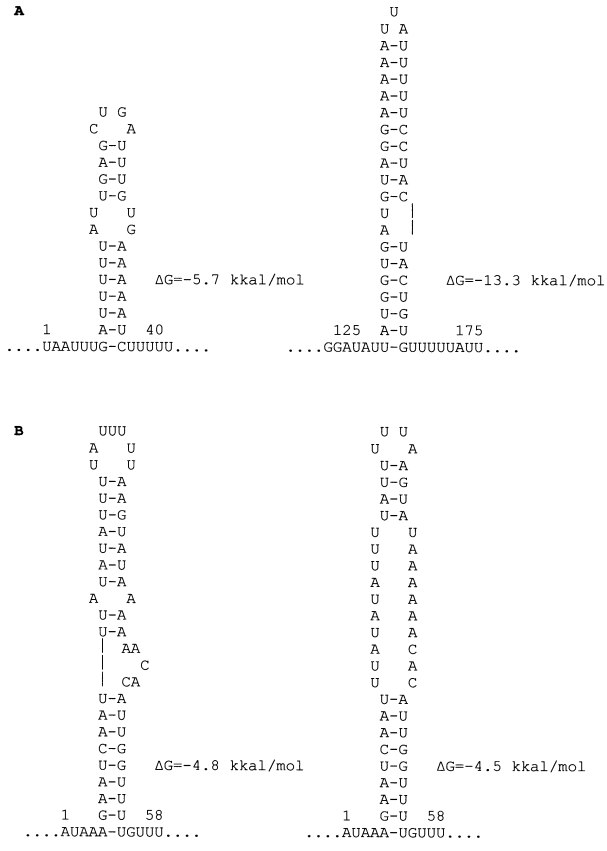

Fig. 6. Potential RNA secondary structures in the *bmpD–bmpC* (A) and *bmpA–bmpB* (B) intergenic regions of *B. burgdorferi*. Structures were drawn and free energies (ΔG) were calculated using the MFOLD RNA secondary structure analysis program (Zuker, 1989). Numbers show the position after the translation termination codon.

(Fig. 2). The transcription of *bmpD* independently from the other members of the *bmp* cluster was not unexpected, because the DNA sequence downstream of its translation termination codon contains several potential rho-independent terminator structures (Fig. 6A) (Platt, 1986). The transcription of *bmpD* by polycistronic messages with *rpsL–rpsG* potentially places the expression of this gene under translational control, as in *E. coli* in which the product of *rpsG* represses its own synthesis and can affect the expression of downstream genes (Saito *et al*., 1994). Similarly, the coupling of the transcription of *bmpD* to the transcription of ribosomal protein genes may make the expression of this gene the first example in *B. burgdorferi* of a structural lipoprotein putatively regulated by the stringent response (Condon *et al*., 1995).

The primer extension experiments resulted in the detection of the transcriptional start sites for *bmpD*, *bmpC* and *bmpA and* allowed us to deduce the promoters from the 5′ DNA sequences of these genes (Fig. 3). These experimentally determined promoters do not coincide with the previously determined putative promoters, underlining a shortcoming of the comparative analysis of DNA sequences to identify regulatory elements in unrelated bacteria (Gelfand, 1999). Nevertheless, these promoters are homologous to the σ^{70} promoters of *E. coli* (Fig. 3B), in agreement with the presence of the gene for σ^{70} factor in the genome of *B. burgdorferi* (Fraser *et al*., 1997), and findings by other investigators indicating that most of the *B. burgdorferi* promoters described up to now correspond to σ^{70}-like promoters (Jonsson *et al*., 1992; Margolis *et al*., 1994; Porcella *et al*., 1996; Cloud *et al*., 1997; Ge *et al*., 1997; Indest *et al*., 1997). Analysis of the DNA sequences upstream of the *bmp* promoters did not detect long stretches rich in T that could have a role in modifying promoter strength, as has been described in the 5′ upstream DNA sequence of the *Borrelia ospA*, *ospB* and *vmp* promoters (Sohaskey *et al*., 1999). However, short T stretches were found in the 5′ upstream region of the *bmpC* gene; their significance for the expression of this gene remains undetermined.

The quantification of *bmp* mRNAs indicated that they display a wide range of transcriptional levels from 65 fg for *bmpA* to 1.1 fg for *bmpC* per 10 ng of total *B. burgdorferi* RNA. The high concentration of the *bmpA* message could be the result of the elevated strength of its promoter, which displays a higher homology with the canonical σ^{70} promoter of *E. coli* than with promoters of the other *bmp* genes (Fig. 3). Although *bmpB* does not have its own promoter and its transcription would be expected to be co-ordinated with the transcription of *bmpA*, the amount of *bmpB* mRNA is only a fraction of that of *bmpA* (Fig. 5). One possible explanation for this difference may be the premature termination of transcription in the *bmpA–bmpB* non-coding region on the short hairpin structures (Fig. 6,B) that could probably contribute to termination of the transcription, resulting in monocistronic *bmpA* mRNA. On the other hand, this difference may be the result of the 3′ degradation of polycistronic *bmpA–bmpB* mRNA (Rauhut and Klug, 1999). The relatively low level of expression of the *bmpD and bmpC* genes could be attributed to the less canonical structure of σ^{70} promoters compared with the promoter of *bmpA* (Fig. 3), and the lower level of expression of *bmpC* as opposed to *bmpD* to the fact that the latter gene is also transcribed as a polycistronic message originated from the *rpsL* gene promoter (Fig. 5). Thus, these results indicate that all four *bmp* genes are expressed at different levels in *B. burdgdorferi* growing *in vitro* and that their regulation differs from that professed by the current paradigm of complementary upregulation/downregulation as in *ospA*/*ospC* gene expression (Schwan *et al*., 1995).

Results indicating that the four *bmp* genes are simultaneously expressed *in vitro* and that this pattern of expression was not modified by changing environmental conditions such as temperature and cell density suggests that these genes might be involved in essential functions. On the other hand, the variations in the transcriptional level of the *bmpC* gene between infectious strains N40 and BL206 and high-passage non-infectious B31 suggest that the expression of this gene may be regulated by unknown mechanisms, as these variations do not result from differences in the *bmpC* promoter region. We have found only a small number of point mutations in the DNA sequence outside the *bmpC* promoter; one was the substitution G → A in the spacer between the −35 and −10 regions that can facilitate DNA melting; the second, which can probably affect the strength of the promoter, was the deletion of G residue from oligoT sequence in the −100 region upstream of *bmpC* transcription start site (Sohaskey *et al*., 1999).

The interpretation of these transcriptional studies is limited by the lack of studies of the half-life of the mRNA and of the correlation between levels of mRNA and synthesis of the Bmp proteins. However, the complexity of the transcription of this paralogous gene family in *B. burgdorferi* growing *in vitro* is apparent from these experiments. We also suggest that the different expression levels of *bmp* genes might be the result of the different strengths of their promoters, but this conclusion is based on the untested assumption that *B. burgdorferi* RNA polymerase has a higher affinity to those promoters that have high homology with *E. coli* σ^{70} promoters. In summary, it would appear that plasmid-encoded and chromosomal-encoded paralogous gene families of *B. burgdorferi* have a complex pattern of differential expression. This, in turn, suggests that, in spite of their homology, their gene products may have dissimilar or complementary functions in the life cycle of *B. burgdorferi*.

These results, the conservation of the genetic structure of the *bmp* gene cluster in *B. burgdorferi sensu lato* and the small number of its members suggest that they are useful models for the study of gene regulation in this bacteria and the potential role of this regulation in bacterial survival and virulence.

Experimental procedures

Bacterial strains and growth conditions

E. coli strain DH5α containing plasmids was grown in Luria–Bertani (LB) medium with ampicillin at concentrations of 100 μg ml^{-1}. Unless otherwise specified, high-passage non-infectious *B. burgdorferi* strain B31 (ATCC 35210), low-passage infectious strain N40 (from Dr Linda Bockenstadt, Yale University) and a recent clinical isolate from human blood sample BL 206 (from Dr Ira Schwartz, New York Medical College) were grown at 32–34°C until the cell density reached 10^7-10^8 cells ml^{-1} in BSK-H medium supplemented with 7% rabbit sera (Sigma) (Indest *et al*., 1997). Borrelial cells were counted by phase-contrast microscopy in a Petroff–Hausser cell counting chamber (Hausser Scientific Partnership). Infectivity of *B. burgdorferi* strains was confirmed by intradermal (i.d.) inoculation of 10^4 spirochaetes per C3H/HeJ mouse (Akins *et al*., 1998) and detection of spirochaetes by blood and ear punch culture and PCR of ear puncture (Sinsky and Piesman, 1989).

Construction of plasmids to generate shorter mRNAs to use as controls for competitive RT–PCR

Previously, the primers were chosen to amplify a central region of each *bmp* gene (Gorbacheva *et al*., 2000) and the *flaB* gene (Sartakova *et al*., 2000). To construct positive controls for competitive RT–PCR, we used pUC19-based recombinant plasmids containing different DNA segments of the *bmp* region of *B. burgdorferi* (Aron *et al*., 1996). DNA manipulations were performed by standard methods (Sambrook *et al*., 1989) using the appropriate restriction enzymes to clone the distinct segments of *bmp* region and to introduce small deletions (88–189 bp; Table 1) into the amplified region of each *bmp* gene. The DNA fragments containing each *bmp* gene with the corresponding internal deletions were cloned into a pBlueScript $SKII^+$-based vector (Borovkov and Rivkin, 1997) under the control of T7 RNA polymerase promoter. The construction of a competitor for the *flaB* gene has been described previously (Sartakova *et al*., 2000). Competitor RNAs smaller than the corresponding wild-type RNAs (Table 1) were synthesized *in vitro* using the Riboprobe *In Vitro* Transcription System (Promega) according to the manufacturer's instructions, treated with RQ1 RNase-free DNase (Promega) for 3 h at 37°C, extracted with phenol–chloroform and precipitated with ethanol. The DNase treatment was repeated if DNA contamination was detected by RT–PCR analysis. Restriction endonucleases, T4 DNA ligase and other enzymes used for cloning were from New England BioLabs. Primers from Table 1 were synthesized by GenoSys Biotechnology.

RT–PCR and competitive RT–PCR

Total RNA from borrelial cells was isolated by the guanidine thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987) and treated to eliminate DNA contamination as described above. One-step RT–PCRs were performed with the Access RT–PCR System (Promega) according to the manufacturer's recommendations. Reaction mixtures (10 μl) contained reaction buffer, 1.5 mM $MgSO_4$, 0.2 mM each dNTP, 0.5 μM each primer and 0.1 u μl^{-1} both AMV reverse transcriptase and *Tfl* DNA polymerase. In the experiments presented in Fig. 2 (RT–PCR for all *bmp* genes and intergenic regions), 100 ng of total *B. burgdorferi* B31 RNA was added per reaction. For competitive RT–PCR assays, serial 10-fold dilutions of total and competitor RNAs were made; the concentration of total RNA chosen was inside the range where there was a direct relationship between the amount of PCR product and the amount of RNA added. This concentration was kept constant in each series of reactions and mixed with different twofold dilutions of competitor RNA. In experiments in which a comparison of gene expression levels was made between different RNA isolates, the amounts of total RNAs containing equal concentrations of *flaB* mRNA were used in each reaction. Both reverse transcription and PCR amplification were carried out in thin-walled tubes (Denville Scientific) in a Rapid Thermal Cycler (Idaho Technology) under the following settings: reverse transcription at 48°C for 45 min, then denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 10 s, 52°C (for *flaB and bmpC* genes) or 56°C (all other genes) for 10 s, 68°C for 30–50 s depending on amplicon size and final extension at 68°C for 5 min. Reactions without reverse transcriptase were used as negative controls for all RT–PCRs to exclude DNA contamination. In competitive RT–PCRs, target RNA and competitor RNA were assumed to be present at equimolar concentrations in those reactions in which they generated bands of similar intensities in agarose gel electropherograms (Zimmerman and Mannhalter, 1997).

Primer extension

The RNA for primer extension experiments was isolated as for RT–PCRs from mid-log phase cultures of *B. burgdorferi*. Primers (Table 1) were end-labelled using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]-ATP (> 3000 Ci $mmol^{-1}$; Amersham) and purified with a QIAQuick Nucleotide Removal Kit (Qiagen). Extension reactions were performed with Primer Extension System AMV Reverse Transcriptase or MMLV Reverse Transcriptase (Promega) as follows: 100 fmol of the primer was annealed with 10–30 μg of total RNA at 56–58°C for 20 min; then, reaction buffer, dNTP and reverse transcriptase were added, and reaction mixtures were incubated at 37°C (with MMLV reverse transcriptase) or 42°C (with AMV enzyme) for 30–45 min. Extension products were resolved in 7% denaturing polyacrylamide gel and visualized by autoradiography according to standard protocols (Sambrook *et al*., 1989). To generate sequence ladders for each extension product, sequencing reactions of plasmid with the cloned *bmp* region of *B. burgdorferi* (Aron *et al*., 1996) were performed with a T7 Sequenase Quick-Denature Plasmid Sequencing Kit and

[^{35}S]-dATPαS (> 1000 Ci mmol^{-1}; Amersham) directed by the same primer used for primer extension.

DNA sequencing analysis

Total DNA from *B. burgdorferi* N40 strain was isolated from a mid-log phase culture; overlapping fragments covering the entire *bmp* region were generated by PCR amplification with primers from Table 1 and purified with QIAquick Gel Extraction Kit (Qiagen). Both strands of each PCR product were sequenced at the Columbia University Cancer Center by U. Beauchamp, and comparative analysis of *B. burgdorferi* B31 and N40 *bmp* regions was performed using OMIGA 1.1 software (Oxford Molecular). The nucleotide sequence of the *B. burgdorferi* N40 *bmp* region has been submitted to GenBank under the accession no. AF288609.

Acknowledgements

We thank Ira Schwartz and Radha Iyer for kindly providing us with a clinical isolate of *Borrelia burgdorferi* BL206, Linda Bockenstadt for low-passage strain N40, Lieselotte Aron for the recombinant plasmids with cloned fragments of *bmp* region and, posthumously, Mark Rivkin for pBlueScript SKII$^+$-based cloning vector. Michael Norgard advised us on quantitative RT–PCR, and Henry P. Godfrey advised on the computer analysis of DNA sequences and in the preparation of the manuscript. Victoria Gorbacheva selected the primers for RT–PCR, and James Jones helped us in the animal experiments. Stuart A. Newman, Harriett V. Harrison and Melody Steinberg contributed to the preparation of the manuscript. This work was supported by Public Health Service grant RO1 AI 43063 to F. C. Cabello.

References

Akins, D.R., Bourell, K.W., Caimano, M.J., Norgard, M.V., and Radolf, J.D. (1998) A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *J Clin Invest* **101**: 2240–2250.

Aron, L., Alekshun, M., Perlee, L., Schwartz, I., Godfrey, H.P., and Cabello, F.C. (1994) Cloning and DNA sequence analysis of *bmpC*, a gene encoding a potential membrane lipoprotein of *Borrelia burgdorferi*. *FEMS Microbiol Lett* **123**: 75–82.

Aron, L., Toth, C., Godfrey, H.P., and Cabello, F.C. (1996) Identification and mapping of a chromosomal gene cluster of *Borrelia burgdorferi* containing genes expressed *in vivo*. *FEMS Microbiol* **145**: 309–314.

Barthold, S.W., Feng, S., Bockenstedt, L.K., Fikrig, E., and Feen, K. (1997) Protective and arthritis-resolving activity in sera of mice infected with *Borrelia burgdorferi*. *Clin Infect Dis Suppl* **1**: S9–S17.

Bono, J.L., Tilly, K., Stevenson, B., Hogan, D., and Rosa, P. (1998) Oligopeptide permease in *Borrelia burgdorferi:* putative peptide-binding components encoded by both chromosomal and plasmid loci. *Microbiology* **144**: 1033–1044.

Borovkov, A.Y., and Rivkin, M.I. (1997) *Xcm*I-containing vector for direct cloning of PCR products. *Biotechniques* **22**: 812–814.

Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., *et al.* (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.

Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162**: 156–159.

Cloud, J.L., Marconi, R.T., Eggers, C.H., Garon, C.F., Tilly, K., and Samuels, D.S. (1997) Cloning and expression of the *Borrelia burgdorferi lon* gene. *Gene* **194**: 137–141.

Condon, C., Squires, C., and Squires, C.L. (1995) Control of rRNA transcription in *Escherichia coli*. *Microbiol Rev* **59**: 623–645.

Cotter, P.A., and Miller, J.F. (1998) *In vivo and ex vivo* regulation of bacteria virulence gene expression. *Curr Opin Microbiol* **1**: 17–26.

Cox, D.L., Akins, D.R., Bourell, K.W., Lahdenne, P., Norgard, M.V., and Radolf, J.D. (1996) Limited surface exposure of *Borrelia burgdorferi* outer surface lipoproteins. *Proc Natl Acad Sci USA* **93**: 7973–7978.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., *et al.* (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390**: 580–586.

Ge, Y., and Charon, N.W. (1997) Identification of a large motility operon in *Borrelia burgdorferi* by semi-random PCR chromosome walking. *Gene* **189**: 195–201.

Ge, Y., Old, I.G., Saint Girons, I., and Charon, N.W. (1997) Molecular characterization of a large *Borrelia burgdorferi* motility operon which is initiated by a consensus σ^{70} promoter. *J Bacteriol* **179**: 2289–2299.

Gelfand, M.S. (1999) Recognition of regulatory sites by genomic comparison. *Res Microbiol* **150**: 755–771.

Gorbacheva, V.Y., Godfrey, H.P., and Cabello, F.C. (2000) Analysis of the *bmp* gene family in *Borrelia burgdorferi*. *J Bacteriol* **182**: 2037–2042.

Harley, C.B., and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**: 2343–2361.

Howe, T.R., LaQuier, F.W., and Barbour, A.G. (1986) Organization of genes encoding two outer membrane proteins of Lyme disease agent *Borrelia burgdorferi* within a single transcriptional unit. *Infect Immun* **54**: 207–212.

Indest, K.J., Ramamoorthy, R., Solé, M., Gilmore, R.D., Johnson, B.J.B., and Philipp, M.T. (1997) Density-dependent expression of *Borrelia burgdorferi* lipoproteins *in vitro*. *Infect Immun* **65**: 1165–1171.

Jonsson, M., Noppa, L., Barbour, A.G., and Bergström, S. (1992) Heterogeneity of outer surface membrane proteins in *Borrelia burgdorferi:* comparison of the *osp* operons of three isolates of different geographic origins. *Infect Immun* **60**: 1845–1853.

McClure, W.R. (1985) Mechanism and control of transcription initiation in prokaryotes. *Annu Rev Biochem* **54**: 171–204.

Margolis, N., Hogan, D., Cieplack, W., Jr, Schwan, T.G., and Rosa, P.P. (1994) Homology between *Borrelia burgdorferi* OspC and members of the family of *Borrelia hermsii* variable proteins. *Gene* **143**: 105–110.

Platt, T. (1986) Transcription termination and the regulation of gene expression. *Annu Rev Biochem* **55**: 339–372.

Porcella, S.F., Popova, T.G., Akins, D.R., Li, M., Radolf, J.D., and Norgard, M.V. (1996) *Borrelia burgdorferi* supercoiled plasmids encode multicopy tandem open reading frames and a lipoprotein gene family. *J Bacteriol* **178**: 3293–3307.

Ramamoorthy, R., and Philipp, M.T. (1998) Differential expression of *Borrelia burgdorferi* proteins during growth *in vitro*. *Infect Immun* **66**: 5119–5124.

Ramamoorthy, R., Povinelli, L., and Philipp, M.T. (1996) Molecular characterization, genomic arrangement, and expression of *bmpD*, a new member of the *bmp* class of genes encoding membrane proteins of *Borrelia burgdorferi*. *Infect Immun* **64**: 1259–1264.

Rauhut, R., and Klug, G. (1999) mRNA degradation in bacteria. *FEMS Microbiol Rev* **23**: 353–370.

Saito, K., Mattheakis, L.S., and Nomura, M. (1994) Post-transcriptional regulation of the *str* operon in *Escherichia coli*. *J Mol Biol* **235**: 111–124.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Plainview, NY: Cold Spring Harbor Laboratory Press.

Sartakova, M., Dobrikova, E., and Cabello, F.C. (2000) Development of an extrachromosomal cloning vector system for use in *Borrelia burgdorferi*. *Proc Natl Acad Sci USA* **97**: 4850–4855.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995) Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc Natl Acad Sci USA* **92**: 2909–2913.

de Silva, A.M., Zeidner, N.S., Zhang, Y., Dolan, M.C., Piesman, J., and Fikrig, E. (1999) Influence of outer surface protein A antibody on *Borrelia burgdorferi* within feeding ticks. *Intect Immun* **67**: 30–35.

Simpson, W.J., Cieplak, W., Schrumpf, M.E., Barbour, A.G., and Schwan, T.G. (1994) Nucleotide sequence and analysis of the gene in *Borrelia burgdorferi* encoding the immunogenic P39 antigen. *FEMS Microbiol Lett* **119**: 381–388.

Sinsky, R.J., and Piesman, J. (1989) Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. *J Clin Microbiol* **27**: 1723–1727.

Sohaskey, C., Zückert, W., and Barbour, A.G. (1999) The extended promoters for two outer membrane lipoprotein genes of *Borrelia* spp. uniquely include a T-rich region. *Mol Microbiol* **33**: 41–51.

Subramanian, G., Koonin, E.V., and Aravind, L. (2000) Comparative genome analysis of the pathogenic spirochetes *Borrelia burgdorferi* and *Treponema pallidum*. *Infect Immun* **68**: 1633–1648.

Suk, K., Das, S., Sun, W., Jwang, B., Barthold, S.W., Flavell, R.A., and Fikrig, E. (1995) *Borrelia burgdorferi* genes selectively expressed in the infected host. *Proc Natl Acad Sci USA* **92**: 4269–4273.

Tilly, K., Fuhrman, J., Campbell, J., and Samuels, D.S. (1996) Isolation of *Borrelia burgdorferi* genes encoding homologues of DNA-binding protein HU and ribosomal protein S20. *Microbiology* **142**: 2471–2479.

Zimmerman, K., and Mannhalter, J.W. (1997) Technical aspects of quantitative competitive PCR. In *The PCR Technique: Quantitative PCR.* Larrick, J.W. (ed.) Natick, MA. Eaton Publishing, pp. 3–17.

Zuker, M. (1989) On folding all suboptimal foldings of an RNA molecule. *Science* **244**: 48–52.