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Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*

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Summary

The paradigm for differential antigen expression in *Borrelia burgdorferi*, the agent of Lyme disease, is the reciprocal expression of its outer surface (lipo)proteins (Osp) A and C; as *B. burgdorferi* transitions from its arthropod vector into mammalian tissue, *ospC* is upregulated, and *ospA* is downregulated. In the current study, using *B. burgdorferi* cultivated under varying conditions in BSK-H medium, we found that a decrease in pH, in conjunction with increases in temperature (e.g. 34°C or 37°C) and cell density, acted interdependently for the reciprocal expression of *ospC* and *ospA*. The lower pH (6.8), which induced the reciprocal expression of *ospC* and *ospA* in BSK-H medium, correlated with a drop in pH from 7.4 to 6.8 of tick midgut contents during tick feeding. In addition to *ospC* and *ospA*, other genes were found to be regulated in reciprocal fashion. Such genes were either *ospC*-like (e.g. *ospF*, *mlp-8* and *rpoS*) (group I) or *ospA*-like (*lp6.6* and *p22*) (group II); changes in expression occurred at the mRNA level. That the expression of *rpoS*, encoding a putative stress-related alternative sigma factor (σ^s), was *ospC*-like suggested that the expression of some of the group I genes may be controlled through σ^s. The combined results prompt a model that allows for predicting the regulation of other *B. burgdorferi* genes that may be involved in spirochaete transmission, virulence or mammalian host immune responses.

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Introduction

Borrelia burgdorferi, the spirochaetal agent of Lyme disease (Steere, 1989), is transmitted when infected *Ixodes* ticks feed on susceptible mammalian hosts; *B. burgdorferi* is thus maintained in nature via a complex enzootic cycle (Steere, 1993). As the spirochaete transitions from its arthropod vector into mammalian tissues, it undergoes dramatic alterations in the expression patterns of its outer surface lipoproteins, the paradigm of which is the reciprocal downregulation and upregulation of the outer surface (lipo)proteins (Osp) A (OspA) (Barbour *et al*., 1983) and C (OspC) (Wilske *et al*., 1993; Stevenson and Barthold, 1994) respectively (Schwan *et al*., 1995; Montgomery *et al*., 1996; de Silva *et al*., 1996). Thus, in flat ticks, spirochaetes in the lumen of the midgut express OspA abundantly but synthesize little, if any, OspC. When ticks engorge, OspA is downregulated, and OspC is upregulated.

OspA has emerged as a particularly important molecule for understanding *B. burgdorferi* membrane biology (Cox *et al*., 1996; Philipp, 1998); its three-dimensional structure has been solved (Li *et al*., 1997; Pham *et al*., 1998), and it also comprises the Lyme disease vaccine recently approved for human use (Steere *et al*., 1998; Steigbigel and Benach, 1998). As such, an understanding of OspA expression, function, membrane topology and role in immune responses continues to be the subject of intensive investigation.

Environmental cues that trigger the differential expression of *B. burgdorferi* genes during the transmission process are just now beginning to be elucidated. Under *in vitro* cultivation conditions, a number of genes, including *ospC*, are induced at elevated temperature (e.g. 37°C), suggesting that a temperature increase in the tick midgut during engorgement contributes to the upregulation of these genes (Schwan *et al*., 1995; Stevenson *et al*., 1995; 1998; Akins *et al*., 1998; Cassatt *et al*., 1998; Yang *et al*., 1999). However, incubation of unfed ticks at 37°C does not induce *ospC* (Schwan *et al*., 1995), suggesting that temperature alone is not sufficient to upregulate *ospC* expression. Recent data suggest that high spirochaete density, at least under *in vitro* cultivation conditions, also influences the upregulation of *ospC* (Indest *et al*., 1997; Ramamoorthy and Philipp, 1998). This notion is consistent

Table 1. pH values of various *Ixodes scapularis* compartments.

Tick compartment	Feeding status	*B. burgdorferi* infection status	*n*[a]	pH[b]
Midgut				
Adult	Unfed	Uninfected	3	7.37 ± 0.04
Adult	Unfed	Infected	4	7.39 ± 0.12
Adult	Fed	Uninfected	3	6.83 ± 0.02
Adult	Fed	Infected	5	6.89 ± 0.08
Nymph	Unfed			ND[c]
Nymph	Fed	Uninfected	5	6.84 ± 0.14
Nymph	Fed	Infected	7	6.83 ± 0.18
Haemolymph				
Adult	Unfed	Uninfected	3	6.82 ± 0.03
Adult	Fed	Uninfected	4	6.78 ± 0.04
Adult	Fed	Infected	5	6.81 ± 0.11
Saliva				
Adult	Unfed	Uninfected	3	9.68 ± 0.05

a. Number of independent measurements.
b. Values represent the mean ± standard error.
c. ND, not determined.

with the fact that spirochaete numbers within the tick midgut increase dramatically during tick engorgement (de Silva and Fikrig, 1995). In addition, de Silva *et al*. (1999) showed that, when ticks fed on OspA-immunized mice, the number of spirochaetes in the midgut not only became significantly reduced, but the remaining organisms failed to produce OspC. Another environmental factor, pH, may also affect the expression of *ospC*; Carroll *et al*. (1999) showed that *B. burgdorferi* did not produce OspC when spirochaetes were cultivated at pH 8.0.

In contrast to *ospC*, *in vitro* expression of *ospA* by *B. burgdorferi* appears to be unaffected by temperature or cell density and, thus, little is known about environmental cues that may modulate the expression of *ospA*. However, under natural conditions, the upregulation of *ospC* appears to be coupled with the downregulation of *ospA* (Schwan *et al*., 1995; Montgomery *et al*., 1996; de Silva *et al*., 1996). The current study was thus designed with two principal objectives. The first was to investigate parameters that might influence the downregulation of *ospA*. The second was to examine whether the upregulation of *ospC* and downregulation of *ospA* are inter-related. In this paper, we present data showing that a modest decrease in environmental pH, in conjunction with elevated temperature and cell density, triggers the reciprocal expression of two groups of *B. burgdorferi* genes, among which are *ospA* and *ospC*. These findings suggest that *ospA* and *ospC* are co-regulated by the same set of environmental stimuli. Our results also engender a new *in vitro* model system in which to study further the molecular mechanisms involved in the regulation of *ospA*, *ospC* and other genes of *B. burgdorferi*. The physiological significance of these findings to the mechanisms governing differential or reciprocal antigen expression in *B. burgdorferi* are discussed.

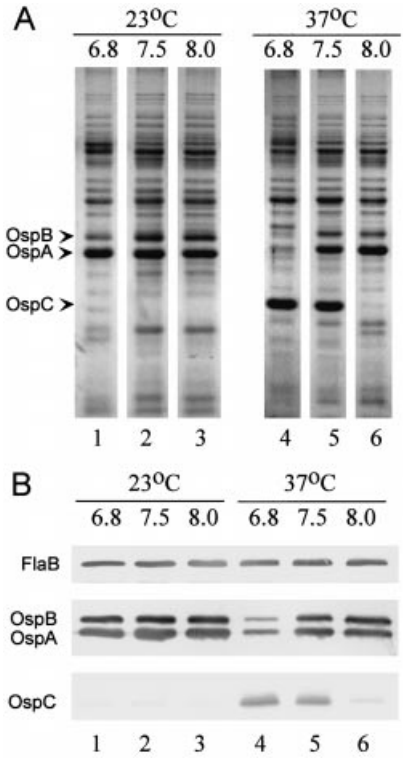

Fig. 1. Influence of temperature and pH on the levels of OspA and OspC in *B. burgdorferi* 297 cultivated in BSK-H medium. Low-passage *B. burgdorferi* was inoculated at a final concentration of 1×10^3 spirochaetes ml^{-1} in BSK-H medium adjusted to various pH values. Cultures were incubated at either 23°C or 37°C and were harvested when the spirochaetes achieved a level of 5×10^7 ml^{-1} (late logarithmic phase).
A. SDS–polyacrylamide gel stained with Coomassie brilliant blue. Polypeptides corresponding to OspA, OspB and OspC are designated by arrowheads on the left. Each gel lane contained protein from 1×10^8 spirochaetes.
B. Immunoblots of samples corresponding to the gel lanes in (A). Antibodies used to detect each antigen (left) are described in the text. To detect FlaB, protein from 2×10^7 spirochaetes was loaded in each gel lane. To observe the actual relative differences between the levels of OspA and OspC, protein from proportionally fewer (1×10^6) spirochaetes was loaded in each gel lane.

Results

pH values of tick tissue compartments

It is known that pH varies among different tick tissue compartments; the pH of the midgut of an unfed *Ixodes* species tick is 7.2–7.6 (Balashov, 1972) and in the range of 9.5 for the saliva of *Amblyomma americanum* ticks (Bowman *et al*., 1995). To examine these values for *I. scapularis* and to assess whether pH represents a varying environmental factor for *B. burgdorferi* during tick feeding, the pH values of various tick tissue compartments were measured under various conditions of tick feeding. The average pH of the midgut contents of unfed adult ticks was about 7.4 (Table 1), consistent with previous findings (Balashov, 1972). The results were the same regardless

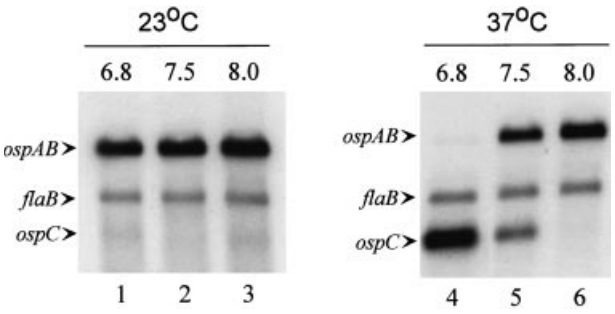

Fig. 2. Northern blot analysis for the expression of *ospA* and *ospC* in *B. burgdorferi*. RNAs from *B. burgdorferi* 297 cultivated at different temperatures (indicated at the top) and pH (indicated above the lanes) were hybridized with probes specific for *ospAB*, *flaB* and *ospC*. The bands corresponding to each transcript are labelled on the left. Assessment of the level of *flaB* mRNA was used as an internal control to ensure equivalent RNA loading among the various gel lanes.

of the *B. burgdorferi* infection status of *I. scapularis*. After 4 days of adult tick feeding, the pH of the midgut contents dropped to 6.8–6.9. The pH values for midgut contents of partially engorged *I. scapularis* nymphs (either uninfected or infected with *B. burgdorferi*) were also 6.8 (Table 1). The pH of adult tick haemolymph was about 6.8, regardless of the feeding status or the *B. burgdorferi* infection status of the ticks (Table 1). The pH of adult tick saliva (unfed; uninfected with *B. burgdorferi*) was approximately 9.7 (Table 1).

OspA *is downregulated during* in vitro *conditions of reduced pH (6.8) and elevated temperature*

The results in Table 1 prompted the question as to whether only modest changes in environmental pH, such as those observed during tick feeding, could affect gene expression in *B. burgdorferi* strain 297. To examine this, BSK-H medium was aliquoted into separate sterile flasks; the pH of each aliquot, before inoculation with *B. burgdorferi*, was adjusted to 6.0, 6.8, 7.5, 8.0 or 9.0. The cultures were then inoculated to 1×10^3 spirochaetes ml^{-1} and incubated at either 23°C or 37°C; the final concentration of cells at harvest was 5×10^7 ml^{-1} (late log phase). Upon cell harvest, the pH of each culture was virtually unchanged, not deviating more than 0.1 pH unit. Cultures at either pH 6.0 or 9.0 did not confer growth after 3 weeks of incubation. The cultures at pH 6.8 grew more slowly than the cultures at pH 7.5, especially those incubated at 23°C.

At the cultivation temperature of 23°C, there were no major alterations in the protein profiles of spirochaetes cultivated under the varying pH conditions (Fig. 1A, lanes 1–3). In particular, levels of OspA and OspC remained constant, as assessed by immunoblotting (Fig. 1B, lanes 1–3). However, when separate cultures were incubated at either 34°C or 37°C and harvested at the late logarithmic stage, there were dramatic alterations in protein profiles of spirochaetes cultivated at pH 6.8, 7.5 and 8.0 (Fig. 1A, lanes 4–6; not shown for 34°C). Most strikingly, OspA was abundant in borreliae grown at pH 7.5 or 8.0 (Fig. 1A and B, lanes 5 and 6), but sharply downregulated during growth of the spirochaetes at pH 6.8 (Fig. 1A and B, lanes 4). In contrast to the downregulation of OspA, OspC was upregulated at the lower culture pH values of 6.8 and 7.5 (Fig. 1A and B, lanes 4 and 5), but was virtually undetectable from the culture grown at pH 8.0 (Fig. 1A and B, lanes 6).

Adjustments in the pH of the BSK-H medium using HCl or NaOH introduced additional Na^+ and Cl^- ions; such adjustments accounted for not more than 10% of the total NaCl concentration in BSK-H medium. Nevertheless, to rule out potential osmolarity effects, spirochaetes were cultivated at either 23°C or 37°C in BSK-H medium containing an additional 15 mM NaCl. Under these conditions, there were no apparent differences observed in the expression of OspA and OspC by *B. burgdorferi* (data not shown).

Regulation of ospA *by pH and temperature occurs at the transcriptional level*

To assess whether the temperature-dependent regulation of *ospA* by pH was at the transcriptional level, total RNA was isolated from cultures of *B. burgdorferi* grown under various conditions and used in Northern blot hybridizations. As shown in Fig. 2, amounts of *ospAB* and *ospC* transcripts remained unchanged under various pH conditions when cultures were incubated at 23°C (Fig. 2, lanes 1–3). However, when the temperature of the cultures was shifted from 23°C to 37°C, the amount of *ospAB* transcript was dramatically reduced for cultures grown at pH 6.8 (Fig. 2, lane 4), whereas the amount of *ospC* transcript was sharply increased (Fig. 2, lane 4). This reciprocal pattern of mRNA levels for *ospAB* and *ospC* was consistent with the protein expression pattern displayed in Fig. 1. Of note, although differences in the quantities of OspC from spirochaetes cultivated at either pH 7.5 or pH 6.8 were not readily apparent (Fig. 1A and B, lanes 4 and 5), differences between the mRNA levels for *ospC* from borreliae grown at 37°C under the two different pH conditions were marked, with greater amounts of *ospC* mRNA from spirochaetes cultivated at pH 6.8 (Fig. 2, compare lanes 4 and 5).

Downregulation of ospA *by temperature and pH is not* B. burgdorferi *strain dependent*

Our findings that culture conditions of pH 8.0 inhibited the expression of *ospC* in *B. burgdorferi* strain 297 (Figs 1 and 2) largely agree with a previously published study of

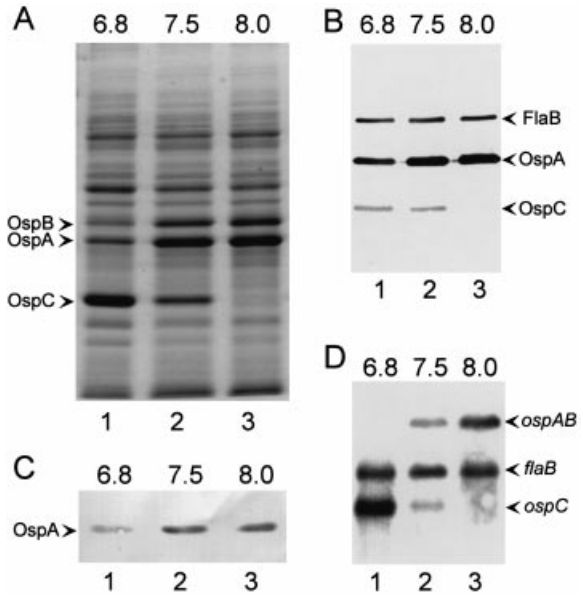

Fig. 3. Influence of pH on the expression of *ospA* and *ospC* in *B. burgdorferi* strain B31 cultivated in BSK-H medium. *B. burgdorferi* B31 was inoculated at a final concentration of 1×10^3 spirochaetes ml^{-1}, incubated at 37°C and harvested at a final concentration of 5×10^7 cells ml^{-1} (late logarithmic phase). The pH of each individual BSK-H culture was adjusted to that labelled above each gel lane.
A. SDS–polyacrylamide gel stained with Coomassie brilliant blue. Polypeptides corresponding to OspA, OspB and OspC are designated by arrows on the left. Each gel lane contained protein from 1×10^8 spirochaetes.
B. Immunoblots of samples corresponding to the gel lanes in (A). A mixture of antibody probes was used to detect the respective antigens (arrows on right). Protein from 1×10^7 spirochaetes was used in each gel lane.
C. Immunoblot as in (B), except that protein from only 5×10^5 spirochaetes was used in each gel lane; this allowed visualization of the different levels of OspA.
D. Northern blot analysis for the expression of *ospA* and *ospC*. Northern blots were performed as described in the legend to Fig. 2. Bands corresponding to each transcript are labelled on the right.

B. burgdorferi strain B31 (Carroll *et al*., 1999). However, the downregulation of *ospA* by any *sensu stricto* strain of *B. burgdorferi*, including strain B31, growing in BSK-H medium has not been reported. Carroll *et al*. (1999) showed that the level of OspA remained unchanged when *B. burgdorferi* strain B31 was cultivated at pH levels of 6.0, 7.0 or 8.0; these findings seem to contradict our observation that strain 297 downregulated its expression of *ospA* under cultivation conditions of reduced pH (6.8). To rule out possible strain effects, we repeated the experiments from Figs 1 and 2 using *B. burgdorferi* strain B31. Under our experimental conditions, levels of OspA were clearly reduced in strain B31 cultures grown at pH 6.8 (Fig. 3A–C, lanes 1), similar to that found for strain 297 (Fig. 1). As in the case of strain 297 (Fig. 2), the downregulation of *ospAB* again occurred at the transcriptional level (Fig. 3D, lane 1).

Relationship between pH and cell density effects on gene regulation in B. burgdorferi

Spirochaetal cell density influences the expression of a number of *B. burgdorferi* genes, including *ospC* (Indest *et al*., 1997; Ramamoorthy and Philipp, 1998). Inasmuch as bacterial cell density also affects the pH of the surrounding medium, we sought to determine (i) the extent to which the pH of BSK-H medium changes during the growth of *B. burgdorferi*; and (ii) whether such changes account for cell density-dependent regulatory processes (Indest *et al*., 1997; Ramamoorthy and Philipp, 1998).

In the mid-logarithmic stage of growth (5×10^6 cells ml^{-1}), the pH of the BSK-H medium remained at 7.5. Under this condition, OspC and another temperature-regulated lipoprotein, Mlp-8 (Yang *et al*., 1999), were barely detectable (Fig. 4A and B, lanes 1). OspC and Mlp-8 were more readily detectable as the culture density increased to 2×10^7 cells ml^{-1} (late logarithmic stage) (Fig. 4B, lane 2), even though the pH of the BSK-H medium was still virtually unchanged. When the *B. burgdorferi* culture achieved a cell density of 8×10^7 cells ml^{-1}, the pH of the BSK-H medium dropped from 7.5 to 7.2, and levels of both OspC and Mlp-8 were increased further (Fig. 4A and B, lanes 3). To test whether reduced pH, in the absence of potential confounding cell density effects, could account for enhanced upregulation of OspC and Mlp-8, *B. burgdorferi* was allowed to grow only to the mid-logarithmic stage in BSK-H medium adjusted to a pH of either 7.2 or 6.8. Under conditions of pH 7.2, both OspC and Mlp-8 were readily detected (Fig. 4A and B, lanes 4), which contrasted with the results for mid-log phase *B. burgdorferi* cultivated under normal BSK-H conditions (pH 7.5) (Fig. 4A and B, lanes 1). This difference was even more pronounced when cultures were grown to the mid-log stage under conditions of pH 6.8–6.9 (Fig. 4A and B, lanes 5). The combined data suggest that increased cell density and elevated temperature are sufficient to induce the expression of *ospC* and *mlp-8*. However, during increases in cell density, the drop in pH from 7.5 to 7.2 that accompanies this cell increase partially contributes to the further upregulation of *ospC* and *mlp-8*.

Identification of other pH-regulated genes in B. burgdorferi

In addition to *ospA*, *ospC* and *mlp-8*, several other genes of *B. burgdorferi* have been shown to be differentially regulated (Champion *et al*., 1994; Akins *et al*., 1995; 1998; Stevenson *et al*., 1995; Suk *et al*., 1995; Wallich *et al*., 1995; Aron *et al*., 1996; Porcella *et al*., 1996; Das *et al*., 1997; Lahdenne *et al*., 1997; Fikrig *et al*., 1998; 1999; Ramamoorthy and Philipp, 1998; de Silva *et al*.,

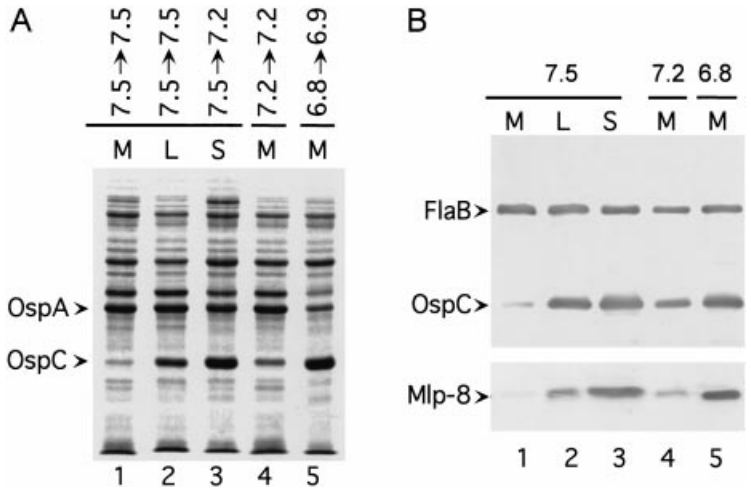

Fig. 4. Influence of pH and cell density on the levels of OspA, OspC and Mlp-8 in *B. burgdorferi* 297. *B. burgdorferi* was inoculated at a final concentration of 1×10^3 spirochaetes ml^{-1} in BSK-H medium adjusted to various pH values. The cultures were incubated at 37°C and harvested at different cell densities, which are labelled above the lanes as M (mid-logarithmic phase; 5×10^6 cells ml^{-1}), L (late logarithmic phase; 2×10^7 cells ml^{-1}) or S (stationary phase; 8×10^7 cells ml^{-1}).
A. SDS–polyacrylamide gel stained with Coomassie brilliant blue. The pH of each culture upon inoculation and the final pH of each culture at cell harvest are labelled above the lanes and separated by arrows. Polypeptides corresponding to OspA and OspC are labelled on the left. Each gel lane contains protein from 1×10^8 spirochaetes.
B. Immunoblots of aliquots from (A). For the detection of FlaB and OspC, protein from 1×10^7 spirochaetes was loaded in each gel lane, which were then probed with a mixture of antibodies directed against FlaB and OspC. For the detection of Mlp-8, which is much less abundant than FlaB and OspC, protein from 2×10^8 spirochaetes was loaded per gel lane and probed with antiserum directed against Mlp-8.

1999). Experiments were thus conducted to infer whether other *B. burgdorferi* genes were influenced by pH. At least four additional pH-dependent proteins were identified by immunoblotting of *B. burgdorferi* cultivated under various pH conditions (Fig. 5). Two of these, outer surface protein F (*ospF*) (Akins *et al*., 1995) and the decorin-binding protein A (*dbpA*) (Guo *et al*., 1995; Hagman *et al*., 1998), were expressed at pH 6.8 or 7.5, but not at pH 8.0 (*ospC*-like), although pH 7.5 was superior to pH 6.8 for the induction of *dbpA*. Two others, the 6.6 kDa lipoprotein (*lp6.6*) (Lahdenne *et al*., 1997) and a 22 kDa protein (not related to P22; Lam *et al*., 1994; Grewe and Nuske, 1996;

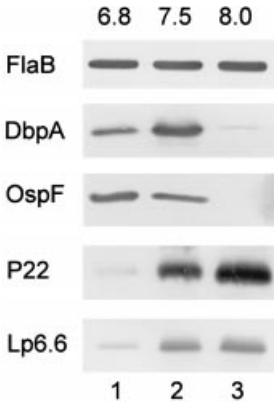

Fig. 5. Other pH-regulated proteins of *B. burgdorferi* 297. Spirochaetes cultivated at 37°C under different pH conditions (labelled above the lanes) were harvested at the late logarithmic stage of growth (5×10^7 ml^{-1}). Whole-cell lysates were then probed with antibodies directed against the respective antigens (indicated on the left). Total protein from approximately 2×10^7 cells was loaded in each gel lane, except for the detection of Mlp-8 and OspF (protein from 2×10^8 cells).

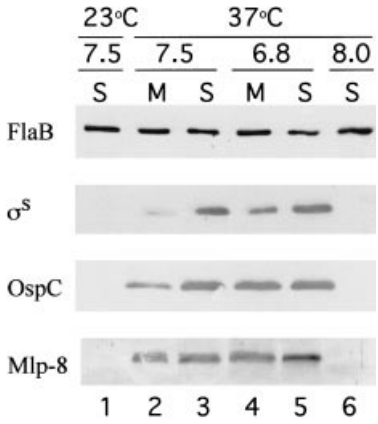

Fig. 6. Multiple environmental factors regulate the expression of σ^S, OspC and Mlp-8 in *B. burgdorferi* 297. Whole-cell lysates from spirochaetes were cultivated at different temperatures (top), different pH levels (6.8, 7.5 or 8.0) and to different cell densities. The stages of growth at the time of cell harvest are indicated above each lane as M (mid-log phase; 2×10^7 cells ml^{-1}) and S (stationary phase; 8×10^7 cells ml^{-1}). Gels were probed with antibodies directed against each respective antigen (designated on the left). For the detection of FlaB and OspC, protein from 1×10^7 cells was used per gel lane. For the detection of σ^S and Mlp-8, protein from 2×10^8 cells was loaded per gel lane.

Akins *et al*., 1998) (*p22*) were downregulated (*ospA*-like). Like *ospA* and *ospC*, genes in both groups were regulated by pH in a temperature-dependent manner; in other words, pH regulation occurred at elevated temperature (i.e. 37°C), but not at lower temperature (i.e. 23°C) (data not shown).

Regulation of rpoS *by multiple environmental factors*

To elucidate further potential mechanisms involved in the regulation of *B. burgdorferi* genes by environmental cues, some putative global regulators identified by theoretical analysis of the *B. burgdorferi* genome sequence (Fraser *et al*., 1997) were also examined. For these studies, we focused on the putative *rpoS* gene, which ostensibly encodes the transcription factor σ^s. In addition to its critical role in stationary phase dynamics, σ^s has been shown to be involved in many organisms in the response to multiple environmental cues (Hengge-Aronis, 1996; 1999). As predicted, the level of σ^s in *B. burgdorferi* was cell density dependent (Fig. 6, lanes 2 and 3). In addition, expression of *rpoS* in *B. burgdorferi* also appeared to be regulated by temperature (Fig. 6; lane 1 versus lane 3), as well as by pH (lanes 2–6). The cell density-, pH- and temperature-dependent regulatory pattern for *rpoS* was similar to the regulation pattern observed for *ospC* and *mlp-8* (Fig. 6).

Discussion

From elegant studies of tick feeding and *B. burgdorferi* transmission, it is well documented that the upregulation of *ospC* is coupled with a concomitant downregulation of *ospA* (Schwan *et al*., 1995; de Silva *et al*., 1996). The upregulation of *ospC* and downregulation of *ospA* has also been observed in selected *B. burgdorferi* model systems. Akins *et al*. (1998) showed a reciprocal pattern of OspA/OspC expression among borreliae cultivated in dialysis membrane chambers implanted into the peritoneal cavities of rats. More recently, Obonyo *et al*. (1999) reported the OspA/OspC paradigm for spirochaetes co-cultivated with tick cells. The complexity of these systems has, however, precluded precise identification of individual parameters that might be involved in the reciprocal regulation of *ospC* and *ospA*. A shift to higher temperature appears to be one important stimulus for triggering the induction of *ospC* (Schwan *et al*., 1995; Stevenson *et al*., 1995). However, no physical parameter influencing the regulation of *ospA* has yet been identified. In the current study, using *B. burgdorferi* cultivated under varying conditions in BSK-H medium, we found that a decrease in pH, in conjunction with increases in temperature and cell density, acted interdependently for the reciprocal expression of *ospC* and *ospA*. This suggests that the same combination of environmental factors can control the expression of both *ospC* and *ospA*, albeit in reciprocal fashion.

A key observation in our study was a lowering of the pH to 6.8 in the midguts of *B. burgdorferi*-infected adult or nymphal ticks upon feeding, suggesting that *B. burgdorferi* is exposed to a signal of decreasing pH conditions during tick feeding. It was not possible to ascertain the pH values of the midgut contents from unfed nymphs, the relevant vector for the transmission of *B. burgdorferi*, because of their exceedingly small size. However, there is no reason to believe that the pH of unfed nymphal midgut contents should deviate from that of adults (i.e. pH 7.4). The pH drop observed within *I. scapularis* tick midguts during engorgement is supported by other studies; tick blood meal digestion occurs at an acidic pH, typically in the range 6.3–6.5 (Coons *et al*., 1986; Mendioloa *et al*., 1996). In fact, major tick midgut digestive enzymes tend to be aspartic proteinases and cysteine proteinases that have pH optimums of about 3.0 (Mendioloa *et al*., 1996). Interestingly, the pH of tick haemolymph was the same as that of the midguts of fed ticks (6.8), suggesting that the lower pH encountered by *B. burgdorferi* during the tick's ingestion of blood is sustained during the spirochaete's migration from the midgut into the haemolymph.

Slight changes in environmental pH have been reported to be sufficient to induce the expression of bacterial virulence factors (Vriesema *et al*., 2000). The findings of a narrow pH drop in the midguts of ticks during feeding prompted us to examine whether reproduction of this pH change in BSK-H medium could affect gene expression in *B. burgdorferi*. Surprisingly, we showed that, when the pH of BSK-H medium was adjusted to the pH of the midgut contents of fed ticks (i.e. pH 6.8), *B. burgdorferi* displayed the predicted reciprocal expression pattern for *ospA* and *ospC*. This phenomenon, however, was temperature dependent, occurring only at elevated (34°C or 37°C) temperature. The temperature dependence of the pH-mediated response appears to be consistent with, and thus physiologically relevant to, the pH and temperature shifts that transpire as ticks take their blood meal. To the best of our knowledge, this is the first study to demonstrate the downregulation of *ospA* by *B. burgdorferi* cultivated alone in artificial medium.

Carroll *et al*. (1999) were the first to draw attention to the importance of pH in gene regulation by *B. burgdorferi* (strain B31). A key finding of their study was a sharp downregulation of *ospC* when spirochaetes were cultivated in BSK-H medium at pH 8.0. We confirmed this observation for *B. burgdorferi* strain 297, another *sensu stricto* strain. In the study by Carroll *et al*. (1999), the authors also cultivated *B. burgdorferi* strain B31 under reduced pH conditions of pH 7.0 or 6.0; under these conditions, a downregulation of *ospA* was not observed. In contrast, we showed that lowering the pH culture

condition to 6.8 sharply downregulated the expression of *ospA* in both the 297 and the B31 strains of *B. burgdorferi*. At least one major difference between the design of our experiments and those of Carroll *et al*. (1999) may have accounted for this disparity. In the study by Carroll *et al*. (1999), spirochaetes were initially subcultured into BSK-H medium at a concentration of 1×10^7 ml^{-1}, a relatively high concentration of cells already nearing the later stages of *in vitro* growth for *B. burgdorferi*. Spirochaetes were then harvested for analysis after achieving a level of 5×10^7 ml^{-1}, representing only slightly more than two cell generations. In our experiments, spirochaetes were subcultured at an initial concentration of 1×10^3 ml^{-1} and harvested when spirochaetes reached 5×10^7 ml^{-1}; this represented approximately 16 generations, starting with organisms derived from early growth phase culture dynamics. Parenthetically, the downregulation of *ospA* expression in the B31 and N40 strains of *B. burgdorferi* in our hands was slightly less marked than for strain 297 (X. Yang and M. V. Norgard, unpublished data). Also, spirochaetes cultivated in BSK II medium (rather than BSK-H medium) did not display pH-dependent downregulation of *ospA* (X. Yang and M. V. Norgard, unpublished observations); the reasons for this disparity are unclear.

Two important studies highlighting the effect of cell density on antigen expression by *B. burgdorferi* have been reported (Indest *et al*., 1997; Ramamoorthy and Philipp, 1998). Consistent with these studies (Indest *et al*., 1997; Ramamoorthy and Philipp, 1998), we have shown that, when the pH of BSK-H cultures was constant, increased cell density indeed contributed to the induction of *ospC* and *mlp-8*. The precise mechanism of this cell density-dependent effect is unknown and, as yet, there is no basis to propose quorum sensing (Hastings and Greenberg, 1999). On the other hand, we also showed that, when cultures were grown only to low cell density under the lower pH values of 7.2 and 6.8, *ospC* expression still increased, as did the expression of *mlp-8*. These results suggest that, at 37°C, minor decreases in environmental pH are sufficient to induce *ospC* and *mlp-8*. Under these same conditions, *ospA* is downregulated, suggesting that *ospC* and *ospA* are co-regulated.

In addition to *ospC* and *ospA*, we identified five additional *B. burgdorferi* genes whose control patterns could be divided into two distinct groups. Genes within the same group are similar not only with respect to their regulation by pH, but also in their control by another subset of environmental factors. Members of group I, which are *ospC*-like in that they are upregulated at lower pH, include *dbpA* (Guo *et al*., 1995; Hagman *et al*., 1998), *mlp-8* (Yang *et al*., 1999) and *ospF* (Akins *et al*., 1995); these genes are also upregulated by elevated temperature (e.g. 37°C) (Schwan *et al*., 1995; Stevenson *et al*., 1995; Akins *et al*., 1998; Cassatt *et al*., 1998; Yang *et al*., 1999), and at least two of them [e.g. *ospC* (Ramamoorthy and Philipp, 1998) and *mlp-8*] are upregulated at higher cell density. Of note, most of the group I genes have been shown to be expressed during mammalian infection (Akins *et al*., 1995; Cassatt *et al*., 1998; Hagman *et al*., 1998; Yang *et al*., 1999), whereas the group II genes, which are *ospA/B*-like [e.g. *p22* (Akins *et al*., 1998) and *lp6.6* (Lahdenne *et al*., 1997)] are sharply downregulated during the mammalian phase of infection by *B. burgdorferi*. We also showed a close correlation between the expression of borrelial *rpoS* (Fraser *et al*., 1997; Elias *et al*., 2000), encoding the putative global stress response regulator σ^s, and the group I genes; the same environmental conditions induced the expression of *rpoS* and the group I genes. This correlation implies that transcription of at least some of the group I genes may be controlled through *rpoS*. DNA sequence analysis typically cannot discern which genes are σ^s dependent, because promoter elements recognized by σ^s are very similar to those recognized by σ^{70}. The recent successes in the construction of an *rpoS* mutant of high-passage *B. burgdorferi* B31 (Elias *et al*., 2000) and in the development of a new shuttle cloning vector for *B. burgdorferi* (Sartakova *et al*., 2000) may be used in combination for delineating directly the role of σ^s in the regulation of *B. burgdorferi* genes.

That genes within each group are co-regulated in either an *ospC*-like or an *ospA*-like manner suggests that the classical expression paradigm for *ospC*/*ospA* may be extended to include other *B. burgdorferi* genes. This is significant because it may be possible to predict the regulation pattern for other *B. burgdorferi* genes that segregate into one of the two regulatory patterns. For example, one might predict that other group I genes (e.g. *mlp8* and *ospF*), like *ospC*, are not only expressed during the mammalian phase of infection (Akins *et al*., 1998; Yang *et al*., 1999), but are also upregulated during the process of tick engorgement. Conversely, group II genes (e.g. *lp6.6* and *p22*), like *ospA*, may be downregulated in *B. burgdorferi* early in the process of tick feeding (i.e. within tick midguts). In support of these predictions, preliminary data from indirect immunofluorescence microscopy have shown that *mlp-8* (group I) is indeed induced during tick engorgement (X. Yang and M. V. Norgard, unpublished observations). In contrast, *lp6.6* (group II) is expressed solely in unfed tick midguts (G. B. Schoeler and S. K. Wikel, unpublished observations). One of the putative group I genes, however, may not fit the general rule entirely because *dbpA* expression, unlike *ospC*, was substantially reduced at pH 6.8 (relative to pH 7.5). That *dbpA* expression is reduced at pH 6.8 is consistent with the recent finding that *dbpA* is not expressed by *B. burgdorferi* in the midguts of fed ticks (Hagman *et al*., 2000).

Work presented in this study prompts a model for the

molecular basis of reciprocal gene regulation in *B. burgdorferi*. In unfed tick midguts, the concentration of spirochaetes and the environmental temperature should be low, whereas the pH is relatively high (pH 7.4). Under these conditions, levels of expression of the group I genes (e.g. *ospC*, *ospF* and *mlp8*) are reduced, whereas levels of expression of the group II genes (e.g. *ospA/B*, *p22* and *lp6.6*) are high. When ticks begin the process of feeding, the temperature within midguts is ostensibly elevated, at least transiently, from the blood meal, and the pH drops to 6.8. Spirochaetes begin to multiply, resulting in increased cell density. These three factors act in an integrated fashion and are sufficient to induce the reciprocal expression of the group I and group II genes. Regulation of some of the group I genes may also be *rpoS* dependent. This model, which will undoubtedly undergo refinements, nevertheless provides a new framework from which to investigate further the molecular mechanisms involved in the regulation of *ospA*, *ospC* and, ostensibly, other *B. burgdorferi* genes implicated in spirochaete transmission, virulence or mammalian host immune responses.

Experimental procedures

Bacterial strains and culture conditions

Low-passage, virulent *B. burgdorferi* strains 297 and B31 have been described previously (Fraser *et al*., 1997; Hagman *et al*., 1998). Borreliae were cultivated *in vitro* in BSK-H medium (Sigma Chemical) (Pollack *et al*., 1993) under various conditions. Spirochaetes 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 grown to a final concentration of $< 1 \times 10^7$ cells ml^{-1}. To adapt *B. burgdorferi* to culture conditions at 23°C, spirochaetes first were diluted to about 1×10^6 cells ml^{-1} and incubated for 1 week. These 23°C-adapted spirochaetes were then 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 1×10^3 cells ml^{-1} and then incubated at either 23°C or 37°C. Cultures were allowed to grow to various cell densities, determined by counting spirochaetes via darkfield microscopy. The phase of growth of each culture was extrapolated from growth curves of *B. burgdorferi* incubated under various culture conditions. *Escherichia coli* strain XL1-Blue (Stratagene) was used as a cloning host, and strain DH5α was used as the host for protein purification. *E. coli* cells were cultivated either in yeast–tryptone broth or on yeast–tryptone agar supplemented with 100 μg ml^{-1} ampicillin.

Determination of pH values for various tick compartments

Pathogen-free *I. scapularis* nymphs were derived from a colony maintained in the laboratory of S. K. Wikel. Maintenance and infection of *I. scapularis* with *B. burgdorferi* 297 have been described previously (Yang *et al*., 1999). To obtain partially fed nymphal or adult ticks, ticks were forcibly detached on day 4 of feeding from either C3H/HeJ mice (nymphs) or rabbits (adult ticks). To determine the pH values of tick midgut contents, intact midguts were dissected out under a stereomicroscope, and one to five isolated organs (depending on their sizes) were pooled for measurement (no diluent added). For measuring the pH of tick haemolymph, the dorsal cuticle behind the capitulum was removed to expose the internal tissues, leaving the midgut intact, and the pH microelectrode was inserted directly into the haemolymph compartment; the procedure was carried out under the stereomicroscope to ensure that the tip of the pH probe was fully emerged in the haemolymph fluid. For measuring the pH of tick saliva, adult ticks were first induced to salivate using a procedure described previously (Kaufman, 1976); saliva was collected and pooled. The pH probe used for all determinations was the MI-413S combination pH electrode (Microelectrodes), capable of measuring 0.5 μl.

Northern blot analysis

Total RNA was isolated from *B. burgdorferi* using an Ultraspec RNA Isolation System (Biotecx) according to the manufacturer's protocol. Northern blot analysis was carried out as described previously (Porcella *et al*., 1996). The hybridization probes for the flagellin (*flaB*), *ospA* and *ospC* genes have also been described previously (Porcella *et al*., 1996; Yang *et al*., 1999).

Fusion proteins

A 6-histidine fusion protein consisting of the C-terminal portion (amino acid residues 120–231) of the putative sigma-S (σ^s) gene (*rpoS*) of *B. burgdorferi* (Fraser *et al*., 1997) was generated by polymerase chain reaction (PCR) amplification of genomic DNA from *B. burgdorferi* strain B31. The oligonucleotide primers used for PCR were 5′-AGTGGATCCAGAAAAGAAAATCTAATACTAC-3′ and 5′-GCTAAGCTTCTAGGGACTATTGTCCAGGTTATATC-3′. The resultant PCR fragment contained a *Bam*HI site at the 5′ end and a *Hin*dIII site at the 3′ end. PCR products were restriction enzyme digested with *Bam*HI and *Hin*dIII, and then ligated into the corresponding polylinker sites of the vector pPROEXHTb (Gibco BRL). The resultant fusion protein was purified by affinity chromatography as described previously (Deka *et al*., 1999).

Antibodies and antisera

Rat polyclonal antisera directed against the truncated σ^s protein were prepared according to a previously published protocol (Lahdenne *et al*., 1997). Polyclonal antisera against OspC, DbpA, Lp6.6 and Mlp-8 have been described previously (Lahdenne *et al*., 1997; Akins *et al*., 1998; Hagman *et al*., 1998; Yang *et al*., 1999). Monoclonal antibodies 14D2-27, 8H3-33, 9A4-40 and 10D7-42, directed against OspA, FlaB, p22 and OspF, respectively, have been reported elsewhere (Akins *et al*., 1998).

SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting were carried out as described previously (Yang *et al*., 1999).

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References

Akins, D.R., Porcella, S.F., Popova, T.G., Shevchenko, D., Li, M., Norgard, M.V., *et al*. (1995) Evidence for *in vivo* but not *in vitro* expression of a *Borrelia burgdorferi* outer surface protein F (OspF) homologue. *Mol Microbiol* **18**: 507–520.

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., 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 Lett* **145**: 309–314.

Balashov, Y.S. (1972) Bloodsucking ticks (Ixodoidea) – vectors of diseases of man and animals. *Misc Publ Entomol Soc Am* **8**: 161–376.

Barbour, A.G., Tessier, S.L., and Todd, W.J. (1983) Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. *Infect Immun* **41**: 795–804.

Bowman, A.S., Sauer, J.R., Zhu, K., and Dillwith, J.W. (1995) Biosynthesis of salivary prostaglandins in the lone star tick, *Amblyomma americanum*. *Insect Biochem Mol Biol* **25**: 735–741.

Carroll, J.A., Garon, C.F., and Schwan, T.G. (1999) Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. *Infect Immun* **67**: 3181–3187.

Cassatt, D.R., Patel, N.K., Ulbrandt, N.D., and Hanson, M.S. (1998) DbpA, but not OspA, is expressed by *Borrelia burgdorferi* during spirochetemia and is a target for protective antibodies. *Infect Immun* **66**: 5379–5387.

Champion, C.I., Blanco, D.R., Skare, J.T., Haake, D.A., Giladi, M., Foley, D., *et al*. (1994) A 9.0-kilobase-pair circular plasmid of *Borrelia burgdorferi* encodes an exported protein: evidence for expression only during infection. *Infect Immun* **62**: 2653–2661.

Coons, L.B., Rosell-Davies, E., and Tarnowski, B.I. (1986) Bloodmeal digestion in ticks. In *Morphology, Physiology, and Behavioral Biology of Ticks.* Sauer, J.R., and Hair, J.A. (eds). Chichester: Ellis Horwood, pp. 248–271.

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.

Das, S., Barthold, S.W., Giles, S.S., Montgomery, R.R., Telford, S.R., and Fikrig, E. (1997) Temporal pattern of *Borrelia burgdorferi* p21 expression in ticks and the mammalian host. *J Clin Invest* **99**: 987–995.

Deka, R.K., Lee, Y.-H., Hagman, K.E., Shevchenko, D., Lingwood, C.A., Hasemann, C.A., *et al*. (1999) Physicochemical evidence that *Treponema pallidum* TroA is a zinc-containing metalloprotein that lacks porin-like structure. *J Bacteriol* **181**: 4420–4423.

Elias, A.F., Bono, J.L., Carroll, J.A., Stewart, P., Tilly, K., and Rosa, P. (2000) Altered stationary-phase response in a *Borrelia burgdorferi rpoS* mutant. *J Bacteriol* **182**: 2909–2918.

Fikrig, E., Feng, W., Aversa, J., Schoen, R.T., and Flavell, R.A. (1998) Differential expression of *Borrelia burgdorferi* genes during erythema migrans and Lyme arthritis. *J Infect Dis* **178**: 1198–1201.

Fikrig, E., Chen, M., Barthold, S.W., Anguita, J., Feng, W., Telford, S.R., *et al*. (1999) *Borrelia burgdorferi erpT* expression in the arthropod vector and murine host. *Mol Microbiol* **31**: 281–290.

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.

Grewe, C., and Nuske, J.H. (1996) Immunolocalization of a 22 kDa protein (IPLA7, P22) of *Borrelia burgdorferi*. *FEMS Microbiol Lett* **138**: 215–219.

Guo, B.P., Norris, S.J., Rosenberg, L.C., and Hook, M. (1995) Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. *Infect Immun* **63**: 3467–3472.

Hagman, K.E., Lahdenne, P., Popova, T.G., Porcella, S.F., Akins, D.R., Radolf, J.D., *et al.* (1998) Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two-gene operon and is protective in the murine model of Lyme borreliosis. *Infect Immun* **66**: 2674–2683.

Hagman, K.E., Yang, X., Wikel, S.K., Schoeler, G.B., Caimano, M.J., Radolf, J.D., *et al.* (2000) Decorin-binding protein A (DbpA) of *Borrelia burgdorferi* is not protective when immunized mice are challenged via tick infestation and correlates with the lack of DbpA expression by *B. burgdorferi* in ticks. *Infect Immun* **68**: 4759–4764.

Hastings, J.W., and Greenberg, E.P. (1999) Quorum sensing: the explanation of a curious phenomenon reveals a common characteristic of bacteria. *J Bacteriol* **181**: 2667–2668.

Hengge-Aronis, R. (1996) Back to log phase: σ^S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* **21**: 887–893.

Hengge-Aronis, R. (1999) Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol* **2**: 148–152.

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

Kaufman, W. (1976) The influence of various factors on fluid secretion by *in vitro* salivary glands of ixodid ticks. *J Exp Biol* **64**: 727–742.

Lahdenne, P., Porcella, S.F., Hagman, K.E., Akins, D.R., Popova, T.G., Cox, D.L., *et al.* (1997) Molecular characterization of a 6.6-kilodalton *Borrelia burgdorferi* outer

membrane-associated lipoprotein (lp6.6) which appears to be downregulated during mammalian infection. *Infect Immun* **65**: 412–421.

Lam, T.T., Nguyen, T.-P.K., Fikrig, E., and Flavell, R.A. (1994) A chromosomal *Borrelia burgdorferi* gene encodes a 22-kilodalton lipoprotein, P22, that is serologically recognized in Lyme disease. *J Clin Microbiol* **32**: 876–883.

Li, H., Dunn, J.J., Luft, B.J., and Lawson, C.L. (1997) Crystal structure of Lyme disease antigen outer surface protein A complexed with Fab. *Proc Natl Acad Sci USA* **94**: 3584–3589.

Mendioloa, J., Alonso, M., Marquetti, M.C., and Finlay, C. (1996) *Boophilus microplus*: multiple proteolytic activities in the midgut. *Exp Parasitol* **82**: 27–33.

Montgomery, R.R., Malawista, S.E., Feen, K.J.M., and Bockenstedt, L.K. (1996) Direct demonstration of antigenic substitution of *Borrelia burgdorferi ex vivo*: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. *J Exp Med* **183**: 261–269.

Obonyo, M., Munderloh, U.G., Fingerle, V., Wilske, B., and Kurtti, T.J. (1999) *Borrelia burgdorferi* in tick cell culture modulates expression of outer surface proteins A and C in response to temperature. *J Clin Microbiol* **37**: 2137–2141.

Pham, T.-N., Koide, A., and Koide, S. (1998) A stable single-layer β-sheet without a hydrophobic core. *Nature Struct Biol* **5**: 115–119.

Philipp, M.T. (1998) Studies on OspA: a source of new paradigms in Lyme disease research. *Trends Microbiol* **6**: 44–47.

Pollack, R.J., Telford, S.R., and Spielman, A. (1993) Standardization of medium for culturing Lyme disease spirochetes. *J Clin Microbiol* **31**: 1251–1255.

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.

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., and Fikrig, E. (1995) Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. *Am J Trop Med Hyg* **53**: 397–404.

de Silva, A.M., Telford, S.R., Brunet, L.R., Barthold, S.W., and Fikrig, E. (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *J Exp Med* **183**: 271–275.

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. *Infect Immun* **67**: 30–35.

Steere, A.C. (1989) Lyme disease. *N Engl J Med* **321**: 586–597.

Steere, A.C. (1993) Current understanding of Lyme disease. *Hosp Pract* **28**: 37–44.

Steere, A.C., Sikand, V.K., Meurice, F., Parenti, D.L., Fikrig, E., Schoen, R.T., *et al.* (1998) Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. *N Engl J Med* **339**: 209–215.

Steigbigel, R.T., and Benach, J.L. (1998) Immunization against Lyme disease – an important first step. *N Engl J Med* **339**: 263–264.

Stevenson, B., and Barthold, S.W. (1994) Expression and sequence of outer surface protein C among North American isolates of *Borrelia burgdorferi*. *FEMS Microbiol Lett* **124**: 367–372.

Stevenson, B., Schwan, T.G., and Rosa, P.A. (1995) Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect Immun* **63**: 4535–4539.

Stevenson, B., Bono, J.L., Schwan, T.G., and Rosa, P. (1998) *Borrelia burgdorferi* Erp proteins are immunogenic in mammals infected by tick bite, and their synthesis is inducible in cultured bacteria. *Infect Immun* **66**: 2648–2654.

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

Vriesema, A.J.M., Dankert, J., and Zaat, S.A.J. (2000) A shift from oral to blood pH is a stimulus for adaptive gene expression of *Streptococcus gordonii* CH1 and induces protection against oxidative stress and enhanced bacterial growth by expression of *msrA*. *Infect Immun* **68**: 1061–1068.

Wallich, R., Brenner, C., Kramer, M.D., and Simon, M.M. (1995) Molecular cloning and immunological characterization of a novel linear-plasmid-encoded gene, *pG*, of *Borrelia burgdorferi* expressed only *in vivo*. *Infect Immun* **63**: 3327–3335.

Wilske, B., Preac-Mursic, V., Jauris, S., Hofmann, A., Pradel, I., Soutschek, E., *et al.* (1993) Immunological and molecular polymorphisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. *Infect Immun* **61**: 2182–2191.

Yang, X., Popova, T.G., Hagman, K.E., Wikel, S.K., Schoeler, G.B., Caimano, M.J., *et al.* (1999) Identification, characterization, and expression of three new members of the *Borrelia burgdorferi* Mlp (2.9) lipoprotein gene family. *Infect Immun* **67**: 6008–6018.