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Influence of Cultivation Media on Genetic Regulatory Patterns in *Borrelia burgdorferi*

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Barbour-Stoenner-Kelly II (BSKII) medium and BSKH medium both are routinely used for the cultivation of *Borrelia burgdorferi*. However, heretofore there have been no studies to compare how these two media affect gene expression patterns in virulent *B. burgdorferi*. In the present study, we found that some *B. burgdorferi* strain 297 genes (e.g., *ospA*, *mlp-7A*, *mlp-8*, *p22*, and *lp6.6*) that typically are regulated by temperature or pH displayed their predicted pattern of expression when *B. burgdorferi* was cultivated in BSKH medium; this was not true when spirochetes were cultivated in conventional BSKII medium. The results suggest that BSKH medium is superior to BSKII medium for gene expression studies with *B. burgdorferi*.

The cultivation of virulent *Borrelia burgdorferi* in Barbour-Stoenner-Kelly (BSK) medium (4) was a seminal advance in Lyme disease research. Modifications to BSK medium ultimately led to BSKII medium, which became the mainstay of *B. burgdorferi* cultivation. About 10 years later, Pollack et al. (19) described the standardization of a modified version of BSKII, designated BSKH, in which the composition of BSKII medium was further modified to contain bovine serum albumin (BSA) and rabbit serum that were prescreened for optimal *B. burgdorferi* growth-supporting characteristics. BSKH medium has since become commercially available (Sigma Chemical Co., St. Louis, Mo.). BSKII and BSKH media are both commonly used for the cultivation of *B. burgdorferi*. However, BSKH medium has gained in popularity, although its cost and limited commercial availability at times have prompted many investigators to continue to rely upon BSKII medium formulated in their own laboratories.

There is compelling evidence that *B. burgdorferi* undergoes a dramatic change in the expression of its outer surface proteins during the different stages of its enzootic life cycle in ticks and mammals. For example, in flat ticks, spirochetes in tick midguts express substantial amounts of the outer surface (lipo)protein A (OspA), with little or no expression of OspC (12, 17, 23). In contrast, when ticks engorge, spirochetes in tick midguts (as well as those deposited into mammalian tissue) downregulate their expression of OspA with a concomitant increase in their expression of OspC (12, 17, 23). Proteins subject to this reciprocal pattern of expression are said to be differentially regulated. Other differentially regulated genes of *B. burgdorferi* include *dbpA* (9, 14), *ospEF* (*erp*) (2, 24), *mlp* (27), and others (reviewed in reference 27). Understanding the molecular mechanisms that govern differential antigen expression is essential for elucidating how genetic regulatory networks influence *B. burgdorferi*'s host range, virulence expression, and possibly even immune evasion.

Studies of gene expression by *B. burgdorferi* cultivated in either BSKII or BSKH medium are contributing towards elucidating factors that trigger the early events of differential antigen expression. In this regard, it already has been shown that temperature shift is one important factor governing key regulatory events in *B. burgdorferi* (10), as in the upregulation of the *ospC*, *mlp*, *dbpA*, and *ospEF* (*erp*) genes (9, 15, 23–25, 27). It additionally has been shown that spirochete cell density and pH also influence gene expression in *B. burgdorferi* (7, 8, 16, 22, 26). More recently, we reported that a combination of reduced pH (pH 6.8) and elevated temperature resulted in a reciprocal pattern of gene expression among two groups of proteins: those whose expression patterns appear to be OspA-like (e.g., P22 and Lp6.6) and those whose expression patterns seem to be OspC-like (e.g., Mlp8, OspF, and σ^s) (26). Given that a drop in pH, an elevation in temperature, and an increase in spirochete number all ostensibly occur in the midguts of ticks as they take their blood meal (11, 13, 26), it is plausible that the combination of these three parameters plays an important role in the control of differential antigen expression in *B. burgdorferi*.

The *mlp* (for multicopy lipoprotein) gene family, formerly known as the 2.9 lipoprotein gene family (5, 21), is one of the paralogous gene families encoded on the multicopy cp32/cp18 plasmids in *B. burgdorferi*. Previously we showed that three newly identified *mlp* genes, *mlp8*, *mlp9*, and *mlp10*, in *B. burgdorferi* strain 297 were all upregulated by increased temperature when *B. burgdorferi* was cultivated in BSKH medium, as well as when *B. burgdorferi* was cultivated in dialysis membrane chambers implanted into rat peritoneal cavities (i.e., when *B. burgdorferi* was grown in a mammalian host-adapted state) (1, 27). This result led us to hypothesize that perhaps all members of the *mlp* family are temperature regulated, a contention consistent with the observation that the *mlp* gene homologs in *B. burgdorferi* strain B31 also appear to be temperature regulated (20). On the other hand, we previously reported that one of the *mlp* genes, *mlp-7A* (previously designated 2.9-7A), was

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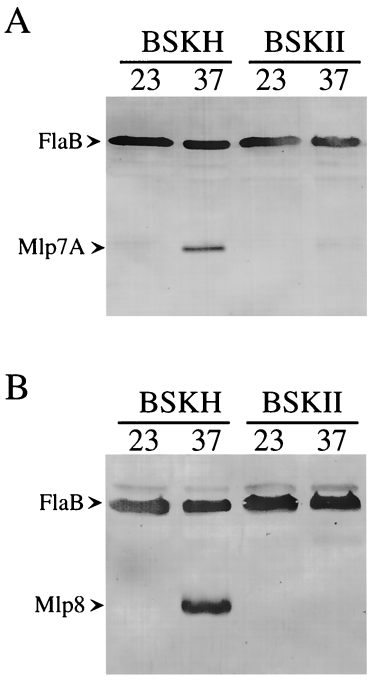

FIG. 1. Influence of BSKH or BSKII medium on the levels of Mlp-7A or Mlp-8 expressed in *B. burgdorferi* 297 cultivated at either 23 or 37°C. *B. burgdorferi* adapted at 23°C was inoculated at a final concentration of 1×10^3 spirochetes per ml, and 23 or 37°C cultures were harvested at a density of 5×10^7 cells per ml (late logarithmic phase). Protein from 5×10^7 spirochetes was loaded in each sodium dodecyl sulfate-polyacrylamide gel lane and subjected to immunoblot assay. The antibodies used to detect FlaB and Mlp-8 were described previously (27). Rat monospecific polyclonal antiserum to an epitopic region of Mlp-7A was generated using a method analogous to that used to create antiserum against Mlp-8 (27). (A) Immunoblot using a mixture of antibodies against FlaB and Mlp-7A. (B) Immunoblot using a mixture of antibodies against FlaB and Mlp-8. Immunoblotting for FlaB was performed to confirm that the numbers of spirochetes loaded in the gel lanes were substantially equivalent.

not upregulated when spirochetes were temperature shifted to 37°C in BSKII medium (1). *mlp-7A* was upregulated, however, when *B. burgdorferi* 297 was cultivated in dialysis membrane chambers implanted into rat peritoneal cavities (1). When in vitro cultivation experiments were later repeated using commercially available BSKH medium (Sigma Chemical Co.), it subsequently was found that *mlp-7A* was induced by elevated temperature (Fig. 1). This inconsistency prompted us to examine more systematically potential differences in gene expression by *B. burgdorferi* cultivated in either BSKII or BSKH medium.

Influence of BSKH or BSKII medium on gene expression in *B. burgdorferi* induced by temperature shift. BSKH medium was purchased from Sigma Chemical Co. (product no. B-8291). BSKII medium was prepared as described by Barbour (4), with the exception that gelatin was omitted from the formulation; BSA (fraction V) was purchased from Sigma Chemical Co. (product no. A-4503), and rabbit serum was obtained from Pel-Freez Biologicals (Rogers, Ark.) (product no. 31126-5). Low-passage, virulent *B. burgdorferi* strain 297 (18) first was adapted at 23°C for 1 week in BSKH medium (26) and then was subcultured at a final cell density of 10^3 spirochetes per ml in either BSKH or BSKII medium. The cultures were then incubated at either 23 or 37°C until the cell density reached approximately 5×10^7 spirochetes per ml.

Upon inspection of the cultures via dark-field microscopy, spirochetes cultured at either temperature in BSKH medium appeared to be more motile than those growing in BSKII medium. Comparative growth curves also revealed that *B. burgdorferi* replicated more rapidly in BSKH medium than in BSKII medium (not shown), as has been noted by others (19). Among spirochetes cultivated in BSKII medium and assessed by immunoblot assay (27), Mlp-7A was undetectable under either 23 or 37°C incubation conditions (Fig. 1A), consistent with our previous report (1). However, when spirochetes were cultivated in BSKH medium, a dramatic increase in the level of Mlp-7A was detected among spirochetes incubated at 37°C, whereas Mlp-7A remained undetectable within *B. burgdorferi* cultivated at 23°C (Fig. 1A). This same temperature-dependent expression pattern for Mlp-8, one of the more abundant Mlp lipoproteins that is temperature regulated (26, 27), again was found among spirochetes cultivated in BSKH but not BSKII medium (Fig. 1B). These results suggest that contrary to an earlier report (1), *mlp-7A* indeed is upregulated by temperature shift, as are other *mlp* genes (20, 27). Of note, although OspC was readily detectable among *B. burgdorferi* organisms cultivated in BSKII medium (Fig. 2A, lanes 4 and 5), OspC levels were substantially increased when borreliae were harvested from BSKH medium at equivalent cell densities (Fig. 2A, lanes 1 and 2). This suggests that *ospC* expression also is promoted by BSKH medium. Higher levels of OspC could be achieved, however, when *B. burgdorferi* was allowed to reach maximal cell densities in BSKII medium (e.g., ca. 10^8 spirochetes per ml) (not shown), a finding that was corroborated at the mRNA level (Fig. 3). Nonetheless, the combined data imply that BSKII medium generally may not be optimal for assessing temporal changes in gene expression by *B. burgdorferi*.

Influence of BSKH or BSKII medium on gene expression in *B. burgdorferi* induced by changes in environmental pH. Recently, we reported that a group of genes, including *ospA*, *p22*, and *lp6.6*, are downregulated under the combined culture conditions of low pH (pH 6.8) and elevated temperature (37°C) (26). Given our findings regarding the influence of BSKII or BSKH medium on genes induced by temperature shift (Fig. 1), it was reasonable to assess whether the influence of pH on *B. burgdorferi* gene regulation was similarly affected by BSKH or BSKII medium. *B. burgdorferi* was cultivated in either BSKII or BSKH medium at 37°C that was preadjusted to either pH 6.8, 7.5, or 8.0 (26). The level of OspA was sharply reduced among spirochetes cultivated in BSKH medium at pH 6.8 (Fig. 2A and B, lanes 1). However, this downregulation of *ospA* did not occur when cells were cultivated in BSKII medium (Fig. 2A and B, lanes 4). Northern blot assay, performed as previously described (21), confirmed that a reduction in OspA mRNA occurred among both late-logarithmic- and stationary-phase *B. burgdorferi* organisms cultured in BSKH medium (pH 6.8; 37°C) but not among those cultured in BSKII medium (Fig. 3). Although late-logarithmic borreliae cultivated in BSKII medium expressed markedly less *ospC* mRNA than those cultivated in BSKH medium (consistent with the differential protein profiles in Fig. 2A, lanes 1 and 4), higher *ospC* mRNA

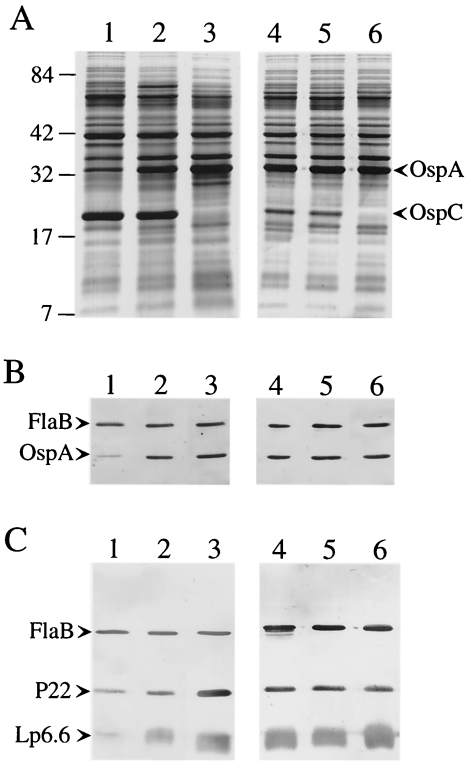

FIG. 2. Influence of BSKH (lanes 1 to 3) or BSKII (lanes 4 to 6) medium on the levels of OspA, P22, and Lp6.6 expressed in *B. burgdorferi* 297 cultivated under various pH conditions. Media were preadjusted to either pH 6.8 (lanes 1 and 4), 7.5 (lanes 2 and 5), or 8.0 (lanes 3 and 6) as reported elsewhere (26). *B. burgdorferi* 297 was then inoculated at a final concentration of 1×10^3 spirochetes per ml, incubated at 37°C, and harvested at a density of 5×10^7 spirochetes per ml. (A) Sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie brilliant blue; each gel lane contained protein from 5×10^7 spirochetes. Protein molecular mass standards (in kilodaltons) are indicated at the left. Polypeptides corresponding to OspA and OspC are labeled at the right. (B) Immunoblot of samples used in panel A, except that gel lanes contained protein from 5×10^6 spirochetes (in order to visualize the relative differences in OspA levels); the antibodies used to detect the respective antigens were described previously (26). (C) Same as panel B, except that protein from 10^7 spirochetes was loaded in each gel lane. Immunoblotting for FlaB (panels B and C) was performed to confirm that the numbers of spirochetes loaded in the gel lanes were substantially equivalent.

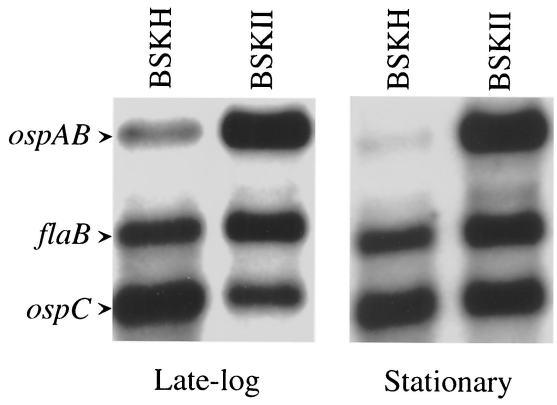

FIG. 3. Northern blot analysis of the expression of *ospAB*, *flaB*, and *ospC* in *B. burgdorferi* during the late logarithmic (5×10^7 spirochetes/ml) and stationary (1×10^8 spirochetes/ml) phases of growth. RNA from *B. burgdorferi* 297 cultivated at each stage of growth in either BSKH or BSKII medium at 37°C (pH 6.8) was hybridized with probes specific for either *ospAB*, *flaB*, or *ospC*. The methods for mRNA isolation and Northern blot assays have been described previously (21, 27). Three micrograms of total RNA was loaded in each gel lane. The three hybridization probes were mixed together in the Northern blot assays. The bands corresponding to each transcript are labeled at the left. Assessment of the level of *flaB* mRNA was used as an internal control to indicate equivalent RNA loading among the various gel lanes.

levels (substantially equivalent to those of borreliae from BSKH medium) eventually could be achieved if spirochetes were allowed to reach the stationary phase of growth in BSKII medium (Fig. 3). This suggests that the upregulation of *ospC* among logarithmic-phase borreliae replicating in BSKII medium is inefficient relative to comparable spirochetal growth and adaptation in BSKH medium. The downregulation expression pattern observed for *ospA* also was observed for two other pH-regulated genes, *p22* and *lp6.6* (26), among *B. burgdorferi* organisms grown in low-pH BSKH medium (Fig. 2C, lane 1) but not among spirochetes cultivated in BSKII medium (Fig. 2C, lane 4). The combined results suggest that genetic regulatory events in virulent *B. burgdorferi* are strongly influenced not only by changes in environmental stimuli (e.g., temperature and pH) but also by the composition of the culture medium.

BSA influences the results of gene expression studies with *B. burgdorferi* cultivated in BSKII medium. BSKH and BSKII media are similar in that they contain a number of complex nutrients, about 5% (wt/vol) BSA, and 6% (vol/vol) rabbit serum (4, 19). The overall importance of serum as a growth supplement for *B. burgdorferi* was highlighted in a study by Alban et al. (3), who showed that serum elimination resulted in marked alterations in spirochetal morphology and protein profiles. It also has been reported that different batches of BSA and rabbit serum vary significantly in their abilities to support the growth of *B. burgdorferi* (4, 6). In particular, Barbour (4) pointed out that some lots of fraction V BSA, but not high-grade BSA, better supported the growth of *B. burgdorferi*. Callister et al. (6) additionally showed that BSA is a significant factor affecting the ability of BSK medium to support the growth of *B. burgdorferi*. Along these lines, commercial BSKH medium is prepared by screening different batches of fraction V BSA as well as rabbit serum for their promotion of vigorous motility and rapid growth by *B. burgdorferi* (19). As such, BSKH can be viewed as a quality-controlled version of BSKII. Ideally, prescreened lots of BSA and rabbit serum should be used when formulating BSKII medium, but it is not practical for most laboratories to perform batch performance testing each time that BSKII medium is needed. This is underscored by the fact that there have been no studies showing that such measures are essential for obtaining valid results from *B. burgdorferi* gene regulation studies.

To garner further evidence for the potential importance of BSA and/or rabbit serum in gene regulation studies with *B. burgdorferi*, we prepared four variations of BSKII medium. The first was made using conventional BSA (fraction V) (Sigma Chemical Co.; product no. A-4503) and rabbit serum (Pel-Freez Biologicals; product no. 31126-5). The second type con-

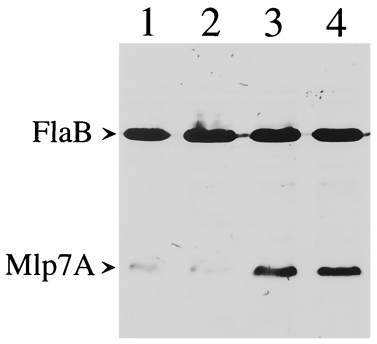

FIG. 4. Influence of BSA and rabbit serum in BSKII medium on the level of Mlp-7A expressed by *B. burgdorferi*. *B. burgdorferi* 297 adapted at 23°C was inoculated at a final concentration of 1×10^3 spirochetes per ml, incubated at 37°C, and harvested at a final density of 5×10^7 cells per ml (late logarithmic phase). Protein from 5×10^7 spirochetes was loaded in each gel lane, and the samples were then subjected to immunoblot assay. The antibodies used to detect each antigen were as for Fig. 1A. Lane 1, spirochetes cultivated in BSKII medium formulated with conventional (nonprescreened) BSA and rabbit serum. Lane 2, spirochetes grown in BSKII medium containing the same nonprescreened BSA but the rabbit serum used to make commercial BSKH medium. Lane 3, borreliae cultivated in BSKII medium formulated with conventional rabbit serum but the BSA used in the commercial preparation of BSKH medium. Lane 4, spirochetes grown in BSKII medium containing both the quality-controlled rabbit serum and quality-controlled BSA, thereby making it substantially equivalent to commercial BSKH medium. Immunoblotting for FlaB was performed to confirm that the numbers of spirochetes were essentially equal within the gel lanes.

tained the same BSA but contained the rabbit serum (Sigma; product no. R-7136) used in the preparation of commercial BSKH medium. The third type was formulated using conventional rabbit serum (Pel-Freez) but BSA (Sigma Chemical Co.; product no. A-9056) of a lot used in commercial BSKH medium. The fourth type of BSKII medium contained both of the quality-controlled lots of rabbit serum and BSA from Sigma Chemical Co., thereby making it substantially equivalent to commercially available BSKH medium. These media were inoculated with *B. burgdorferi*, incubated at 23°C, and then temperature shifted to 37°C (as for Fig. 1). As assessed at the protein level by immunoblotting, BSKII medium formulated with conventional (nonprescreened) BSA and rabbit serum did not support the temperature-induced expression of *mlp-7A* (Fig. 4, lane 1), as shown previously (Fig. 1A). Replacement of the conventional rabbit serum with the quality-controlled (prescreened) rabbit serum also did not trigger a response by *mlp-7A* to temperature (Fig. 4, lane 2). In contrast, when spirochetes were cultivated in BSKII medium with conventional rabbit serum but containing prescreened BSA, the production of Mlp-7A in response to temperature induction was readily apparent (Fig. 4, lane 3), as it was in medium containing both the prescreened rabbit serum and BSA (lane 4). These results suggest that the quality of the BSA supplement in BSKII medium plays an essential role in revealing key regulatory aspects of gene expression by virulent *B. burgdorferi*.

The contents of BSA that account for the variation in results from gene expression studies with *B. burgdorferi* remain obscure. However, it is noteworthy that we have observed no significant difference in the gene expression pattern (e.g., downregulation of *ospA*-like genes and upregulation of *ospC*-like genes) by virulent *B. burgdorferi* growing in either BSKII or BSKH medium when spirochetes were allowed to replicate within dialysis membrane chambers implanted into rat peritoneal cavities (1, 26, 27) (data not shown). Factors that account for these reciprocal patterns of gene expression as *B. burgdorferi* replicates in dialysis membrane chambers also remain unknown, but it is tempting to speculate that one or more might be those that confer to BSA the ability to promote similar genetic regulatory patterns. If so, it would be reasonable to hypothesize that such factors may be small enough to diffuse freely from rat peritoneal fluid into the dialysis membrane chambers (ca. 7,000-Da exclusion limit) during the *B. burgdorferi* cultivation process.

Regardless of the components within BSA that promote spirochetal growth and natural gene expression patterns in *B. burgdorferi*, our observations sound a cautionary note regarding the selection of medium used in in vitro gene regulation studies with virulent *B. burgdorferi*. In this regard, two lines of evidence suggest that spirochetes cultivated in BSKH medium are phenotypically more representative of their native state than they are when cultivated in BSKII medium that has not been properly formulated with BSA. First, spirochetes cultivated in BSKH medium were more motile and active in replication than organisms cultivated in BSKII medium. Second, a pattern of gene expression associated with borrelial transmission (e.g., downregulation of *ospA* and upregulation of *mlp* [12, 17, 27]) can be reproduced by cultivating *B. burgdorferi* in BSKH medium but not in BSKII medium. Thus, it appears that BSKH medium generally is superior to BSKII medium for studies of gene regulation in *B. burgdorferi*. Replacement in BSKII medium of the conventional BSA with a preevaluated lot of BSA restored the expected pattern of gene expression, at least with respect to the expression of *mlp-7A*. Implicit in these findings is that if investigators choose not to use commercially available BSKH medium, then BSKII medium should be formulated with a preevaluated lot of BSA for gene regulation studies with *B. burgdorferi*. One way of potentially evaluating the suitability of investigator-formulated BSKII medium would be to assess the pH-mediated regulation of *ospA* and the temperature-mediated regulation of one or more of the *mlp* genes prior to performing other gene regulation experiments with *B. burgdorferi*.

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