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Serum-starvation-induced changes in protein synthesis and morphology of *Borrelia burgdorferi*

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It has been demonstrated previously that motile *Borrelia burgdorferi* cells transform into non-motile cyst-forms when incubated for several weeks in BSKII (a complex medium) lacking rabbit serum. *B. burgdorferi* cells cannot synthesize fatty acids *de novo* and serum is thought to provide a source of fatty acids and lipids. When *B. burgdorferi* cells were serum-starved in defined RPMI medium, ~90% of the cells formed spherical cysts within 48 h. Cyst formation was inhibited by tetracycline. Cyst opening and recovery of vegetative cells was rapidly induced by the addition of either BSKII or rabbit serum. The percentage of viable cells recovered from cysts ranged from 2·9% to 52·5%. Viability was inversely proportional to cyst age. Protein synthesis by *B. burgdorferi* during serum starvation was examined by labelling cells with Tran^{35}S-Label and analysing the labelled proteins by two-dimensional gel electrophoresis and fluorography. The synthesis of over 20 proteins was induced during serum starvation. Western blots of proteins from vegetative cells and cysts probed with sera from either *B. burgdorferi*-infected humans or monkeys revealed that several cyst proteins were antigenic. These data suggest that cells of *B. burgdorferi*, although possessing a small genome and extremely limited biosynthetic capabilities, rapidly respond to conditions of serum starvation by inducing changes in protein synthesis and cell morphology. This study may help explain how cells of *B. burgdorferi* can survive periods of nutrient deprivation in different hosts and host tissues.

Keywords: *Borrelia burgdorferi*, spirochaete, serum starvation, cysts, Lyme disease

INTRODUCTION

Lyme disease, a multisystem illness, is a tick-borne bacterial disease caused by the spirochaete *Borrelia burgdorferi* (Burgdorfer *et al.*, 1982). Following transmission of the spirochaetes to humans from ticks, Lyme disease can manifest itself in many different ways. Initially, the disease affects the skin, often with the development of a transient rash, erythema chronicum migrans. Other symptoms in the early stages of the disease can include fever, dizziness and chronic fatigue. Late-stage symptoms may include acute or chronic arthritis in large joints, meningoencephalitis, Bell's palsy, or myocarditis (Steere, 1989). *B. burgdorferi* cells have been isolated from the blood, cerebrospinal fluid (CSF), synovial fluid and skin specimens of Lyme disease patients (Johnson & Norton Hughes, 1992).

B. burgdorferi cells alternate between tick vectors (members of the *Ixodes ricinus* complex) and mammals. Therefore, these spirochaetes are adapted to survive in a number of different environments. Further, *B. burgdorferi* cells exhibit substantial changes in protein synthesis and gene expression and antigenicity during different stages of their zoonotic life cycle (Akins *et al.*, 1998; Das *et al.*, 1997; Fikrig *et al.*, 1998; Schwan *et al.*, 1995; de Silva *et al.*, 1996; de Silva & Fikrig, 1997). For example, two major outer-membrane proteins (OspA and OspC) are differentially synthesized within the tick host. OspA is abundant on the surface of spirochaetes within the midguts of unfed ticks (de Silva *et al.*, 1996) whereas OspC is more readily detected on the surface of spirochaetes in fed ticks (Schwan *et al.*, 1995). Additionally, several *B. burgdorferi* genes appear to be

Abbreviations: CSF, cerebrospinal fluid; MPN, most probable number; SSP, serum starvation protein.

expressed only within infected mammals (de Silva & Fikrig, 1997; Akins *et al.*, 1998; Suk *et al.*, 1995).

Brorson & Brorson (1997) demonstrated that cells of *B. burgdorferi* undergo a morphological transformation from motile spirochaetes into non-motile, spherical cyst-forms when incubated for ~4 weeks in Barbour–Stoenner–Kelly (BSKII) medium without the addition of rabbit serum, a routinely added media supplement thought to provide cells with a source of fatty acids (Barbour & Hayes, 1986). Cyst-forms have also been observed in Lyme disease patient tissues (Hulinska *et al.*, 1994; Aberer *et al.*, 1997). It has been reported that these forms of *B. burgdorferi* are viable and capable of transforming back into motile spirochaetes, although survival was not accurately quantified (Brorson & Brorson, 1997). The significance of these forms remains to be seen, but it has been suggested that cysts may represent a different stage in the life cycle of the spirochaetes or possibly play a role in human disease (Brorson & Brorson, 1997, 1998a, b).

In the work described here, we studied cyst formation under defined conditions to determine: (1) the kinetics of cyst formation, (2) the viability of cyst-forms, and (3) whether cysts form as the result of a starvation-induced programme involving differential protein synthesis. Our study suggests that *B. burgdorferi* cells possess a complex starvation response that involves loss of motility, induction of protein synthesis, and morphological changes.

METHODS

Bacterial strains and culture conditions. The high-passage strain *B. burgdorferi* B31 and the low-passage strain T15 were routinely grown at 33 °C in 10 ml portions of BSKII broth (Barbour, 1984) in 16 × 125 mm screw-capped tubes. BSKII is a complex medium that contains 6% rabbit serum in addition to other metabolites.

Serum starvation and recovery experiments. Exponential-phase cells (50 ml) grown in BSKII medium were centrifuged (9000 *g*, 10 min) at 4 °C. Supernatants were removed and cells were resuspended in 80 ml RPMI-1640 Select-Amine (RPMI) (Life Technologies). RPMI is a defined medium containing glucose, vitamins, and all 20 of the protein amino acids. Cultures were incubated at 33 °C in 100 ml screw-cap culture bottles (Fisher) at a concentration of $1{\cdot}5 \times 10^7$ cells ml^{-1}. Cell density was determined by phase-contrast microscopy using a haemocytometer (Reichart).

For the recovery experiments, exponential-phase cells were prepared and incubated in RPMI as described above. Viability of cells at various time points was determined by a three-tube most-probable-number (MPN) technique (American Public Health Association, 1975). Briefly, serum-starved cells (1 ml) were removed from RPMI by centrifugation (14000 *g*, 1 min), resuspended in BSKII (1 ml), and serially diluted (serial tenfold dilutions) into BSKII. Each dilution was done in triplicate. Tubes (1 ml) were incubated at 33 °C for up to 2 weeks or until growth was observed, whichever was first. The percentage of recovery was estimated by a standard method (American Public Health Association, 1975).

Electron microscopy. Vegetative cells and cysts were fixed by the addition of EM-grade formaldehyde (1% final concentration) to the culture medium. Fixed samples were centrifuged at 9000 *g* for 5 min and resuspended in 0·5 ml 3% glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·2). Cells were mounted on 200-mesh carbon-coated grids and negatively stained with 1% phosphotungstic acid or 0·5% ammonium molybdate. The negatively stained preparations were examined and photographed using a JEOL 1200EX transmission electron microscope.

Radioactive labelling and protein precipitation. Exponential-phase cells (50 ml) grown in BSKII were centrifuged (9000 *g*, 4 °C) and resuspended as described above except that methionine was excluded in the formulation of RPMI. After the culture had been allowed to incubate at 33 °C for 5 min, 10 μCi ml^{-1} (370 kBq ml^{-1}) of Tran^{35}S-Label (specific activity 43·48 TBq $mmol^{-1}$; ICN Pharmaceuticals) was added. Cells were labelled for either 2 h or up to 17 h in some cyst preparations where the low rate of incorporation required longer periods of labelling. Radioactive labelling was stopped by adding excess methionine (10 mM). Labelled cells were then centrifuged (9000 *g*, 10 min, 4 °C) and washed twice in 10 mM HEPES buffer (pH 7·6, 4 °C). Cells were disrupted by sonication and protein was TCA-precipitated in order to concentrate the ^{35}S-labelled protein as described previously (Girouard *et al.*, 1993; Scorpio *et al.*, 1994). TCA-precipitated proteins were solubilized in solubilization-reduction mixture (Laemmli, 1970). Radioactive incorporation was determined by liquid scintillation counting of the solubilized protein.

Electrophoresis and Western blotting. Serum-starved (48 h) or exponential-phase cells grown in BSKII were centrifuged (9000 *g*, 10 min) and washed three times in 10 mM HEPES buffer (pH 7·6, 4 °C). Cells were disrupted by sonication and protein concentrations in unlabelled cell extracts were estimated by the method of Bradford (1976) with a Bio-Rad protein assay kit.

One-dimensional SDS-PAGE was performed in 1·5 mm thick slab gels (5% stacking, 10–13% separating) as described by Laemmli (1970), using an SE 600 vertical electrophoresis unit. Equal protein (20 μg) was loaded in each gel lane. The separated proteins were either stained with Coomassie blue or electroblotted to nitrocellulose by a modification of the procedure described by Towbin *et al.* (1979) using a Bio-Rad Transblot Cell (400 mA, 60 min) (Girouard *et al.*, 1993). The nitrocellulose membranes were briefly stained with 0·1% Ponceau S (Sigma) to mark the positions of molecular mass standards and then blocked in Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl; pH 7·4) plus 2% Tween 20 (Sigma) for 20 min. The membranes were washed twice for 5 min in TBS + 0·05% Tween 20. The transferred proteins were reacted overnight at 4 °C with sera from *B. burgdorferi*-infected rhesus monkeys (L913 or K205) (Philipp *et al.*, 1993; Roberts *et al.*, 1998), from a chronically infected Lyme disease patient (J1), or with monoclonal antibodies at the appropriate dilution (see figure legends) in TBS plus 0·05% Tween 20. Monkey sera and patient sera were generous gifts of Mario Philipp (Tulane Regional Primate Research Center, Covington, LA, USA) and Thomas Mather (University of Rhode Island), respectively. Mouse monoclonal antibodies H9724, H5332, 4B8F4 and 149, specific for flagellin, OspA, OspC and GroEL, respectively, were kindly provided by Barbara Johnson (Division of Vector-Borne Diseases, Ft Collins, CO, USA).

Proteins were separated by isoelectric focusing (IEF) using the Multiphor II system (Pharmacia) with Immobiline DryStrips (pH 3–10) according to the manufacturer's protocol. Equal radioactive counts of ^{35}S-labelled proteins (solubilized after TCA precipitation) were loaded onto IEF gels and gels were

run to equilibrium at 20 °C. IEF gels were then incubated for 10 min in equilibrium buffer (50 mM Tris pH 8·8, 6 M urea, 30%, v/v, glycerol, 2% SDS) before being loaded onto a vertical SDS-PAGE gel. Vertical electrophoresis (the second dimension) was performed using the SE 600 apparatus as described above. Separated proteins were visualized by fluorography as previously described (Carreiro *et al.*, 1990). Dried gels were exposed to Fuji RX film (Fisher) at −70 °C for 3–7 d. Fluorograms were analysed by laser densitometry using a Molecular Dynamics Personal Densitometer (Molecular Dynamics) and Image QuaNT v.5.0 software, as previously described (Garcia *et al.*, 1997).

MALDI-MS protein identification. Following two-dimensional PAGE separation of labelled proteins and autoradiography, the protein corresponding to serum starvation protein s (SSP s) was cut from the gel and subjected to in-gel trypsin digestion and MALDI-MS protein identification as described by Williams *et al.* (1996).

RESULTS

Morphological transformation to cysts during serum starvation

In a recent study, Brorson & Brorson (1997) demonstrated that *B. burgdorferi* cells transform from vegetative spirochaetes into spherical 'cyst-forms' when incubated in BSKII medium lacking rabbit serum (BSKII − S). We confirmed these observations. Usually, 30–50% of cells incubated in BSKII − S formed cyst-like structures over 2–4 weeks. However, BSKII is not a defined medium and does contain BSA (fraction V, 50 g l^{-1}). In an effort to better define cyst formation as a response to serum or fatty acid starvation, *B. burgdorferi* B31 cells were incubated in RPMI. One hour after the onset of serum starvation, cells lost normal motility at one or both poles and began twisting into knots. Within 24 h, cells starved of serum were completely non-motile and 30–40% had begun to encyst. After 48 h incubation in RPMI, ~90% of serum-starved cells had formed cysts (Fig. 1). In contrast, control cells incubated in RPMI plus 6% rabbit serum (RPMI + S) remained motile and no cysts were observed. Additionally, while spirochaetes incubated in RPMI + S decreased the pH of the medium from 7·6 to 6·8, those incubated in RPMI decreased the pH from 7·6 to 7·3. Cells incubated in RPMI + S became non-motile after 5–6 d but did not form cysts. Comparable cyst formation was also observed when the low-passage strain T15 was used; therefore, all subsequent experiments were performed using high-passage strain B31. Cyst formation was inhibited by the addition of tetracycline (150 μg ml^{-1}) to RPMI.

Negatively stained preparations of *B. burgdorferi* cysts were observed by electron microscopy and compared to vegetative cells (Fig. 1). In contrast to typical helical vegetative cells, most 48 h serum-starved cells were coiled within a membrane. The overall shape was roughly spherical, often with one end of the coiled spirochaete protruding (Fig. 1C). Additionally, we sometimes observed portions of outer membrane 'beading' away from a pole of the cell (Fig. 1C).

Determination of viability during serum starvation

When rabbit serum or BSK was added to RPMI containing 48 h serum-starved cells, the cysts opened within 10 s to yield intact, but non-motile spirochaete cells (Fig. 2). A newly emerged spirochaete with an empty cyst is shown in Fig. 2C. Cysts did not open when 20% sucrose was added to the culture. Cells began to regain motility 12–15 h after emerging from the cysts. Since RPMI + S is not a growth medium for *B. burgdorferi*, viability experiments were performed by MPN determinations in BSKII as described in Methods. The data presented in Table 1 show that *B. burgdorferi* cells could be grown from cultures starved for serum for up to 8 d. Cells starved for 2 weeks were not recoverable even after > 30 d incubation in BSKII. In contrast, no viable cells could be recovered by 5 d from cultures incubated in RPMI + S, nor could viable cells be recovered from cultures starved in HEPES buffer after

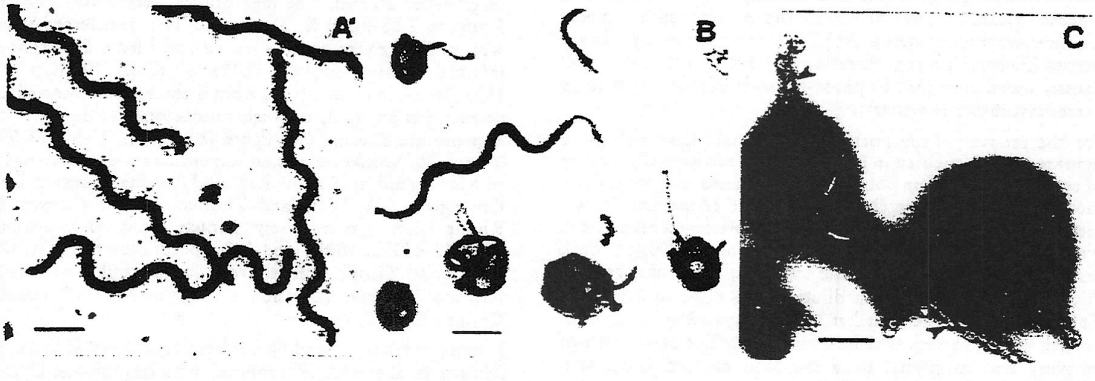

Fig. 1. Transmission electron micrographs demonstrating cyst formation of *B. burgdorferi* in response to serum starvation. A, Typical vegetative spirochaetes observed in BSKII or RPMI + S. B, C, Cyst forms occurring after 48 h incubation in RPMI. Arrowheads indicate membrane 'beading'. Bars, 2 μm (A and B); 1 μm (C).

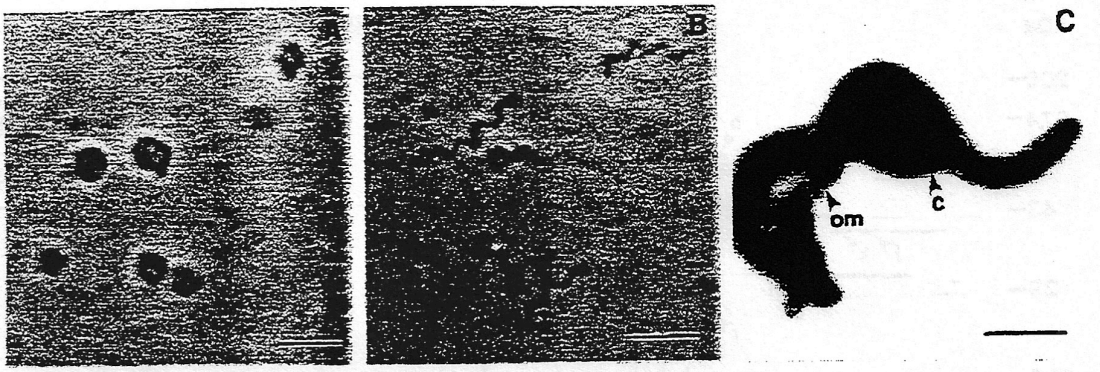

Fig. 2. Morphology of *B. burgdorferi* in a 48 h serum-starved culture before (A) and after (B and C) addition of rabbit sera. Panels A and B are phase-contrast micrographs that show identical fields, allowing the comparison of individual cells. Panel B was photographed <1 min after the addition of rabbit serum. Panel C is a transmission electron micrograph showing a newly emerged spirochaete. Arrowheads indicate an empty cyst (c) and an outer membrane (om). Bars, 5 μm (A and B); 2 μm (C).

Table 1. Recovery of viable cells

Recovery of viable cells was determined by MPN analysis as described in Methods.

Medium	Time (d)	Percentage viability ± SD*	Motility†
RPMI	0	100·0 ± 0	+
	2	52·5 ± 13·4	−
	3	18·2 ± 12·5	−
	5	15·7 ± 12·5	−
	8	2·9 ± 1·9	−
RPMI+S	0	100·0 ± 0	+
	2	56 ± 8·5	+
	5	0 ± 0	−
HEPES	0	100·0 ± 0	+
	1	0 ± 0	−

*Percentage viability = (viable cells as determined by MPN technique)/(direct microscopic count at $t = 0$). The data shown are averaged from two independent experiments.

†Motility was determined by microscopic observation prior to MPN experiments.

1 d (Table 1). It is important to note that spirochaetes incubated in HEPES buffer or distilled water formed round cells that clumped together. These structures resembled spheroplasts and were repeatedly demonstrated to be nonviable by MPN determinations.

Protein synthesis during serum starvation

Changes in protein synthesis by *B. burgdorferi* cells during serum starvation were determined by labelling cells with Tran^{35}S-Label and analysing the labelled proteins by two-dimensional gel electrophoresis and fluorography. Proteins from serum-starved cells (labelled at $t = 0$–2 h and 2–19 h) were compared to proteins labelled in RPMI+S ($t = 0$–2 h). Protein synthesis patterns observed by two-dimensional gel electrophoresis in RPMI+S ($t = 0$–2 h) were similar to those observed when cells were labelled in BSKII for 18 h. Examination of two-dimensional fluorograms revealed that over 20 proteins were induced in serum-starved cells (Fig. 3). The approximate molecular masses and pI values of proteins induced at least twofold during serum starvation are listed in Table 2. The positions of known antigens (OspA, Fla, OspC, GroEL) identified by monoclonal antibodies are also indicated in Fig. 3. Of the identified antigens, only OspA appeared to be upregulated during serum starvation. Examination of the fluorograms presented in Fig. 3 revealed that there was a time-dependent synthesis of proteins after the initiation of serum starvation. Several serum-starvation proteins (SSPs) were synthesized early ($t = 0$–2 h) during starvation (e.g. SSP e, j, m, n, o, u, v). Other SSPs were synthesized predominantly at later times during starvation (e.g. SSP a, c, f, h, q, s, t). One SSP (SSP s) was identified by MALDI-MS as a homologue of VlsE (Table 2), a protein shown to be involved in antigenic variation of *B. burgdorferi* cells (Zhang *et al.*, 1997).

Antigenicity of cyst proteins

Whole-cell protein extracts from vegetative cells and 48 h cysts were separated by SDS-PAGE, electroblotted onto nitrocellulose, and probed with sera from Lyme-disease-infected patients or monkeys. Representative Western blots probed with sera from monkeys with either early stage (L913) or late-stage (K205) Lyme disease (Philipp *et al.*, 1993), or with serum from a chronically infected human patient (J1) are shown in

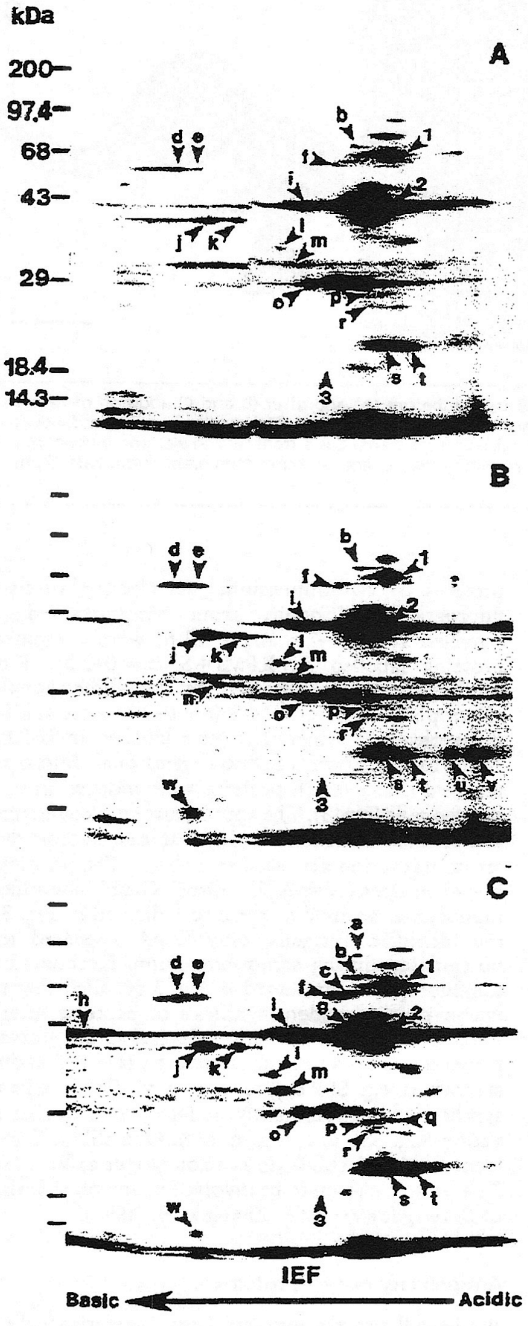

Fig. 3. Two-dimensional fluorograms of proteins synthesized in: A, RPMI+S, $t = 0$–2 h; B, RPMI, $t = 0$–2 h; and C, RPMI, $t =$ 2–19 h. Lower-case letters indicate the location of induced proteins. The positions of known protein antigens, GroEL, flagellin and OspC, determined by two-dimensional Western blots using monoclonal antibodies (1:5000 dilution) are indicated by numbers 1, 2 and 3, respectively. The position of another known antigen, OspA, is indicated by the letter 'o' as it is one of the induced proteins. The molecular mass standards, identified in kDa in panel A, are indicated in panels B and C by their location only.

Table 2. Proteins induced in response to serum starvation

Induced SSP*	Mol mass (kDa)	pI	Relative induction in RPMI†	
			0–2 h	2–19 h
a	89	5·8	0	+
b	66	5·9	0	+
c	58	6·0	0	+
d	57	8·4	0	+
e	57	8·1	+	+
f	57	6·1	+	+
g	43	5·9	0	+
h	41	10	0	+
i	41	6·7	0	+
j	37	7·9	+	+
k	37	7·4	+	+
l	34	7·0	+	+
m	32	6·9	+	+
n	31	7·7	+	0
o‡	29	6·9	+	+
p	28	5·7	0	+
q	28	5·7	0	+
r	27	5·7	0	+
s‡	24·5	5·5	+	+
t	24	5·0	+	+
u	24	4·3	+	0
v	24	4·0	+	0
w	13·5	8·0	+	+

* Proteins are designated by lower-case letters (a–w) as shown in Fig. 3.

† Relative induction of SSPs determined by comparison of two-dimensional fluorograms of proteins from cells incubated and labelled in RPMI and RPMI+S. Induced proteins are indicated by (+); proteins synthesized at similar or reduced levels are indicated by (0). Determinations were based on at least three separate trials for each time point.

‡ SSP o and SSP s have been identified as OspA and a VlsE homologue, respectively.

Fig. 4. While Coomassie-blue-stained SDS gels prepared from vegetative cells or cysts were virtually indistinguishable (data not shown), the Western blots displayed consistent differences between the protein antigens recognized in vegetative cells and cysts. For example, several low-molecular-mass bands (~16–25 kDa) were consistently more reactive in cyst preparations. Additionally, examination of two-dimensional fluorograms showed that four proteins in the 16–25 kDa range were upregulated in cysts (Fig. 3). These two observations suggested that some or all of these low-molecular-mass proteins might be identical and antigenic. Preliminary

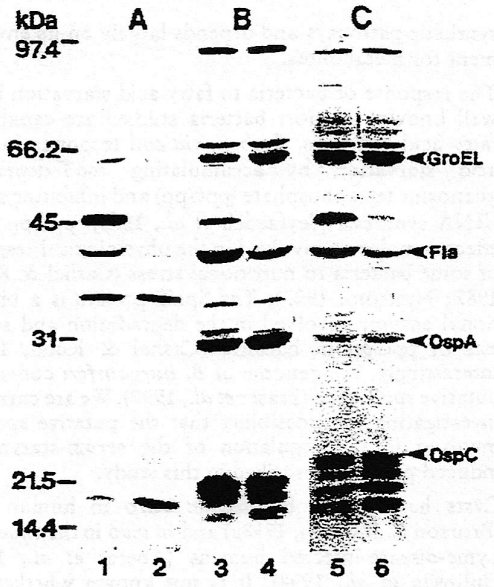

Fig. 4. Western blots of *B. burgdorferi* proteins from vegetative cells (1, 3, 5) and serum-starved cells (2, 4, 6) reacted with sera from monkeys with either early-stage (L913) or late-stage (K205) Lyme disease (Philipp *et al.*, 1993) (panels A and B, respectively), or with serum from a chronically infected human patient (J1) (panel C). The monkey sera and human sera were diluted 1:2000 and 1:2500, respectively. The positions of known antigens GroEL, Flagellin, OspA and OspC are indicated by arrows; they were detected with monoclonal antibodies (1:5000 dilution).

two-dimensional Western blots showed that at least two of these proteins were recognized by serum from a chronically infected monkey (data not shown). In contrast, both the 46 kDa and 41 kDa (flagellin) protein bands exhibited less reactivity to sera from humans or monkeys in blots prepared from cysts, suggesting that both proteins were present in decreased amounts in cysts.

DISCUSSION

B. burgdorferi grows in both tick and mammalian hosts during its infection cycle. The ability to survive and grow in disparate hosts clearly displays the adaptability of this microbe. *B. burgdorferi* possesses a genome of only ~1000 kbp, encoding very limited biosynthetic capabilities (Fraser *et al.*, 1997). The lack of many biosynthetic pathways explains why growth *in vitro* requires a complex medium supplemented with animal serum (BSKII). The serum is thought to provide the cells with a source of fatty acids, since they lack the ability to synthesize fatty acids *de novo* (Fraser *et al.*, 1997). It is not yet known how these fastidious organisms survive periods of starvation such as that encountered during the tick moult, between tick blood meals. In this report, we demonstrate that *B. burgdorferi* cells respond to serum starvation by inducing a starvation response that results in the transformation of motile helical vegetative cells into non-motile, spherical cysts containing tightly coiled spirochaetes. This starvation-response programme also includes the coordinated synthesis of over 20 proteins. Some of these proteins are antigenic in mammalian hosts.

It has previously been reported that *B. burgdorferi* spirochaetes develop into cysts *in vitro* when they are incubated either in BSKII−S for several weeks (Brorson & Brorson, 1997) or in CSF (37 °C) for 24 h (Brorson & Brorson, 1998a). We began our study by observing cyst formation in BSKII−S. We estimate that only 30–50% of the cells transform into cysts after 4 weeks. We hypothesize that cells form cysts in response to starvation for serum or the abundant fatty acids and lipids in serum. Slow formation of cysts in BSKII−S is probably due to the presence of some fatty acids or lipids in the large amount of BSA (50 g l^{-1}) in the medium. We thought that elimination of all fatty acids and lipids from the medium would increase both the rate of cyst formation and the percentage of cells which undergo the transformation. Using RPMI without the addition of serum, cells become non-motile within a few hours and 90% of vegetative spirochaetes form cysts within 48 h. A cyst appears to contain an intact, coiled non-motile spirochaete within a membrane (Fig. 1). It is unclear whether the membrane is part of the original outer membrane of the vegetative cell or whether it is constructed during cyst formation.

Cyst formation is an active cellular response to serum starvation. The addition of tetracycline inhibits cyst formation, demonstrating that cyst formation requires protein synthesis and that cysts are not merely degenerative forms. Further, the temporal pattern of protein synthesis during cyst formation strongly suggests that encystment is a global response to a specific starvation (see Fig. 3 and Table 2). Additionally, cysts are able to survive longer than *B. burgdorferi* cells incubated in either similar media plus serum or buffer alone (Table 1). In contrast to a recent report that describes rapid cyst formation in distilled water (Brorson & Brorson, 1998b), we did not observe the formation of viable cysts in HEPES or distilled water. We did observe that *B. burgdorferi* incubated for more than 1 h in distilled water or HEPES buffer formed rounded, clumping, spheroplast-like cells, but these structures were consistently shown to be nonviable by quantitative methods. When serum or BSK is added to these types of cell structures they appear to rupture, releasing cytoplasmic contents, unlike the emergence of intact spirochaete-shaped cells that we observed (Fig. 2B, C).

Morphological changes by *B. burgdorferi* cells in response to adverse environmental conditions have been described by others (Barbour & Hayes, 1986; Brorson & Brorson, 1997, 1998a; Burgdorfer & Hayes, 1989; Kersten *et al.*, 1995; Preac Mursic *et al.*, 1989). For example, the formation of vesicles or blebs and gemmae

has been shown to occur when cells are exposed to physiological stress such as changes in pH, depletion of metabolites, and ageing (Burgdorfer & Hayes, 1989) or exposure to antibiotics (Kersten *et al.*, 1995; Preac Mursic *et al.*, 1989). Little is known about the physiological role of these forms, although blebs and gemmae have been shown to contain DNA (Garon *et al.*, 1989) and may be involved in the exchange of genetic information. Unlike cyst-forms, blebs and gemmae have not been shown to be viable, capable of transforming back into motile, vegetative cells. We have also observed blebs and gemmae in our cultures, particularly when cells are exposed to antibiotics and extreme pH stress, but it is clear that these vesicles are not cysts.

In addition to loss of motility and cyst formation, *B. burgdorferi* cells responded to serum starvation by inducing the synthesis of a number of proteins (Table 2). Using monoclonal antibodies to well-characterized antigens, we identified one of the starvation-induced proteins (SSP o; see Table 2). It appears that SSP o (OspA) synthesis increases about twofold when compared to cells incubated in RPMI+S. The upregulation of OspA during serum starvation is not surprising since OspA is the major outer-membrane protein detected in unfed ticks. Additionally, it has been suggested that OspA and OspC are inversely regulated by nutrient availability (Das *et al.*, 1997; Schwan *et al.*, 1995). However, an increased accumulation of OspA was not detected by Western blot analysis (Fig. 4). This may be due to either increased turnover of OspA during cyst formation or the loss of OspA-containing membrane during cyst formation (Fig. 2). We successfully identified another SSP (SSP s) by MALDI-MS as a homologue of VlsE, a protein shown to be involved in antigenic variation of *B. burgdorferi* cells (Zhang *et al.*, 1997). VlsE and its role in starvation-induced forms warrant further study. It is tempting to speculate that some of the proteins upregulated during serum starvation regulate this response.

Bacteria have evolved elaborate strategies that enable them to survive periods of starvation (Siegele & Kolter, 1992). Examples of morphological changes that occur in response to starvation include the formation of endospores (Losick & Youngman, 1984), ultramicrocells (Kjelleberg *et al.*, 1987) and fruiting bodies (Kaiser, 1984). Bacterial cells have also been shown to alter fatty acid composition, surface properties and cell walls in response to starvation (Kjelleberg *et al.*, 1987; Nyström & Kjelleberg, 1989). Further, the carbon-starvation response of *Vibrio* sp. strain S14 has been shown to be dependent upon a *spoT* homologue (Ostling *et al.*, 1996). As in *B. burgdorferi* cyst formation, the *Vibrio* S14 carbon-starvation response (or ultramicrocell formation) involves: (1) loss of motility, (2) change in morphology to a spherical form, and (3) expression of a number of genes. Burgdorfer & Hayes (1989) suggested that cyst formation of *B. burgdorferi* cells may be part of a complex developmental cycle. An adaptive response by *B. burgdorferi* to periods of starvation would seem to be essential for an organism that lacks critical biosynthetic pathways and depends largely on its environment for metabolites.

The response of bacteria to fatty acid starvation is not well known, as most bacteria studied are capable of fatty acid synthesis. *Escherichia coli* responds to fatty acid starvation by accumulating *spoT*-dependent guanosine tetraphosphate (ppGpp) and inhibiting stable rRNA synthesis (Seyfzadeh *et al.*, 1993). ppGpp is an effector molecule involved in the physiological response of some bacteria to nutritional stress (Cashel & Rudd, 1987; Nyström, 1993). The SpoT protein is a bifunctional enzyme involved in the degradation and synthesis of ppGpp in bacteria (Cashel & Rudd, 1987). Interestingly, the genome of *B. burgdorferi* contains a putative *spoT* gene (Fraser *et al.*, 1997). We are currently investigating the possibility that the putative *spoT* is involved in the regulation of the serum-starvation-induced proteins described in this study.

Cysts have been observed *in vitro* in human CSF (Brorson & Brorson, 1998a) and *in vivo* in the tissues of Lyme-disease-infected humans (Aberer *et al.*, 1997; Hulinska *et al.*, 1994). It is not known whether the cysts observed in those studies are the same as the cysts described in this report. However, if viable cysts form in the body, they may represent a strategy that facilitates the survival of *B. burgdorferi* cells during nutritionally adverse conditions in host tissues. By forming cysts, it is also conceivable that *B. burgdorferi* cells evade detection by the immune system. We have not yet investigated the surface properties of cysts; thus, the clinical significance of these forms is not clear. However, Hulinska *et al.* (1994) demonstrated that the surface of cyst-forms found in the erythema chronicum migrans of infected-human tissue was non-reactive to antibodies against OspA, whereas the content of the cysts did react with OspA. It would be interesting to determine whether the surfaces of cysts described in this study are antigenically different from the surface of vegetative cells.

This study presents data describing the physiological response of *B. burgdorferi* to a specific starvation. Our data suggest that *B. burgdorferi* cells, although possessing a small genome and extremely limited biosynthetic capabilities, rapidly respond to conditions of serum starvation by inducing a programme that involves loss of motility, change in morphology, and rapid induction of proteins.

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