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Cystic forms of *Borrelia burgdorferi* sensu lato: induction, development, and the role of RpoS

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Summary. It has been demonstrated recently that cells of *Borrelia burgdorferi* sensu lato, the etiological agent of Lyme disease, transform from mobile spirochetes into nonmotile cystic forms in the presence of certain unfavourable conditions, and that cystic forms are able to reconvert to vegetative spirochetes in vitro and in vivo.

The purpose of this study was to investigate the kinetics of conversion of borreliae to cysts in different stress conditions such as starvation media or the presence of different antibiotics. Using the same experimental conditions we also investigated the possible role in cyst formation of RpoS, an alternative sigma factor that controls a regulon in response to starvation and transition to stationary phase.

We observed that β-lactams penicillin G and ceftriaxone, the antibiotics of choice in Lyme borreliosis treatment, favoured the production of cysts when used with serum-depleted BSK medium. In contrast, we observed a low level of cyst formation in the presence of macrolides and tetracyclines.

In order to elucidate the role of the *rpoS* gene in cyst formation we analyzed the reaction of the *rpoS* mutant strain in comparison with its wild-type in different conditions. Under the same stimuli, both the wild-type borrelia and the *rpoS* knock-out isogenic strain produced cystic forms with similar kinetics, thus excluding the participation of the gene in this phenomenon.

Our findings suggest that cyst formation is mainly due to a physical-chemical rearrangement of the outer membrane of *Borrelia burgdorferi* sensu lato leading to membrane fusion and controlled by different regulation mechanisms.

Key words: *Borrelia burgdorferi*, cystic forms, starvation, antibiotics, *rpoS* gene.

Introduction

Lyme borreliosis is a tick-borne zoonosis which develops through a peculiar polyphasic course, and one of the crucial questions still unanswered concerns the persistence of the infection. One of the arguments waiting to be clarified concerns "cystic forms" of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) and their possible role in resistance to antibiotic treatment, frequent relapses of the disease and spirochete survival in infected ticks. The first accurate report focusing on cystic forms was published in 1997 [1]. Similar and probably equivalent borrelial structures (gemmae, spheroplasts, L-forms) were described earlier [2–6] but were associated with spirochetal cells undergoing degenerative changes in a hostile environment (ambient changes, antibiotic treatment, host immune defence activity, aging of in vitro borrelial culture etc.). Brorson et al. demonstrated that normal motile borreliae could develop from *B. burgdorferi* cysts and that the cysts were metabolically active, at least under in vitro conditions [2]. Further studies showed that motile spirochetes also matured from cysts formed in distilled water, even after being kept in such a medium for over a month [7]. Recent studies demonstrated that cyst suspensions of different ages can retain infectivity and induce illness in vivo, in a mouse model [8]. These experiments clearly indicate that cystic forms are not only a sign of spirochetal degeneration but might represent a state or phase of low metabolic activity of *B. burgdorferi* bacterial cells that allows the spirochete to survive in a hostile environment until conditions are favourable for the borreliae to grow and replicate again.

The purpose of this study was to investigate the kinetics of conversion of borreliae to cysts in different stress conditions such as starvation media or the presence of different antibiotics. In addition, we investigated the possible role of the *rpoS* gene in cyst formation in the same experimental conditions. In *Escherichia coli,* RpoS controls a regulon of more than 30 genes, positively or negatively regulated, in response to starvation and transition to stationary phase [9, 10]. *E. coli* and other bacteria respond to nutrient starvation by entering a metabolic state referred to as stationary phase allowing them to survive environmentally unfavourable conditions such as oxidative stress, heat, high salts, and near-UV radiation [11–13]. Since cystic forms are a consequence of some unfavourable conditions, we also wanted to investigate the possible involvement of the *rpoS* gene in cyst formation. Therefore, we analyzed the conversion of borreliae to cystic forms in a *B. burgdorferi* sensu stricto *rpoS* mutant strain (M), with the *rpoS* locus inactivated by allelic exchange [14], in comparison with the *rpoS* wild-type organism (W).

Materials and methods

Borrelial strains and culture conditions

The borreliae used were *Borrelia garinii* strain BITS and *Borrelia burgdorferi* sensu stricto B31-A59 (wild-type; W) and its derived isogenic strain B31-A74 (mutant strain; M) with the *rpoS* locus inactivated by allelic exchange with *gyrB*^{r} as a selectable marker, kindly provided by A. F. Elias [14]. Bacteria were cultured to a density of approximately 10^7 bacteria/ml in BSK-H medium (Sigma) supplemented with 6% rabbit serum.

Induction of cystic forms

The cultures were centrifuged at 3200 x *g* for 30 min at room temperature and resuspended in starvation media such as BSK-H or RPMI 1640 (Sigma) to a final concentration of 1–2×10^7 bacteria/ml. Where appropriate, media were supplemented with antibiotics: penicillin G, ceftriaxone or erythromycin (Sigma) at a concentration of 2MIC, corresponding to 0.06 µg/ml for ceftriaxone and erythromycin and 0.12 µg/ml for penicillin G, as already determined [15–17]. The number of spirochetes and the development of cysts were monitored at selected time intervals by dark-field microscopy.

Verification of inactivation of the rpoS gene in mutant strain B31-A74

In order to confirm the persistence of *rpoS* gene inactivation in strain B31-A74 in different growth phases and during cyst formation, we amplified the region flanking the *gyrB*^{r} insertion using primers rpoS-F1 and rpoS-B1, kindly provided by A. F. Elias. Briefly, 1 ml of culture was washed twice in PBS, resuspended in 50 µl of H_2O, boiled for 10 min, and used as the template source. Amplification was carried out in a 50 µl mix as previously described [14]. Reaction conditions were 94 °C for 1 min, and then 30 cycles at 94 °C for 1 min, 50 °C for 0.45 min, and 68 °C for 3 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Results

Cyst induction in starvation media

Cyst formation was assayed in strain BITS, employing two different starvation media, serum-depleted BSK and RPMI. The slope of the growth curve is shown in Fig. 1: in BSK medium borreliae grew as living vegetative forms for the first two days and cysts were absent. During the following four days the number of borreliae was reduced by one logarithm and on the sixth day some cystic forms appeared. Subsequently the number of vegetative spirochetes continuously decreased and cyst density increased in parallel reaching a maximum on day 23. When the experiments were performed in RPMI borreliae were not able to grow, so we could only observe a loss in the number of viable bacteria, whereas cysts increased after 9 days, but their concentration was always below that reached in BSK-serum. This could be a consequence of the lower number of borreliae present in the first days of the culture that could convert to cysts in the following days of incubation. These results indicated that the best medium for producing cystic forms was BSK-serum; consequently this starvation medium was used throughout the investigation.

Induction of cyst formation under starvation and in the presence of different antibiotics

In order to analyse the influence of different antibiotics in cyst development, we followed the reaction in the presence of β-lactam antibiotics or macrolides at concentrations of 2MIC in BSK-complete or BSK-serum as starvation medium (Fig. 2a–h). Again no cyst formation was observed in BSK-complete medium in the first seven days, whereas in BSK-serum the cysts appeared after 3–4 days. The addition of β-lactam antibiotics penicillin G and ceftriaxone greatly stimulated cyst formation: in fact when added to both complete and starvation media at a

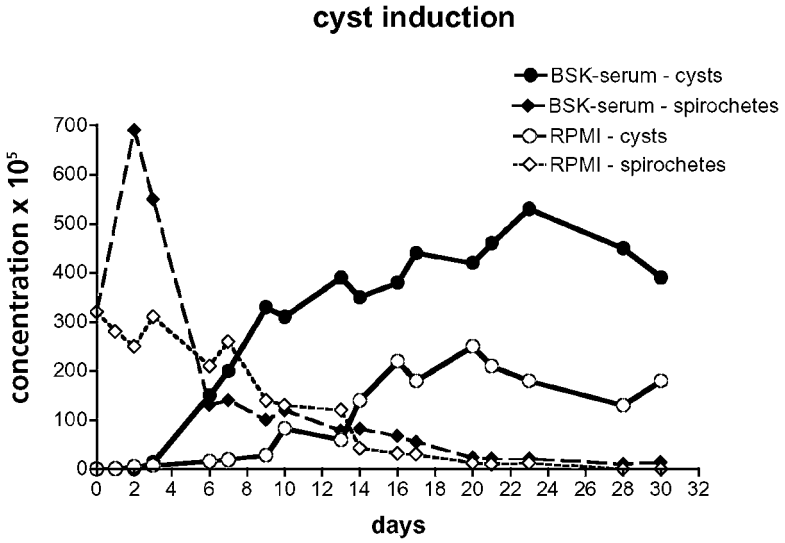
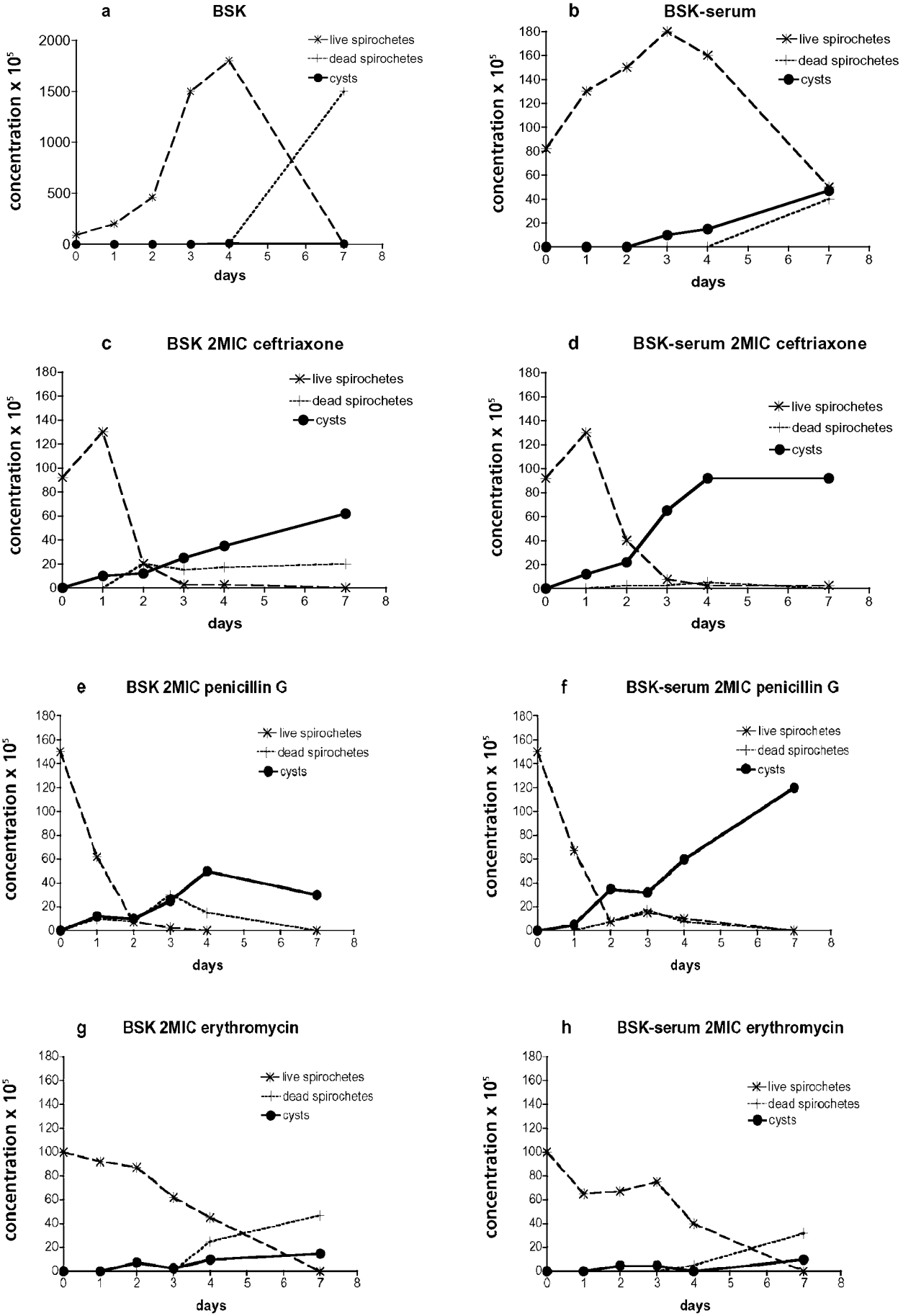


Fig. 1. Strain BITS was assayed for cyst formation in different starvation media: BSK depleted of serum (-serum) and RPMI, for one month. Borreliae and cysts were counted by dark-field microscopy


a
BSK
b
BSK-serum
c
BSK 2MIC ceftriaxone
d
BSK-serum 2MIC ceftriaxone
e
BSK 2MIC penicillin G
f
BSK-serum 2MIC penicillin G
g
BSK 2MIC erythromycin
h
BSK-serum 2MIC erythromycin
live spirochetes
dead spirochetes
cysts
concentration x 10⁵
days

concentration of 2MIC such compounds induced the development of cysts from the first day of incubation (Fig. 2 c–f). It is noteworthy that in serum-depleted BSK medium (Fig. 2 c, d) the number of cysts counted by microscopy was two or three times higher compared with the same medium without ceftriaxone or penicillin G. Therefore β-lactam antibiotics increased cyst production in addition to the effect of starvation medium. In contrast, very few cysts were observed in the presence of erythromycin in either medium, or with other antibiotic inhibitors of protein synthesis such as doxycycline or minocycline (data not shown).

Induction of cyst formation from wild and mutant strains

To test the possible involvement of the *rpoS* gene in cyst development we assayed the two borrelia strains B31-A59 (W), the wild type *rpoS* +, and *rpoS*-inactivated B31-A74 (M) in BSK-serum medium. As shown in fig. 3a both strains produced a lower concentration of cysts than strain BITS and showed a parallel course of cyst production over time. A similarity between the two strains was also observed in an inductive medium such as BSK-serum containing β-lactam antibiotics (Figs. 3b, 4c). In these conditions both strains developed cysts faster than in BSK-serum alone. This phenomenon was already appreciable after 3 days' incubation and increased until day 5, when the spirochetes were all dead. It is noteworthy that, in the presence of penicillin G (Fig. 3b), from day 8 of culture there was a decrease in cyst concentration contemporary with the reappearance of vegetative spiral forms in both W and M strains. Since penicillin G undergoes inactivation during 8 days of culture, the reappearance of the spiral vegetative forms of *Borrelia burgdorferi* and their multiplication is certainly due to the reconvertion of cysts to the viable spirochete forms.

Persistence of rpoS inactivation in A74 (M) during cyst formation

In order to check the persistence of *rpoS* inactivation in the mutant strain, we amplified the chromosomal sequences flanking the *gyrB*^{r} insertion within the *rpoS* locus. PCR reactions were performed on borreliae in different growth phases: early, mid-log, late-log and stationary phases that corresponded to 10^5, 10^6, 5×10^7 and 10^8 borreliae/ml respectively (Fig. 5). The same reaction was used to analyze *rpoS* inactivation during cyst formation after 10, 21, and 32 days of incubation in BSK-serum containing a concentration of 10^6, 10^7, and 2×10^7 cysts/ml respectively. As shown in Fig. 5, all the amplified segments of the B31-A74 culture were of about 3000 bp which corresponds to a fragment of *rpoS* gene plus the 2 kb insertion of *gyrB*^{r}, confirming the inactivation of *rpoS* gene in all the conditions. PCR of the wild type strain B31-A59 produced an amplicon of only 1200 bp (Fig. 4), which corresponds to the length of the fragment without the insertion.

Discussion

Different aspects of the formation of so-called cystic forms of *Borrelia burgdorferi* have been studied by several authors [1–7,18]:

- morphology and morphological evolution using electron microscopy;
- induction by some stress conditions such as hyperosmolarity, age, starvation, antibiotics;
- reconversion to vegetative helical spirochetes in vitro [8].

The majority of the reports agree [2, 4, 6, 18] that these morphological variations develop in vitro under starvation and other critical environmental conditions. Recent work published by our group [8] demonstrated that cystic forms were able to reproduce the infection in a mouse model confirming that they are able to reconvert also in vivo.

In this study, in order to determine the possible involvement of the regulator gene *rpoS* in cyst formation, we set up starvation conditions with added antibiotics, which forced the formation of cysts and the elimination of vegetative forms. One observation we made is that β-lactams penicillin G and ceftriaxone, the antibiotics of choice in Lyme borreliosis treatment, favoured the production of cysts when used at concentrations of 2MIC in serum-depleted BSK medium. We also observed a similar trend in the presence of the clinical therapeutic dose of ceftriaxone (13–15 μg/ml) [19], with a conversion of borreliae to a comparable cyst concentration (data not shown). It is possible that at subinhibitory concentrations β-lactams induce cyst formation also in vivo: in such conditions, as in those occurring at the end of treatment, cysts could germinate to vegetative forms, and this phenomenon could contribute to recurrence of the infection.

Clear evidence of a strong stimulation of cystic forms was not observed when borreliae were tested in the presence of substances that block protein synthesis. In fact we observed a low level of cyst formation not only in the presence of macrolides but also in the presence of tetracyclines such as doxycycline or minocycline (data not shown). This is in contrast to the data of Scott Alban et al. [18] and Kersten et al. [6], who reported cyst formation as a sporadic phenomenon occasionally observed in the presence of tetracycline. We hypothesize that the action of β-lactams in comparison with antibiotics acting on bacterial ribosomes is due to the specific inhibition of peptidoglycan biosynthesis, which results in a destabilization of cell structure and wall disruption, thus favouring the assembly of cystic forms. Similar conclusions were reached by DeLoney et al. [20] regarding the transformation of vegetative to coccoid forms of *Helicobacter pylori* treated with β-lactams in vitro.

Fig. 2. Cyst formation from strain BITS in BSK (**a**), BSK-serum as starvation medium (**b**), and in the presence of 2MIC ceftriaxone (0.06 μg/ml) (**c**, **d**), 2MIC penicillin G (0.12 μg/ml) (**e**, **f**), and 2MIC erythromycin (0.06 μg/ml) (**g**, **h**). Borreliae and cysts were counted by microscopy for seven days

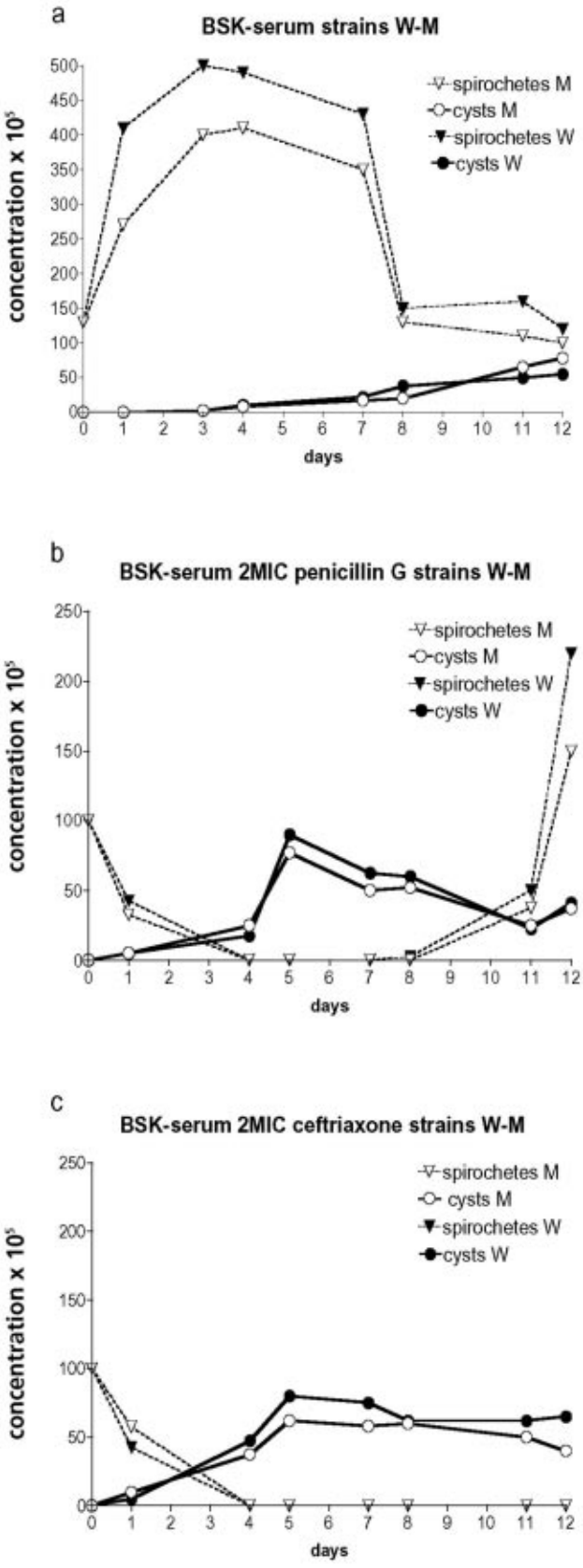


Fig. 3. Strains B31-A59 (W) and B31-A74 (M) were examined for cyst formation in BSK-serum medium (**a**) with 2 MIC penicillin G (0.12 μg/ml) concentration (**b**) and with 2MIC ceftriaxone (0.06 μg/ml) concentration (**c**)

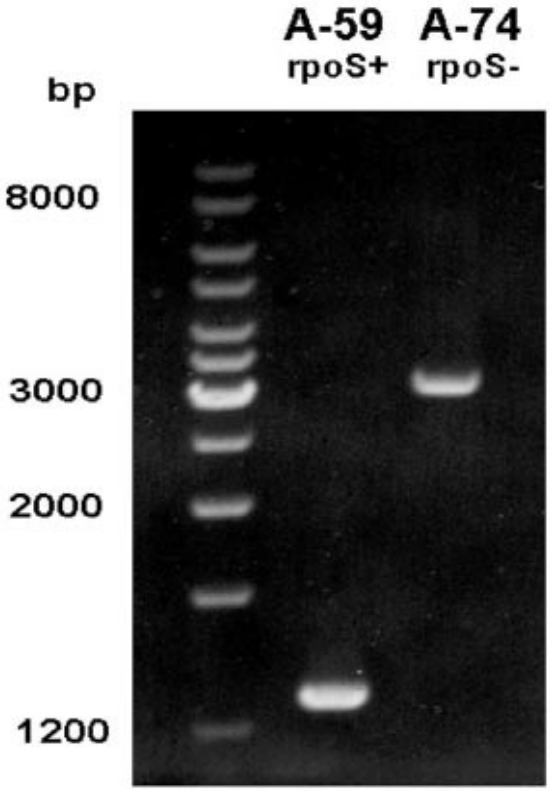


Fig. 4. Confirmation of *gyb*^{r} insertion in the *rpoS* chromosomal locus by PCR. Genomic DNA from *Borrelia burgdorferi* sensu stricto clones B31-A74 (Mutant) and B31-A59 (Wild). DNA was amplified by PCR with the primer set rpoS-F1 and rpoS-B1 flanking the *gyrb*^{r} insertion in the *rpoS* gene, analyzed by agarose gel electrophoresis, and visualized with ethidium bromide. The positions of DNA size standards are indicated on the left

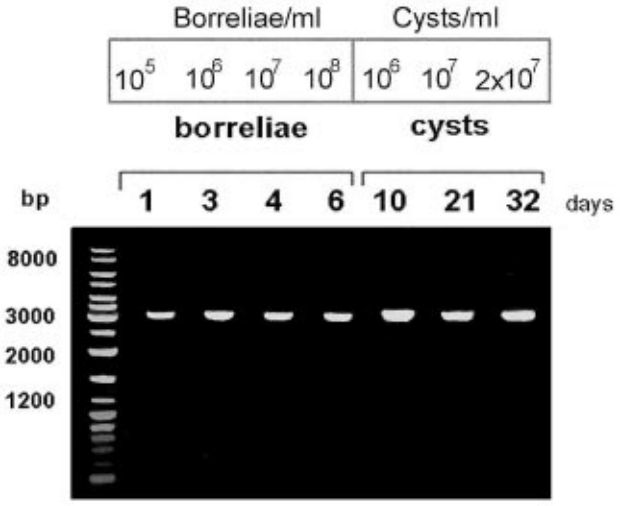


Fig. 5. Persistence of *rpoS* inactivation in strain A-74 (mutant) in different growth phases: early (10^5 borreliae/ml), mid log (10^6 borreliae/ml), late log (5×10^7 borreliae/ml) and stationary phases (10^8 borreliae/ml), and during cyst formation in different maturation times (10, 21, and 32 days of incubation) and concentrations (10^6, 10^7, 2×10^7). PCR was performed as already described in the legend to Fig. 4. The positions of DNA size standards are indicated on the left

In order to elucidate the role of gene regulation in the phenomenon of cyst formation we analyzed the possible involvement of the *rpoS* gene. RpoS is a regulator of a central importance for gene expression during the transition into stationary phase and starvation in many bacteria, and borrelial RpoS controls a regulatory pathway involving at least 7 proteins [21] and increases osmotic resistance during the starvation period [14].

We analyzed the reaction of the *rpoS* mutant strain, the first strain of *Borrelia burgdorferi* with a chromosomal gene inactivated, in comparison with its wild-type in different conditions.

Under the same stimuli, both the wild-type of borrelia and the *rpoS* knock-out isogenic strain produced cystic forms with similar kinetics, thus excluding the participation of this gene in the phenomenon. Similar observations have been reported for other bacteria such as *Campylobacter jejuni*: this microorganism develops variant cell types known as "coccoid forms" with different phenotypic characteristics from the vegetative forms even though the *rpoS* gene is not present in the genome of the species [22].

Our findings, together with other observations that spiral to cyst transformation occurs in a very short time under iposmotic [7] and oxidative stress (data not shown), suggest that the phenomenon is mainly due to a physical-chemical rearrangement of the outer membrane of *Borrelia burgdorferi*, leading to membrane fusion and controlled by different regulation mechanisms.

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