Sleeper cells: The stringent response and persistence in the *Borreliella* (*Borrelia*) *burgdorferi* enzootic cycle

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Summary

Infections with tick-transmitted *Borreliella* (*Borrelia*) *burgdorferi*, the cause of Lyme disease, represent an increasingly large public health problem in North America and Europe. The ability of these spirochetes to maintain themselves for extended periods of time in their tick vectors and vertebrate reservoirs is crucial for continuance of the enzootic cycle as well as for the increasing exposure of humans to them. The stringent response mediated by the alarmone (p)ppGpp has been determined to be a master regulator in *B. burgdorferi*. It modulates the expression of identified and unidentified open reading frames needed to deal with and overcome the many nutritional stresses and other challenges faced by the spirochete in ticks and animal reservoirs. The metabolic and morphologic changes resulting from activation of the stringent response in *B. burgdorferi* may also be involved in the recently described non-genetic phenotypic phenomenon of tolerance to otherwise lethal doses of antimicrobials and to other antimicrobial activities. It may thus constitute a linchpin in multiple aspects of infections with Lyme disease borrelia, providing a link between the micro-ecological challenges of its enzootic life-cycle and long-term residence in the tissues of its animal reservoirs, with the evolutionary side-effect of potential persistence in incidental human hosts.

Introduction

Borreliella (*Borrelia*) *burgdorferi* (Adeolu and Gupta, 2014; Barbour *et al.*, 2017), the cause of Lyme disease, has a small but complex genome consisting of one large linear chromosome and multiple linear and circular plasmids, together comprising approximately 1,520 kbp with a G+C content of 28.6% (Fraser *et al.*, 1997; Casjens *et al.*, 2000). This spirochete has two two-component regulatory systems (TCS), three sigma factors, is totally dependent on anaerobic glycolysis to generate ATP (i.e., it has no enzymes of the tricarboxylic cycle), and as such, is unable to synthesize de novo amino acids, nucleotides and fatty acids (Fraser *et al.*, 1997; Radolf *et al.*, 2012; Corona and Schwartz, 2015). It is thus a fastidious auxotroph whose nutritional requirements are still undetermined (Gherardini *et al.*, 2010; Corona and Schwartz, 2015).

Despite the paucity of TCS compared to other bacterial pathogens, *B. burgdorferi* is still able to overcome the challenges encountered in infecting, colonizing and surviving long-term in ticks and vertebrates (Radolf *et al.*, 2012; Corona and Schwartz, 2015). The regulatory axis mediated by the histidine kinase 1 (Hk1)-response regulator 1 (Rrp1) TCS is involved in tick colonization (Rogers *et al.*, 2009; Freedman *et al.*, 2010; He *et al.*, 2011; Pappas *et al.*, 2011; Caimano *et al.*, 2015). It generates the second messenger cyclic diguanylate monophosphate (c-di-GMP) and stimulates utilization of glycerol and other functions necessary for survival in ticks (Rogers *et al.*, 2009; Freedman *et al.*, 2010; He *et al.*, 2011; Pappas *et al.*, 2011; He *et al.*, 2014; Novak *et al.*, 2014). In contrast, the regulatory axis mediated by RpoN, RpoS and the Hk2-Rrp2 TCS modulates expression of genes essential for tick transmission and mammalian infection (Hübner *et al.*, 2001; Caimano *et al.*, 2004; Fisher *et al.*, 2005; Caimano *et al.*, 2007; Smith *et al.*, 2007; Boardman *et al.*, 2008; Ouyang *et al.*, 2008; Ouyang *et al.*, 2012). This second TCS regulatory axis also represses glycerol utilization and many other functions needed by *B. burgdorferi* for proliferation in ticks but not in the mammalian host where glucose, a

preferred carbon source, is readily available (Caimano *et al.*, 2007; Corona and Schwartz, 2015).

In many pathogens, the ancestral stringent response triggered by amino acid starvation and mediated by the alarmones guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp or “magic spots”) is involved in coordinated regulation of many genes and regulatory and metabolic pathways (Fig. 1) (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Boutte and Crosson, 2013; Hauryliuk *et al.*, 2015; Liu *et al.*, 2015; Steinchen and Bange, 2016). The stringent response links cell division, bacterial growth, intermediary metabolism, chemotaxis and motility, morphotypic transformations, and virulence properties necessary to survive environmental challenges. In some bacteria, (p)ppGpp is mainly synthesized and hydrolyzed by two enzymes, RelA and SpoT, in others chiefly by a single bifunctional enzyme, Rel or RSH (RelA/SpoT homolog), with both activities. Cytoplasmic redundant short alarmone synthetases and GTPases in some bacteria provide additional paths to (p)ppGpp regulation (Gaca *et al.*, 2015a; Gaca *et al.*, 2015b). The global changes in transcription seen with the stringent response are due to allosteric changes in RNA polymerase that modify its specificity for different promoters and are caused by the interaction of (p)ppGpp with the RNA polymerase β' and ω subunits and with the small protein DksA (Mallik *et al.*, 2006; Doniselli *et al.*, 2015; Ross *et al.*, 2016). In *E. coli*, the ability of (p)ppGpp to repress or trigger transcription of different promoters is provided by the presence of specific DNA sequences called discriminators (Potrykus and Cashel, 2008).

The limited number of TCS in *B. burgdorferi* suggests that alternative global regulators able to sense environmental conditions will be prominent in modulating gene expression in this organism (Radolf *et al.*, 2012; Corona and Schwartz, 2015; Caimano *et al.*, 2016). Extrapolations based on results obtained with *E. coli* and other bacteria can provide important clues to this analysis, with the understanding that the functions and interactions of borrelial orthologs might not always solely be determinable by extrapolation from these other organisms

(Hyde *et al.*, 2006; Caimano *et al.*, 2007). We long ago suggested that the stringent response was likely to be involved in the ability of *B. burgdorferi* to survive and persist in its vector and vertebrate hosts (Godfrey *et al.*, 2002). Several studies have subsequently confirmed that the stringent response is a global regulator in *B. burgdorferi*, and in fact is the only such regulator that can simultaneously modulate DNA replication, synthesis of stable RNAs (tRNA and rRNA), and synthesis and translation of mRNA in this organism (Fig. 1) (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). We now review evidence revealing that the stringent response plays an important role in the perpetuation of the *B. burgdorferi* enzootic cycle, suggest where it may exert its functions in the enzootic cycle, and indicate how these might be recruited to pathological ends in hosts outside it (Figs. 2 and 3)**.**

Rel_{Bbu} and (p)ppGpp in *B. burgdorferi*

B. burgdorferi contains a single rel_{Bbu} gene (BB0198) transcribed from its own σ^{70} promoter (Fraser *et al.*, 1997). It encodes a bifunctional enzyme able to synthesize and hydrolyze (p)ppGpp and complement *E. coli* mutants unable to produce (p)ppGpp (Bugrysheva *et al.*, 2003; Bugrysheva *et al.*, 2005; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). Null mutants of rel_{Bbu} of non-infectious *B. burgdorferi* B31 and infectious N40 and B31-5A4 strains failed to generate (p)ppGpp, confirming its unique responsibility for the presence of the alarmone in this bacterium (Bugrysheva *et al.*, 2005; Drecktrah *et al.*, 2015). Growth of *B. burgdorferi* B31 in nutrient-limited RPMI media in the absence of rabbit sera and in the presence of tick saliva led to increases in the transcription of rel_{Bb} and levels of (p)ppGpp, decreased synthesis of motility genes and the appearance of round forms (Alban *et al.*, 2000; Concepcion and Nelson, 2003; Drecktrah *et al.*, 2015). Experiments with wild-type infectious *B. burgdorferi* B31-5A4 and its null rel_{Bbu} mutant and complemented derivatives confirmed the induction of (p)ppGpp under nutrient depletion and indicated that these markers decreased but did not totally disappear when *B. burgdorferi* cells were returned to an enriched media (Drecktrah *et al.*, 2015). Moreover, both

transcriptional and post-transcriptional expression of Rel_{Bbu} was modulated by the host environment during growth in rat peritoneal chambers, in the presence of tick cells, and in ticks. Although regulation and the levels of (p)ppGpp appeared to be strain dependent, (p)ppGpp was always present in *B. burgdorferi* B31 and N40 growing in BSK-H (Bugrysheva *et al.*, 2002; Bugrysheva *et al.*, 2003; Bugrysheva *et al.*, 2005). Further experiments are needed to characterize the nutritional requirements of *B. burgdorferi* and the stimuli that trigger the stringent response in various environments.

Absence of (p)ppGpp in a *B. burgdorferi* 297 rel_{Bbu} null mutant grown in BSK-H media was associated with a paradoxical growth deficit resulting from the slowing of cell division, most dramatically at the stationary phase (Bugrysheva *et al.*, 2005). Transition to stationary phase from exponential phase in this mutant was associated with abnormally high levels of rRNA, similar to that seen during unbalanced growth of the relaxed *E. coli* mutant (Gallant and Cashel, 1967; Lazzarini *et al.*, 1971; Bugrysheva *et al.*, 2011). This suggests that the observed growth deficit in the stationary phase may be due to aberrant gene expression and may indicate that (p)ppGpp is an important regulator of balanced growth in *B. burgdorferi* (Fig. 1) (Bugrysheva *et al.*, 2005; Potrykus *et al.*, 2011; Bugrysheva *et al.*, 2011). Furthermore, a *B. burgdorferi* B31-5A4 null rel_{Bbu} derivative grown in RMPI media to stationary phase lost viability and exhibited a significant number of borrelial round bodies, thus confirming the presence of these structures in the life cycle of the organism (Brorson and Brorson, 1997; Alban *et al.*, 2000; Dunham-Ems *et al.*, 2012; Drecktrah *et al.*, 2015).

The stringent response is a global regulator in *B. burgdorferi*

A global gene regulation pattern corresponding to a potential stringent response was observed in early microarray studies of wild type *B. burgdorferi* 297 exposed to in vitro growth conditions that mimicked those in ticks (Revel *et al.*, 2002). Comparative microarray analysis of this strain and its rel_{Bbu} null mutant found altered expression of many genes in both the exponential (6%)

and stationary phases (20%) of growth (Bugrysheva *et al.*, 2015). Analysis of similar mutant derivatives of *B. burgdorferi* B31-5A4 by RNA-seq showed the stringent response to be an important global regulator, especially in the stationary phase. In these latter studies, (p)ppGpp could shift gene expression both positively and negatively from that characteristic of stationary phase to that of starvation and recovery (Drecktrah *et al.*, 2015). The *B. burgdorferi* stringent response modulated expression of genes mediating DNA synthesis and repair, proteins synthesis, cell division and cell envelope synthesis, motility and chemotaxis and intermediary metabolism (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). Lipoprotein genes, including several adhesins and a decorin binding protein (*dbpB*) as well as the antigenic variation surface exposed lipoproteins (VslEs) were also modulated by (p)ppGpp in the stationary phase (Drecktrah *et al.*, 2015).

The *B. burgdorferi* stringent response also modulates expression of regulatory and structural genes affecting important biochemical pathways such as carbon source and amino acid metabolism (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). For example, lack of synthesis of (p)ppGpp was accompanied by increased transcription of the genes of the stringent response regulator DksA, σ^{70} and the Rrp1/Hk1 TCS. This suggests some repression of these global regulators during the stringent response (Bugrysheva *et al.*, 2015). Transcription of genes encoding regulatory proteins CsrA, RpoS and BosR were also modulated by the borrelial stringent response, implying that (p)ppGpp may modulate gene expression indirectly through other global regulators (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). Metabolic genes positively regulated during the stringent response included those involved in metabolism of glycerol, a sugar utilized by *B. burgdorferi* in ticks, and genes involved in oligopeptide transport, as would be expected as a response to lack of nutrients (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). Genes negatively regulated by the stringent response in *B. burgdorferi* included those involved in the metabolism of chitobiose, an alternative sugar also present in ticks and utilized by *B. burgdorferi* and the mevalonate pathway genes *hmg* and *mvaA* involved in cell wall and

membrane synthesis (Fig. 2) (Rhodes *et al*., 2009; Van Laar *et al*., 2012; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015).

The alarmone (p)ppGpp is thus an important global regulator of *B. burgdorferi*. It can modulate expression of approximately 30% of its genes during both exponential and stationary phases of growth and during shifts to and from starvation and repletion, and redirect borrelia metabolism accordingly (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). Although more than half of regulated *B. burgdorferi* genes encode currently hypothetical proteins, the patterns of altered regulation of known genes in this organism generally correspond to those associated with the stringent response in other bacteria (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Boutte and Crosson, 2013; Hauryliuk *et al.*, 2015; Liu *et al.*, 2015). The short half-life of (p)ppGpp means that reversal of the *B. burgdorferi* stringent response triggered by starvation and other still uncharacterized stimuli is rapid once their stimuli subside which helps it quickly accommodate to the microenvironmental changes associated with borrelial residence in ticks and vertebrates (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015; Bergkessel *et al.*, 2016). *B. burgdorferi*, like other bacteria, produces basal detectable levels of (p)ppGpp in the absence of nutritional stimuli (Bugrysheva *et al.*, 2005; Drecktrah *et al.*, 2015; Gaca *et al.*, 2015a; Gaca *et al.*, 2015b); the function of these basal levels of (p)ppGpp in global gene regulation deserves further investigation.

The stringent response during acquisition and transmission of *B. burgdorferi* by *Ixodes* larvae and nymphs

After feeding to repletion on an infected mammalian host, each larva contains about 500 *B. burgdorferi* per tick (Piesman *et al.*, 1990; Soares *et al.*, 2006). By 10 days, this rises to less than 3,000 just before molting into nymphs is completed and the reservoir blood is absorbed. Once molting is completed, the concentration of *B. burgdorferi* in the unfed nymphs is less than

100 organisms per tick, probably because of a lack of nutrients and exposure to the tick's innate immune responses and microbiome-mediated antibacterial properties (Piesman *et al.*, 1990; Soares *et al.*, 2006; Narasimhan *et al.*, 2014). In contrast, there is a much larger range in numbers of *B. burgdorferi* in feeding nymphs during transmission, with less than 100 spirochetes in the flat nymph at the start of feeding to close to 10^5 at repletion (Fig. 2) (Piesman *et al.*, 1990). These changes appear to be tied to the increase and/or decrease of nutritional components provided by the blood meal, the blood meal's antibacterial properties, and antibacterial components present in the tick (Piesman *et al.*, 1990; Soares *et al.*, 2006; Pal and Fikrig, 2010). Involvement of the stringent response in the ability of *B. burgdorferi* to traverse tick metamorphosis and transmission to a new host (Fig. 2) is suggested by its ability to regulate growth and modulate expression of genes involved in glycerol and chitobiose metabolism, both carbon sources crucial to its survival in larvae and nymphs (Fig. 1) (He *et al.*, 2011; Pappas *et al.*, 2011; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). In fact, *B. burgdorferi* rel_{Bbu} null mutants unable to produce (p)ppGpp acquired by nymphs from infected mice could not reach sufficiently high concentrations to permit transmission and completion of the enzootic cycle (Drecktrah *et al.*, 2015).

We suggest that the *B. burgdorferi* stringent response is unlikely to be active in feeding larvae during acquisition because blood entering the tick gut contains amino acids, glucose, other sugars and fatty acids (Fig. 2) (Radolf *et al.*, 2012; Corona and Schwartz, 2015). By the time larvae finish feeding and molting, the blood meal has been digested and its nutrients completely absorbed. This lack of nutrients stimulates the stringent response and results in non-dividing borrelia with inhibited growth and sluggish motility in the flat nymphs (Dunham-Ems *et al.*, 2009; Pal and Fikrig, 2010; Corona and Schwartz, 2015). After the flat nymphs start to feed, we assume that the stringent response continues for a short period of time but is blunted by the blood entering the tick gut. The hypothesized active *B. burgdorferi* stringent response in the first 24-48 h of nymph feeding would coincide with observed borrelial cell blebs, transient round

forms and formation of biofilm-like networks and aggregates, and expression of borrelial adhesins for tick epithelial cell receptors together with adhesion-mediated motility through the gut epithelial cells (Fig. 2) (Pal *et al.*, 2004; Ferullo and Lovett, 2008; Traxler *et al.*, 2008; Srivastava and de Silva, 2009; Dunham-Ems *et al.*, 2009; Zhang et al., 2011; Dunham-Ems *et al.*, 2012; Meriläinen *et al*., 2015; Gupta *et al.*, 2016). It is plausible that the stringent response mediates these processes since the stringent response in *B. burgdorferi* (like that in other bacteria) modulates expression of genes of the mevalonate pathway involved in cell wall morphogenesis, inhibition of motility, and formation of aggregates, biofilms and quorum sensing (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Boutte and Crosson, 2013; Arnold *et al.*, 2015; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015; Gupta *et al*., 2016). After 48 h of feeding, as nutrients from the blood meal begin to be utilized by tick gut epithelial cells and increased nutrients are available to the spirochetes, the stringent response will subside, and spirochetes will begin to divide and rapidly reach high concentrations (Piesman *et al.*, 1990; Dunham-Ems *et al.*, 2009). By the time spirochetes reach the basement membrane of the tick gut epithelia, motility has been reactivated, and a fraction of motile borrelia migrate from the gut to the haematocele and the salivary glands (Fig. 2) (Dunham-Ems *et al.*, 2009; Dunham-Ems *et al.*, 2012).

These hypothesized shifts in activity of the *B. burgdorferi* stringent response are consistent with experimentally observed changes in transcription of genes associated with sugar utilization (Pappas *et al.*, 2011; He *et al.*, 2011; Bugrysheva *et al.*, 2015; Corona and Schwartz, 2015; Drecktrah *et al.*, 2015). Borrelia preferentially utilize glucose provided in feeding ticks by ingested blood (von Lackum and Stevenson, 2005; Corona and Schwartz, 2015). The suggested activation of the stringent response in flat nymphs is associated with glycerol being used as a carbon source (He *et al.*, 2011; Pappas *et al.*, 2011). With the hypothesized blunting of the stringent response as feeding in nymphs is being completed, utilization will shift from glycerol to glucose and chitobiose (Fig. 2) (Traxler *et al.*, 2006; Pappas *et al.*, 2011; Corona and

Schwartz, 2015). The latter sugars only become available at this stage because of the reorganization of the peritrophic membrane produced by the incoming blood and by the sloughing of tick gut intestinal cells (Fig. 2) (Zhu *et al.*, 1991; Dunham-Ems *et al.*, 2009; Pal and Fikrig, 2010; Pappas *et al.*, 2011; Dunham-Ems *et al.*, 2012; Corona and Schwartz, 2015).

Regulation of these metabolic shifts in *B. burgdorferi* by other global regulators including RpoS, BosR, BadR and c-di-GMP as well as by (p)ppGpp underlines their relevance for the bacterial life cycle in the tick (Hyde *et al.*, 2006; Hyde *et al.*, 2010; Freedman *et al.*, 2010; He *et al.*, 2011; Pappas *et al.*, 2011; Sze *et al.*, 2012; Miller *et al.*, 2013; Sze *et al.*, 2013; Corona and Schwartz, 2015; Caimano *et al.*, 2015; Ouyang and Zhou, 2015; Caimano *et al.*, 2016). The progressive variations described here, in cell division, growth, morphotypes, motility, carbon source utilization and potential tolerance to noxious stimuli all appear to be necessary for borrelia to complete the tick cycle (Fig.2) (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). The stimuli responsible for the stringent response in their transit through the ticks, although currently undetermined, can be reasonably assumed (as in other bacteria) to result from a lack of amino acids required for protein synthesis (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Boutte and Crosson, 2013; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). This supposition is supported by the fact that the stringent response positively regulates the Opp transporter systems needed for the transport of oligopeptides, the intracellular source of these amino acids (Wang *et al.*, 2002; Medrano *et al.*, 2007; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). The stringent response could also be generated by as yet uncharacterized mechanisms including the availability, or lack of, other nutrients such as fatty acids, sugars, metal ions, oxidative stress and changes in osmolarity or pH (Revel *et al.*, 2002; Potrykus and Cashel, 2008; Boutte and Crosson, 2013; Bontemps-Gallo *et al.*, 2016).

The stringent response and *B. burgdorferi* residence in vertebrate reservoirs

The emergence and spread of *B. burgdorferi* infections depends on its ability to take up residence in its tick vectors and vertebrate hosts for extended periods of time (i.e., its permanence) in a fashion that ensures continuity of the enzootic cycle (Radolf *et al.*, 2012; Schotthoefer and Frost, 2015; Steere *et al.*, 2016). While the estimated numbers of spirochetes in the salivary glands of nymphs during transmission are approximately 60 per gland, only some of them are injected and only a fraction of those injected are infectious (Leuba-Garcia *et al.*, 1998; Ohnishi *et al.*, 2001; Lima *et al.*, 2005). This suggests that borrelia undergo a proliferative burst after a low dose inoculation in the skin (Fig. 3) (Leuba-Garcia *et al.*, 1998; O'Rourke *et al.*, 2013; Stupica *et al.*, 2015). This has been directly confirmed both in humans with Lyme disease and in *Peromyscus* and *Mus musculus* mice infected with *B. burgdorferi* by either feeding nymphs or needle injection (Piesman *et al.*, 1987; Piesman, 1989; Barthold *et al.*, 1991; Liveris *et al.*, 2002; Barthold *et al.*, 2010b; Li *et al.*, 2011).

The few spirochetes inoculated in the mouse dermis are exposed to appreciable concentrations of tissue nutrients and glucose, their preferred carbon source (von Lackum and Stevenson, 2005; Corona and Schwartz, 2015), and the stringent response probably abates. The rate of cell division in the skin rapidly increases; concentrations of 1 x 10^4 to 1 × 10^5 per mg tissue are reached in a few days, with dissemination towards the periphery of the site of inoculation (Liveris *et al.*, 2002; O'Rourke *et al.*, 2013; Stupica *et al.*, 2015). This growth is accompanied by active motility mediated by chemotaxis and hematogenous and lymphatic spread to distant organs (Barthold *et al.*, 1991; Wang *et al.*, 2001; Wang, 2002; Barthold *et al.*, 2010a; Kumar *et al.*, 2015). Another burst of cell division and growth occurs on colonization of organs a few days later, and densities of approximately 1 x 10^4 spirochetes per mg tissue can be reached in this phase of infection (Fig. 3) (Barthold *et al.*, 1991; Wang *et al.*, 2001; Wang, 2002; Barthold *et al.*, 2010b).

In time, bacterial multiplication subsides, probably as result of antibacterial immune responses and nutrient limitations. Months after infection, only scattered borrelia are detected in

some organ refugia by light microscopy and PCR (Fig. 3) (Barthold *et al.*, 1991; Zeidner *et al.*, 2001; Barthold *et al.*, 2010a). In animal models of *B. burgdorferi* infection, there is a predilection of the spirochetes for collagenous tissues, which may be the result of borrelial adhesins for receptor molecules on the cells of these tissues (Coburn *et al.*, 2013; Imai *et al.*, 2013; Brissette and Gaultney, 2014; Caine and Coburn, 2015; Kumar *et al.*, 2015; Wager *et al.*, 2015; Zhi *et al.*, 2015) and/or the presence of antibodies preventing multiplication and invasion of other tissues and organs (Barthold *et al.*, 1991; Bockenstedt *et al.*, 2001; Barthold *et al.*, 2006; Barthold *et al.*, 2010b).

In mice, resurgence in the number of *B. burgdorferi* can occur many months after infection as antibody levels begin to wane (Barthold *et al.*, 1993; Barthold *et al.*, 2010b). The sparse numbers of bacteria in these tissues display a quiescent state with low motility and no evidence of multiplication, but are probably viable as evidenced by their transcriptional competence that may permit their resurgence under some conditions (Barthold *et al.*, 1993; Liang *et al.*, 2004; Cabello *et al.*, 2007; Barthold *et al.*, 2010b; Imai *et al.*, 2013). In this apparently quiescent state in the collagenous tissues, it is expected that the stringent response will be activated because of nutrient limitation, and transport mechanisms for amino acids and other molecules will be stimulated (Wang *et al.*, 2002; Medrano *et al.*, 2007; Potrykus and Cashel, 2008; Boutte and Crosson, 2013; Hauryliuk *et al.*, 2015; Liu *et al.*, 2015; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). This quiescent state could have the side-effect of making the borrelia tolerant to antimicrobials and immune activity and lead to spirochetal persistence (Fig. 3) (Lusitani *et al.*, 2003; Barbour, 2012; Feng *et al.*, 2014; Caskey and Embers, 2015; Sharma *et al.*, 2015; Feng *et al.*, 2015a).

This pattern of infection in the mammalian host suggests that following transmission of *B. burgdorferi* into the dermis by feeding nymphs, the stringent response is turned off because sufficient levels of nutrients are available in the dermis, blood and other host tissues (Corona and Schwartz, 2015). This could possibly account for the finding that mice were not infected

following injection of a low concentration (1 x 10^4 cells) of a *B. burgdorferi* 297 rel_{Bbu} mutant unable to synthetize (p)ppGpp (Bugrysheva *et al.*, 2005). However, a similar *B. burgdorferi* mutant of a different strain (*B. burgdorferi* B31-5A4) was infectious at higher doses of organisms (1 x 10^5 to 1 x 10^6) (Drecktrah *et al.*, 2015). The potential relevance of rel_{Bbu} and (p)ppGpp for *B. burgdorferi* infection and continued presence in the mouse reservoir may thus be dependent on both the inoculated dose and the *B. burgdorferi* strain involved (Bugrysheva *et al.*, 2003; Bugrysheva *et al.*, 2005; Drecktrah *et al.*, 2015). The ability of the stringent response to coordinate the changes needed by borrelia to transition rapidly from actively multiplying to quiescent states and back in vertebrate reservoirs and to insure its persistence and availability for the vector during acquisition argues for its role in this stage of the enzootic cycle (Fig. 3) (Barthold *et al.*, 1991; Bugrysheva *et al.*, 2003; Bugrysheva *et al.*, 2005; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015).

Could the borrelial stringent response mediate antimicrobial tolerance and persistence as an adaptation for the enzootic cycle?

While still a matter of dispute, there are numerous reports of antimicrobial treatment unable to completely eliminate *B. burgdorferi* from the tissues of experimentally infected rodents and non-human primates (Bockenstedt *et al.*, 2002; Hodzic *et al.*, 2008; Wormser and Schwartz, 2009; Barthold *et al.*, 2010b; Barbour, 2012; Embers *et al.*, 2012; Embers and Barthold, 2012; Wormser *et al.*, 2012; Hodzic *et al.*, 2013; Iyer *et al.*, 2013; Hodzic *et al.*, 2014). In some instances, bacteria could be rescued from treated animals by xenodiagnoses; these rescued bacteria were non-culturable and displayed a decreased infectious potential (Bockenstedt *et al.*, 2002; Hodzic *et al.*, 2008; Embers *et al.*, 2012). They appeared to be transcriptionally active in the reservoir, could be transmitted transstadially in ticks after xenodiagnoses, were able to infect SCID mice and produce pathological lesions, and could be transmitted by transplanted tissue

(Hodzic *et al.*, 2008; Barthold *et al.*, 2010b; Embers and Barthold, 2012). This suggests that antimicrobial-tolerant forms described in vitro may have relevance in explaining their occurrence in the mammalian reservoir (Feng *et al.*, 2014; Caskey and Embers, 2015; Sharma *et al.*, 2015; Feng *et al.*, 2015a; Feng *et al.*, 2016).

Borrelia may become phenotypically (non-heritably) tolerant to antimicrobials. For example, calprotectin, a human neutrophil antibacterial protein, both inhibits *Borrelia* growth in vitro and makes the organism tolerant to penicillin (Lusitani *et al.*, 2003; Montgomery *et al.*, 2006). Stationary phase *B. burgdorferi* cells in culture can also become phenotypically tolerant to antimicrobials used in treating Lyme borreliosis such as ceftriaxone, doxycycline, and amoxicillin (Feng *et al.*, 2014; Caskey and Embers, 2015; Sharma *et al.*, 2015; Feng *et al.*, 2015b). This tolerance appears to be a function of both cell concentration and growth phase, since mathematical and experimental analyses show these antimicrobial-tolerant bacteria to represent slow-growing variants whose prevalence is increased in the stationary phase (Feng *et al.*, 2014; Caskey and Embers, 2015; Sharma *et al.*, 2015; Feng *et al.*, 2015a). RNAseq analysis also suggests that borrelia, like other bacteria, probably have multiple and redundant mechanisms to draw on for the development of such tolerance to antimicrobials (Lewis, 2010; Mok *et al.*, 2015; Feng *et al.*, 2015a). Intriguingly, *B. burgdorferi* tolerant to doxycycline and amoxicillin appear to have a pattern of gene modulation similar to that seen with the stringent response (Feng *et al.*, 2015a).

The term “bacterial persistence” is used to describe the ability of pathogenic bacteria (“persisters”) to survive in infected host tissues despite the presence of effective levels of antimicrobials and antibacterial cellular and humoral immunity (Lewis, 2010; Nguyen *et al.*, 2011; Balaban *et al.*, 2013; Amato *et al.*, 2014; Conlon *et al.*, 2015; Michiels *et al.*, 2016). Though its applicability to *B. burgdorferi* has been controversial (Wormser and Schwartz, 2009; Wormser *et al.*, 2012; Iyer *et al.*, 2013), persistence is a widely-accepted phenomenon in microbiology which in some instances can have therapeutic implications (Dahl *et al.*, 2003;

Lewis, 2010; Amato *et al.*, 2014; Zhang, 2014; Putrins *et al.*, 2015; Brauner *et al.*, 2016; Chuang *et al.*, 2016; Corrigan *et al.*, 2016). Persisters, while non-dividing, appear to be metabolically active (Michiels *et al.*, 2016). They are thus similar to viable but non-culturable (VBNC) organisms but are present at a lower concentration than VBNC organisms, and potentially capable of being rescued by media without antimicrobials and generating colonies (Amato *et al.*, 2013; Amato and Brynildsen, 2014; Amato *et al.*, 2014; Ayrapetyan *et al.*, 2015; Orman *et al.*, 2016; Michiels *et al.*, 2016). Persisters also seem to be different from what have been termed dormant bacteria with a decreased rate of metabolism (Kim and Wood, 2017). Clearly, much work needs to be done to clarify these phenotypic differences. It should be noted that although persistence is phenotypic, the presence of persisters can also facilitate emergence of genetically antimicrobial-resistant bacteria, e.g., by mutation (Levin-Reisman *et al*., 2017).

It is also important to note that bacterial persistence with tolerance to antimicrobials may be generated by multiple and redundant mechanisms involving both regulatory and non-regulatory genes (Lewis, 2010; Zhang, 2014 ; Amato and Brynildsen, 2015; Mok *et al.*, 2015; Feng *et al.*, 2015a; Brauner *et al.*, 2016; Kaldalu *et al.*, 2016; Michiels *et al.*, 2016). In *E. coli* and in many other bacteria (e.g., *Salmonella*, *Mycobacterium tuberculosis*), toxin-antitoxin (TA) systems have been widely characterized as responsible for stringent response-mediated extracellular and intracellular persistence (Korch *et al.*, 2003; Germain *et al.*, 2013, 2015; Maisonneuve *et al.*, 2013; Maisonneuve and Gerdes, 2014; Gerdes and Maisonneuve, 2015; Harms *et al.*, 2016). Because *B. burgdorferi* does not appear to have conventional Type I and II TA systems (most probably as a result of gene loss associated with its small genome) (Fraser *et al.*, 1997; Makarova *et al.*, 2009; Harms *et al.*, 2016), unbalanced synthesis and hydrolysis of (p)ppGpp might act in their place to promote a persister phenotype (Amato and Brynildsen, 2015).

Increased frequency of persisters in *B. burgdorferi* might result from increases in the levels of (p)ppGpp generated by spontaneous variations in the synthetic/hydrolytic activity of rel_{Bbu} followed by slow growth and tolerance to antimicrobials (Terekhova *et al.*, 2002; Bugrysheva *et*

al., 2005; Kotte *et al.*, 2014; Bugrysheva *et al.*, 2015; Amato and Brynildsen, 2015; Drecktrah *et al.*, 2015). These borrelia would be expected to have metabolic characteristics of classical bacterial persisters in utilizing glycerol and being tolerant to antimicrobial peptides, variations in osmolarity, and reactive oxygen and nitrogen species (Bugrysheva *et al.*, 2005; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). That such a mechanism might be at work is suggested by the observed increased frequency of persisters in stationary phase cultures of *B. burgdorferi*, since the observed levels of (p)ppGpp in *B. burgdorferi* are higher in this growth phase; possibilities for spontaneous variations in (p)ppGpp levels may also arise under these conditions (Fig. 1) (Bugrysheva *et al.*, 2005; Bugrysheva *et al.*, 2015; Caskey and Embers, 2015; Drecktrah *et al.*, 2015; Feng *et al.*, 2015a; Sharma *et al.*, 2015). Alternatively, (p)ppGpp and DksA might play a role in *Borrelia* comparable to the one they play in *E. coli* where they are involved in mediating the increasing numbers of persisters generated during the diauxic shift from glycerol to trehalose utilization (Amato and Brynildsen, 2015; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015).

The ability to shift between different carbon sources (diauxie), changes in intermediary metabolism, metabolic challenges, and exposure to human sera all appear to play an important role in the evolution of persisters (Amato et al., 2013; Amato et al., 2014; Amato and Brynildsen, 2014; Amato and Brynildsen, 2015; Mok et al., 2015; Putrins et al., 2015; Ayrapetyan et al., 2015). For example, independent modulation of glycerol and trehalose metabolism in *E. coli* is related to the formation of persisters (Spoering *et al.*, 2006; Kuczynska-Wisnok *et al.*, 2015). Such metabolic changes in carbon utilization would be expected to occur in *B. burgdorferi* as it transits the enzootic cycle and would therefore be expected to stimulate both the stringent response and the formation of persisters (Fig. 2) (Tilly *et al.*, 2001; von Lackum and Stevenson, 2005; Rhodes *et al.*, 2009; He *et al.*, 2011; Pappas *et al.*, 2011; Bugrysheva *et al.*, 2015; Corona and Schwartz, 2015; Drecktrah *et al.*, 2015). The role these metabolic alterations may play in the formation of persisters in *B. burgdorferi* therefore deserves detailed examination

(Amato and Bryldnilsen, 2015; Corona and Schwartz, 2015; Troy *et al.*, 2016). In addition, *B. burgdorferi* CgtA is a GTPase of the Obg family involved in (p)ppGpp degradation. In other bacteria it is involved in persistence and might be involved in persistence in *B. burgdorferi* because its repression by the stringent response might increase levels of (p)ppGpp (Drecktrah *et al*., 2015; Verstraeten *et al*., 2015; Gaca *et al*., 2015a; Steinchen and Bange, 2016).

Nutritional fluctuations in vertebrate tissues during development of chronic infections might also trigger the stringent response (Fig. 3) and create a bi-stable heterogeneous population of transcriptionally competent borrelia growing at different rates, with slow growing bacteria becoming tolerant to antimicrobials and innate and adaptive immunity (Kotte *et al.*, 2014; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). In infected vertebrate hosts, such refugia could be found in collagenous and other avascular tissues where borrelia are not multiplying. These tissues would include the aortic root, tendons and entheses associated with joints, and synovial and spinal fluids (Fig. 3) (Barthold *et al.*, 2010b; Barbour, 2012; Bockenstedt *et al.*, 2012; Embers and Barthold, 2012). In these tissues, host antibodies might increase the nutritional stress of borrelia, for example, by blunting uptake of oligopeptides and other essential substrates by Opp and other unknown transporters whose genes are induced by the borrelial stringent response (Wang *et al.*, 2002; Medrano *et al.*, 2007; Barthold *et al.*, 2010a; Barthold *et al.*, 2010b; Raju *et al.*, 2011; Hodzic *et al.*, 2014; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). The stringent response, by its ability to generate persisters, may thus be crucial for progression of *B. burgdorferi* through its enzootic cycle.

Conclusions and outstanding questions

The ability of *B. burgdorferi* to utilize the stringent response to mediate metabolic shifts during cycling between its tick vector and its vertebrate reservoir likely involves coordination between global regulators such as Rel, RpoS, BosR, c-di-GMP, BadR, CsrA, and DksA (Tilly *et al.*, 2001; Miller *et al.*, 2013; Novak *et al.*, 2014; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015; Corona

and Schwartz, 2015; Ouyang and Zhou, 2015; Caimano *et al.*, 2016). The role played by c-di-GMP in the Hk1-Rrp1 pathway in motility and in glycerol and chitobiose metabolism suggests that it has perhaps parallel and coordinated functions to that of (p)ppGpp, albeit most probably in response to different stimuli (Rhodes *et al*., 2009; Sultan *et al.*, 2010; He *et al.*, 2011; Pappas *et al.*, 2011; Sze *et al.*, 2013; Corona and Schwartz, 2015; Caimano *et al.*, 2016). In *M. smegmatis*, for example, increased levels of (p)ppGpp and c-di-GMP act coordinately to decrease motility, facilitate aggregation and biofilm formation, and increase tolerance to antimicrobials (Gupta *et al.*, 2016). Similarly, since null mutants of rel_{Bbu} and RpoS increase the frequency of round form morphotypes in vitro and in vivo (Dunham-Ems *et al.*, 2012; Drecktrah *et al.*, 2015), they might also act coordinately at some stage to generate various morphotypes during *B. burgdorferi* migration in nymphal ticks (Dunham-Ems *et al.*, 2012; Harms *et al.*, 2016).

The global regulator DksA is an important factor in persistence and virulence in a number of pathogens (Azriel *et al.*, 2015; Amato and Brynildsen, 2015; Holley *et al.*, 2015), and while the stringent response in *B. burdorgferi* modulates production of DksA, it is not known whether the regulation achieved by (p)ppGpp in borrelia is due to interactions with DksA and RNA polymerase, or whether, as in other bacteria, an independent alternative DksA regulon exists (Bugrysheva *et al.*, 2015; Holley *et al.*, 2015). Similarly, identification of sequences functionally homologous to discriminators in the promoters of *B. burgdorferi* need further study (Potrykus and Cashel, 2008). The stringent response, like other global regulators of *B. burgdorferi*, can regulate expression of a succession of genes between the different stages of infection in ticks and mammals, with many common genes expressed across different stages (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015; Iyer *et al.*, 2015). This ability creates a continuum of gene regulation in response to nutritional and other challenges, and insures the successful perseverance of the bacteria in its very demanding and complex enzootic cycle. It is yet another example of the role the stringent response has in maintenance of the enzootic cycles of vector-transmitted pathogens (Charity *et al.*, 2009; Sun *et al*., 2009).

Both CsrA and the stringent response can modulate borrelial motility (Sze *et al.*, 2011; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015). In *E. coli*, CsrA is also involved in glucose utilization through the phosphotransferase system, a system also present in *B. burgdorferi* (Corona and Schwartz, 2015; Leng *et al.*, 2016). Because the networks of these two global regulators are heavily interlinked in other bacteria (where they also regulate virulence) (Edwards *et al.*, 2011; Vinella *et al.*, 2012; Romeo *et al.*, 2013; Vakulskas *et al.*, 2015), future investigations of the interactions between CsrA and the borrelial stringent response would be of great interest. The relevance of BadR, the growth phase regulator that upregulates expression of Rel and downregulates RpoS expression in *B. burgdorferi* should also be explored given the centrality of the stringent response and its potential interconnections with the two well characterized *B. burgdorferi* TCS axes (Miller *et al.*, 2013; Ouyang and Zhou, 2015; Iyer and Schwartz, 2016). As BadR and (p)ppGpp both repress expression of genes involved in chitobiose utilization, it would clearly be relevant to ascertain whether they do so independently or if they constitute an epistatic regulatory cascade (Miller *et al.*, 2013; Bugrysheva *et al.*, 2015; Ouyang and Zhou, 2015). In *B. burgdorferi* as in other bacteria, (p)ppGpp might be involved in global regulation by directly binding to proteins and modifying their function, and pppGpp and ppGpp may have different and independent regulatory roles (Rymer *et al.*, 2012; Mechold *et al.*, 2013; Liu *et al.*, 2015). Global regulation in *B. burgdorferi* by (p)ppGpp could also be mediated by its ability to modulate RNA transcription initiation of promoters depending on nucleotide concentrations (Krasny and Gourse, 2004; Hauryliuk *et al.*, 2015) as a function of its ability to modify the GTP/ATP ratio by consumption of GTP during its synthesis and its inhibition of GTPases such as CgtA as is the case with other bacteria (Kriel *et al.*, 2014; Hauryliuk *et al.*, 2015; Drecktrah *et al.*, 2015; Verstraeten *et al.*, 2015; Gaca *et al.*, 2015a).

If the stringent response is utilized in the enzootic life cycle of borrelia as we and others have suggested (Godfrey *et al.*, 2002; Bugrysheva *et al.*, 2005; Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015; Caimano *et al.*, 2016), its mobilization in different developmental stages

and tissue types in its arthropod hosts and primary mammalian reservoirs can be expected to have been under strong evolutionary pressure with respect to tissue tropism and timing (Radolf *et al.*, 2012; Caimano *et al.*, 2016; Steere *et al.*, 2016). In dead-end mammalian hosts such as humans which are not critical to the spirochete's propagation, the spirochete's ability to evade immune responses and antimicrobial treatment and take up residence in refugia would, by implication, be a side-effect of selection on other traits (a "spandrel" in the terminology of Gould and Lewontin (1979)). Its incidental origin would make it no less of a potential clinical problem if it were found to be involved in manifestations of late Lyme disease such as arthritis and post-treatment Lyme disease syndrome (Steere *et al.*, 1994; Chandra *et al.*, 2011; Arvikar and Steere, 2015; Steere *et al.*, 2016).

Readily detectable borrelia tolerant to antimicrobials in suspension cultures and biofilms in vitro (Terekhova *et al.*, 2002; Caskey and Embers, 2015; Sharma *et al.*, 2015; Feng *et al.*, 2015a), detection of *B. burgdorferi* gene expression in tick stages (Iyer *et al.*, 2015), development of models of infection for vertebrate reservoirs, including potential refugia for persister spirochetes (Akins *et al.*, 1998; Zambrano *et al.*, 2004; Cabello *et al.*, 2007; Iyer *et al.*, 2015), and the appearance of apparently quiescent round forms under several kinds of environmental stresses (Brorson and Brorson, 1997; Alban *et al.*, 2000; Dunham-Ems *et al.*, 2012; Drecktrah *et al.*, 2015; Meriläinen et al., 2015; Feng *et al.*, 2016) suggest that mechanisms of these potentially clinically relevant phenomena may eventually be discovered (Bockenstedt *et al.*, 2012; Marques *et al.*, 2014; Steere *et al.*, 2016). For example, the genetic and metabolic make-up of non-culturable *B. burgdorferi* tolerant to antimicrobials rescued from animals by xenodiagnoses could provide insight into the relationship between tolerance and resistance to these compounds (Bockenstedt *et al.*, 2002; Embers *et al.*, 2012; Marques *et al.*, 2014). The use of recently developed genomic tools (Lybecker *et al.*, 2014; Arnold *et al.*, 2016; Wright *et al.*, 2016; Adams *et al.*, 2017), should permit ready isolation of multiple mutants of *B. burgdorferi* global regulators including rel_{Bbu} that will allow assessment of their hierarchic order

in gene regulation by epistasis and their role in borrelial permanence in the enzootic cycle and borrelial persistence in vitro and in vivo (Avery and Wasserman, 1992; Phillips, 2008). Dissection of these networks of interactions among global regulators of *B. burgdorferi* is essential for understanding the ability of this organism to persist in its hosts, its vectors and its enzootic cycle (Corona and Schwartz, 2015; Iyer *et al.*, 2015), and will be critical to informing the design of relevant vaccines and antimicrobials (Wexselblatt *et al.*, 2013; Syal *et al.*, 2017).

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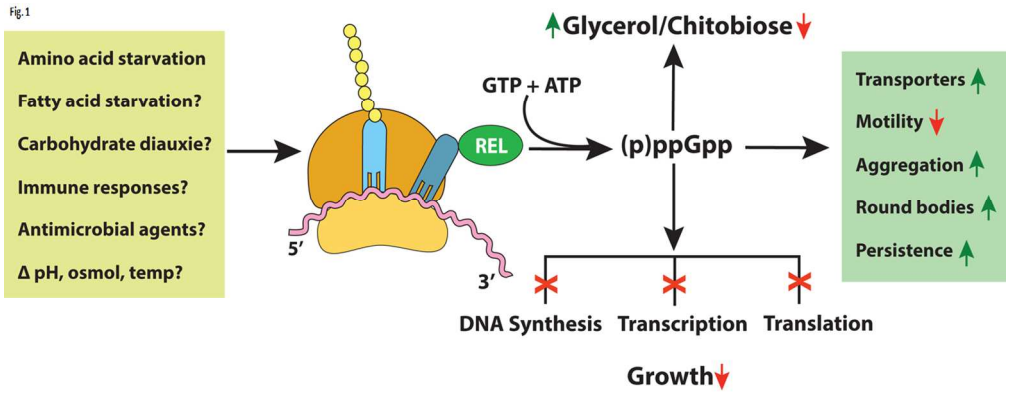
Figure Legends

Fig. 1. The stringent response in *B. burgdorferi*. Potential triggers and consequences of the stringent response that could facilitate borrelial adaptation to microenvironmental challenges in the mammal and in the tick vector. Factors shown in the box on the left induce the activity of Rel_{Bbu}, which converts ATP and GTP into the alarmone (p)ppGpp. The effects on the bacteria include altered rates of growth and motility, regulation of transport of metabolites, shifts in sugars (diauxie), amino acids and lipid utilization, and different morphotypes. Many of these changes enable persistence in ticks and mammals and the progression and maintenance of the enzootic cycle.

Fig. 2. *Hypothetical role of the B. burgdorferi* stringent response in the *I. scapularis* reservoir and vector. Modulation of bacterial growth mediated by the stringent response is crucial for its adaptation to nutritional challenges. Internal organs of the tick are shown in the central panel (dark red: gut; light green: salivary gland). In *feeding larvae* during acquisition of spirochetes (light blue circle), rapid growth in the gut (cell layer and magnified inset) results from attenuation of the stringent response within bacterial cells. In *flat nymphs* (bright green circle)., high levels of (p)ppGpp within the bacteria together with other regulatory molecules stimulate utilization of glycerol and decreased spirochete motility, and appearance of persister cells in the gut lumen via the stringent response. This state continues at the early transmission stage (yellow oval) (1) in the *feeding nymph* where the stringent response might be involved in

spirochete bleb formation, generation of reversible epithelium-associated biofilm-like spirochete networks, round forms and persisters in the gut (cell layers and insets). Later (2), attenuation of the stringent response associated with irruption of blood into the tick gut activates spirochete motility at the basement membrane and migration to the haemocele and the salivary glands (3). Degradation of the peritrophic membrane, produced by enzymes from gut cells and the blood, generates chitobiose, the metabolism of which is derepressed by attenuation of the stringent response and low levels of (p)ppGpp. The shift from glycerol utilization to chitobiose utilization may also be a stimulus for the generation of persister cells. ▲ = (p)ppGpp in borrelia cells

Fig. 3. Hypothetical role of the *B. burgdorferi* stringent response in the *P. leucopus* reservoir. Transmission of *B. burgdorferi* into the dermis of mice, usually by *I. scapularis* nymphs, generates an acute infection. In the dermal environment with high levels of glucose and other nutrients, borrelia begin to display an attenuated stringent response with low levels of (p)ppGpp, which in turn enables rapid borrelial division, multiplication and motility. These rapidly dividing and motile bacteria subsequently invade adjacent areas of the dermis, bloodstream and various organs, reaching relatively high concentrations (outer pink circle, attenuated stringent response). After several weeks, as a result of the immune response, borrelia disappear from blood, and few remain in connective tissues. These low numbers of borrelia display an activated stringent response and high levels of (p)ppGpp in response to nutritional depletion and other stimuli, including the potential ability of the immune response to block the uptake of nutrients by borrelial transporters. These spirochetes will probably be slow moving, transcription competent and persisters (inner orange circle, high concentration of (p)ppGpp). ▲ = (p)ppGpp in borrelia cells



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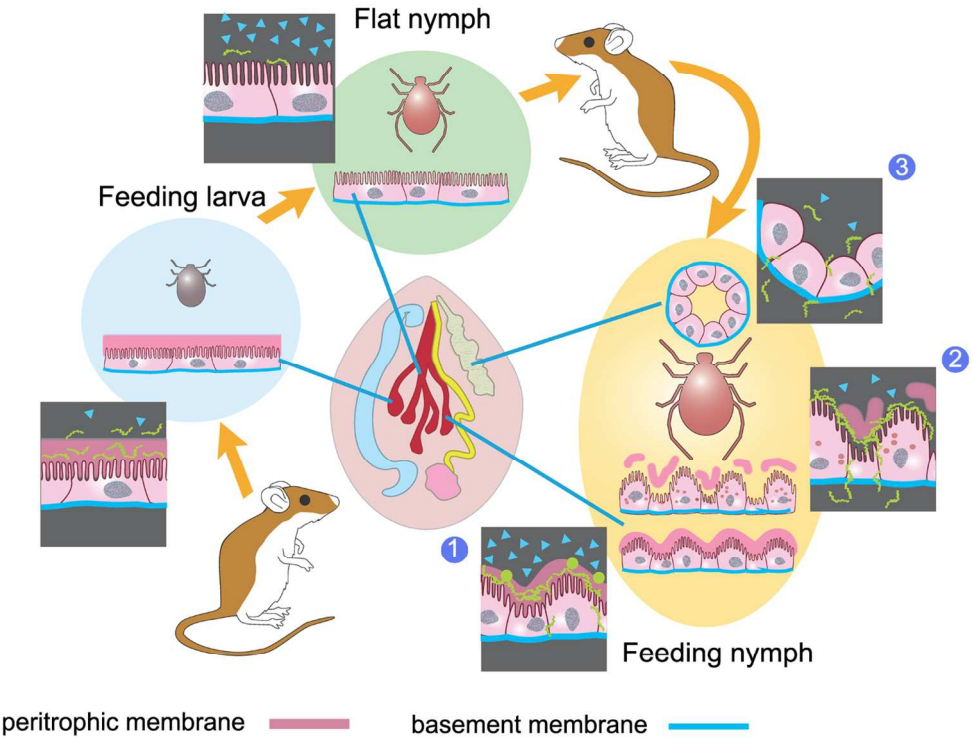

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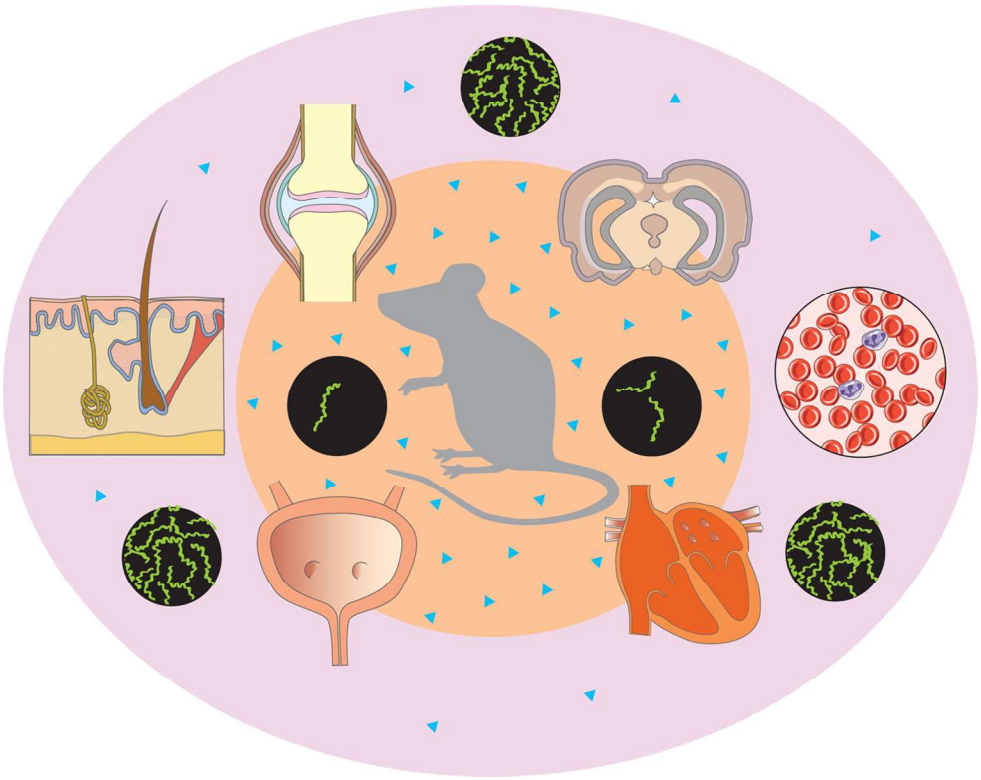

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