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Gene Regulation and Transcriptomics

D. Scott Samuels[1,*], **Meghan C. Lybecker**[2], **X. Frank Yang**[3], **Zhiming Ouyang**[4], **Travis J. Bourret**[5], **William K. Boyle**[5], **Brian Stevenson**[6], **Dan Drecktrah**[1], **Melissa J. Caimano**[7]

[1]Division of Biological Sciences, University of Montana, Missoula, MT 59812, USA

[2]Department of Biology, University of Colorado, Colorado Springs, CO 80918, USA

[3]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN4 6202, USA

[4]Department of Molecular Medicine, University of South Florida, Tampa, FL 33612, USA

[5]Department of Medical Microbiology and Immunology, Creighton University, Omaha, NE, 68105 USA

[6]Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky School of Medicine, Lexington, KY 40536, USA

[7]Departments of Medicine, Pediatrics, and Molecular Biology and Biophysics, UConn Health, Farmington, CT, USA

Abstract

Borrelia (*Borreliella*) *burgdorferi*, along with closely related species, is the etiologic agent of Lyme disease. The spirochete subsists in an enzootic cycle that encompasses acquisition from a vertebrate host to a tick vector and transmission from a tick vector to a vertebrate host. To adapt to its environment and persist in each phase of its enzootic cycle, *B. burgdorferi* wields three systems to regulate the expression of genes: the RpoN-RpoS alternative sigma (σ) factor cascade, the Hk1/Rrp1 two-component system and its product c-di-GMP, and the stringent response mediated by Rel_{Bbu} and DksA. These regulatory systems respond to enzootic phase-specific signals and are controlled or fine-tuned by transcription factors, including BosR and BadR, as well as small RNAs, including $DsrA_{Bb}$ and Bb6S RNA. In addition, several other DNA-binding and RNA-binding proteins have been identified, although their functions have not all been defined. Global changes in gene expression revealed by high-throughput transcriptomic studies have elucidated various regulons, albeit technical obstacles have mostly limited this experimental approach to cultivated spirochetes. Regardless, we know that the spirochete, which carries a relatively small genome, regulates the expression of a considerable number of genes required for the transitions between the tick vector and the vertebrate host as well as the adaptation to each.

[*]Corresponding author: samuels@mso.umt.edu.

Introduction

The Lyme disease spirochete *Borrelia* (*Borreliella*) *burgdorferi*, and closely related species *B. afzelii, B. garinii*, *B. bissettii*, and *B. mayonii*, are maintained in an enzootic cycle involving a tick vector and a vertebrate host (Figure 1) (Lane et al., 1991; Radolf et al., 2012; Caimano et al., 2016). The spirochetes regulate the expression of their genes in a phase-specific fashion as they traverse this cycle in nature, moving back and forth between the tick and a vertebrate. *Ixodes* larvae feed on an infected host and acquire the spirochetes, which take up residence in the midgut. Following the molt, nymphs feed on a vertebrate and the spirochetes exit the midgut, migrate through the hemocoel, reach the salivary glands, and transmit to the naïve host (Ribeiro et al., 1987; Dunham-Ems et al., 2009). The spirochete survives in these two disparate habitats, and moves between them at the proper time, by sensing biological cues that alter the expression of the requisite collection of genes. Different 'modulons' are expressed as *B. burgdorferi* cycles from tick to vertebrate and vertebrate to tick, and adapts to these different environments (Iyer et al., 2015). The physiological signposts of the enzootic cycle, molecular cues prompting the spirochete to regulate its gene expression, have not been fully delineated nor have the mechanisms of signal integration and transduction to the regulatory machinery been elucidated. Schwan et al. (1995) uncovered temperature as an environmental signal; these seminal findings suggested that the increased temperature of the warm blood meal regulates the levels of outer membrane lipoproteins during tick feeding. *B. burgdorferi* is exposed to a variety of other factors that vary during the enzootic cycle and have been shown to affect gene expression, including pH (Carroll et al., 1999; Yang et al., 2000), dissolved oxygen (Seshu et al., 2004a) and carbon dioxide (Hyde et al., 2007), transition metals (Troxell et al., 2013), nutrients (Bugrysheva et al., 2015; Drecktrah et al., 2015), osmolarity (Bontemps-Gallo et al., 2016), weak organic acids (Dulebohn et al., 2017), and short-chain fatty acids (Lin et al., 2018). Nevertheless, uncertainty remains regarding the relative roles of each and their interactions.

This review covers our current understanding of gene regulation during the enzootic cycle; since the original chapter was written (Skare et al., 2010), a number of recommended reviews have been published (Samuels, 2011; Radolf et al., 2012; Kung et al., 2013; Troxell and Yang, 2013; Groshong and Blevins, 2014; Caimano et al., 2016; Iyer and Schwartz, 2016; Samuels and Samuels, 2016; Ye et al., 2016; Lybecker and Samuels, 2017; Stevenson and Seshu, 2018). We begin the review by introducing the enzyme catalyzing transcription, and invoke the maxim *spirochetes do it differently*, as the *B. burgdorferi* RNA polymerase is unusually, albeit not uniquely, dependent on manganese (Boyle et al., 2020). We know of three main gene regulatory systems that are in play throughout the enzootic cycle: the RpoN-RpoS alternative sigma (σ) factor cascade; the Hk1/Rrp1 two-component system and its second messenger c-di-GMP; and stringent response regulators, Rel_{Bbu} and DksA, and the alarmone (p)ppGpp. Orthologs of several transcriptional regulators that have been thoroughly characterized in model microorganisms are involved in modulating these gene regulatory networks, although in ways that, more often than not, are utterly idiosyncratic for this deeply branching group of bacteria (Samuels and Radolf, 2009). We discuss nucleic-acid binding proteins and mention a relatively recently described signaling molecule. We

then take up post-transcriptional mechanisms, focusing on small regulatory RNAs, before turning our attention to what has been learned via high-throughput transcriptomic approaches.

RNA polymerase

The transcription of genes in *B. burgdorferi* is carried out by the RNA polymerase holoenzyme consisting of subunits encoded by *rpoA* (α subunit), *rpoB* (β subunit), *rpoC* (β′ subunit), and *rpoZ* (ω subunit), which constitute the core RNA polymerase, along with one of three σ factors encoded by *rpoD* (housekeeping sigma factor σ^{70} or σ^{D}), *rpoN* (σ^{N} or σ^{54}), or *rpoS* (σ^{S} or σ^{38}). It has the same $\alpha_2\beta\beta'\omega$ plus σ architecture as other bacterial RNA polymerases. The σ subunit is required for recognition of gene promoters and separation of the two strands of the double helix (Marchetti et al., 2017). The σ subunit associates with the $\alpha_2\beta\beta'\omega$ core to initiate transcription and then elongation proceeds, often after the σ subunit dissociates from the holoenzyme. The largest subunit, β′, houses the active site that catalyzes RNA synthesis. The ω subunit promotes RNA polymerase activity in other bacteria (Mathew and Chatterji, 2006; Kurkela et al., 2020); however, its role in the *B. burgdorferi* RNA polymerase has yet to be characterized.

Unlike prototypical bacterial RNA polymerases that require magnesium for activity (Sosunov et al., 2003), the enzymatic activity of the *B. burgdorferi* RNA polymerase is manganese-dependent (Boyle et al., 2020). Manganese-dependent RNA polymerases have been found in other bacteria, including *Clostridium acetobutylicum*, *Bacillus subtilis*, and *Lactobacillus curvatus* (Rutberg and Armentrout, 1972; Stetter and Zillig, 1974; Pich and Bahl, 1991). The molecular basis of the manganese requirement for the *B. burgdorferi* RNA polymerase is unclear, as the conserved catalytic site amino acids within the β′ subunit are identical to those found in both magnesium- and manganese-utilizing bacteria. The spirochete regulates the cellular concentrations of manganese through the BmtA transporter (Ouyang et al., 2009a; Wagh et al., 2015), and alterations in extracellular levels of manganese affect the expression of a variety of virulence genes (Troxell and Yang, 2013; Troxell et al., 2013). Changes in *B. burgdorferi* gene expression in response to lower concentrations of manganese may be the consequence of reducing the bioavailability of manganese required for RNA polymerase activity, resulting in transcriptional stalling as described in other bacteria (Rutberg and Armentrout, 1972; Stetter and Zillig, 1974; Borbely and Schneider, 1988; Pich and Bahl, 1991; Sosunov et al., 2003; Poranen et al., 2008; Agapov et al., 2017).

The enzymatic activity of the *B. burgdorferi* RNA polymerase also responds to temperature and pH (Boyle et al., 2020), which is notable because the environmental temperature and pH vary as *B. burgdorferi* traverses its enzootic cycle (Carroll et al., 1999; Schwan and Piesman, 2000; Yang et al., 2000). *In vitro* transcription assays initiated from RpoD (σ^{70})-dependent promoters using the *B. burgdorferi* RNA polymerase core and recombinant RpoD σ factor showed RNA polymerase activity is affected by temperature with maximal activity at 37°C. Similarly, RNA polymerase activity is pH-dependent within the physiological range experienced by *B. burgdorferi* with the lowest activity observed at pH 6.8 and the highest activity at pH 7.5 (Boyle et al., 2020). These *in vitro* experiments suggest regulation of *B.*

burgdorferi gene expression in response to vector and host environments might be a consequence, at least partially, of biochemical changes in RNA polymerase activity.

During growth of *B. burgdorferi in vitro*, RpoD (σ^{70}) is the primary σ factor responsible for the initiation of transcription, as the alternative σ factors, RpoN (σ^{N}) and RpoS (σ^{S}), are either inactive or present at low levels in the cell during logarithmic growth of *B. burgdorferi* (Hübner et al., 2001; Burtnick et al., 2007; Bontemps-Gallo et al., 2016). Sites of RpoD-dependent transcriptional initiation were mapped by *in vitro* transcription reactions initiated by recombinant RpoD (Adams et al., 2017; Boyle et al., 2020). The ability of *B. burgdorferi* to complete its enzootic cycle is dependent on RNA polymerase utilization of RpoN and RpoS (see the next section on the RpoN-RpoS alternative σ factor cascade). The expression of *rpoN* and *rpoS*, along with corresponding changes in RpoN- and RpoS-dependent gene expression, occur in response to environmental signals. While much of the work on gene regulation has focused on the impact of transcription factors, alternative σ factors and small RNAs in *B. burgdorferi*, the intracellular environment, including pH, temperature, and manganese availability, may directly alter RNA polymerase function along with the interactions of gene regulatory systems with RNA polymerase.

RpoN-RpoS alternative σ factor cascade

The RpoN-RpoS alternative σ factor cascade has undoubtedly been the most studied gene regulatory pathway in *B. burgdorferi* (Samuels, 2011; Radolf et al., 2012; Samuels and Samuels, 2016; Ye et al., 2016; Stevenson and Seshu, 2018) (Figure 2). It plays a vital role in the enzootic cycle of *B. burgdorferi* by activating transcription of genes that are essential for transmission and vertebrate host infection, while repressing genes that are required for spirochete survival in the tick vector (Hübner et al., 2001; Caimano et al., 2004; Caimano et al., 2005; Fisher et al., 2005; Caimano et al., 2007; Ouyang et al., 2008; Dunham-Ems et al., 2012; Grove et al., 2017; Arnold et al., 2018). The housekeeping σ factor (e.g., σ^{70} in *E. coli*) is responsible for transcription of most genes (see the section on RNA polymerase above). On the other hand, alternative σ factors recognize different promoters and allow for transcription of a subpopulation of genes in response to environmental or developmental signals (Kazmierczak et al., 2005; Österberg et al., 2011). RpoN (σ^{N} or σ^{54}; *bb0450*) and RpoS (σ^{S} or σ^{38}; *bb0771*) are the only two alternative σ factors encoded in the *B. burgdorferi* genome (Fraser et al., 1997). RpoN directly controls *rpoS* expression, which constitutes the RpoN-RpoS σ factor cascade (or RpoN-RpoS pathway).

RpoN is a unique σ factor that does not belong to the σ^{70} (RpoD) family (Reitzer and Schneider, 2001; Wigneshweraraj et al., 2008). RpoN-dependent gene expression possesses several distinctive features. First, RpoN-holoenzyme ($E\sigma^{54}$) exclusively requires a transcriptional activator, called bacterial enhancer binding protein (bEBP) (Bush and Dixon, 2012). *B. burgdorferi* has a single bEBP, Rrp2 (Yang et al., 2003). Second, RpoN recognizes a highly conserved −24/−12 promoter sequence that differs dramatically from the −35/−10 promoter sequence recognized by σ^{70} family σ factors (Studholme and Buck, 2000). Third, RpoN-dependent gene activation requires ATP binding (although not hydrolysis). As such, RpoN controls the expression of only a few genes in most bacteria (Reitzer and Schneider, 2001; Kazmierczak et al., 2005). In *B. burgdorferi*, the only gene with an RpoN-type

promoter identified is *rpoS*, which has a perfect −24/−12 promoter sequence (Hübner et al., 2001; Smith et al., 2007).

RpoS from *B. burgdorferi* is evolutionarily distinct from the prototypical RpoS of γ-proteobacteria (Chiang and Schellhorn, 2010). However, it retains key features of its namesake, including the presence of a region σ1.2 and promoter recognition via an extended −10 (Eggers et al., 2006; Caimano et al., 2019). In most bacteria, RpoS serves to globally regulate gene expression to adapt to environmental stresses, such as entering stationary growth phase (reviewed in Hengge-Aronis, 2002a). RpoS accumulates following low or high temperature, low pH, high osmolarity, oxidative stress, UV exposure, and carbon starvation, resulting in regulation of genes to address the stressful conditions and prevent cellular damage. Yet, in *B. burgdorferi*, RpoS controls expression of enzootic phase-specific genes rather than the general stress response, and is required for infection of the vertebrate host (Caimano et al., 2004; Caimano et al., 2019) as well as transmission during tick feeding (Fisher et al., 2005; Dunham-Ems et al., 2012). RpoS in *B. burgdorferi* induces vertebrate host-specific genes and represses tick vector-specific genes (Hübner et al., 2001; Caimano et al., 2004; Caimano et al., 2005; Fisher et al., 2005; Caimano et al., 2007; Ouyang et al., 2008; Grove et al., 2017; Arnold et al., 2018) and, thus, RpoS has been designated the "gatekeeper" of the enzootic cycle (Figure 1) (Caimano et al., 2007; Mulay et al., 2009; Caimano et al., 2019).

RpoN and RpoS constitute a σ factor cascade

The discovery of the RpoN-RpoS σ factor cascade by Hübner et al. (2001) was made possible by inactivation of the *rpoS* and *rpoN* genes in an infectious *B. burgdorferi* strain. Both *rpoS* and *rpoN* mutants showed a striking phenotype: loss of production of OspC and DbpB/A, two of the abundant surface antigens. Abrogation of OspC and DbpB/A in the *rpoN* mutant was due to abolished production of RpoS, and constitutive expression of *rpoS* in the *rpoN* mutant rescued OspC and DbpB/A production. RpoN controlling *rpoS* activation appeared to be direct, as the *rpoS* gene has a perfect consensus −24/−12 RpoN-dependent promoter sequence (Hübner et al., 2001; Burtnick et al., 2007; Lybecker and Samuels, 2007; Smith et al., 2007). Further biochemical and genetic analyses indicated that the −24/−12 RpoN-dependent promoter is the major promoter responsible for cell density-dependent *rpoS* expression (Burtnick et al., 2007; Lybecker and Samuels, 2007; Smith et al., 2007).

To date, *rpoS* is the only gene identified whose transcription has been demonstrated to be directly controlled by RpoN in *B. burgdorferi*. Virtually all differentially expressed genes in the *rpoS* and *rpoN* mutants are overlapping (Ouyang et al., 2008), supporting the hypothesis that RpoN modulates *B. burgdorferi* genes mainly through RpoS; however, other data suggest that RpoN and RpoS can regulate different groups of genes under certain conditions (Fisher et al., 2005; Arnold et al., 2018). In addition, a second, RpoD (σ^{70})-dependent, promoter (−35/−10) has been reported upstream that transcribes a longer *rpoS* mRNA (Lybecker and Samuels, 2007). Translation of this long *rpoS* mRNA is regulated by the small RNA (sRNA) $DsrA_{Bb}$ (Lybecker and Samuels, 2007) and may explain the controversy regarding the RpoN-dependent and RpoS-dependent transcriptomes (see section below on Small regulatory RNAs and RNA chaperones). There is evidence that RpoS is post-

transcriptionally regulated by BBD18 encoded on the linear plasmid lp17, although the mechanistic details are unknown (Dulebohn et al., 2014) (Figure 2).

Genes controlled by RpoN-RpoS σ factor cascade

The first genes identified as being under the control of RpoN-RpoS σ factor cascade were *ospC* and *dbpBA* (Hübner et al., 2001); microarray and RNA-seq analyses have revealed that the RpoN-RpoS σ factor cascade influences the expression of more than 100 genes (Fisher et al., 2005; Caimano et al., 2007; Boardman et al., 2008; Ouyang et al., 2008; Arnold et al., 2018; Caimano et al., 2019) (see the section on Transcriptomics below). Many of the vertebrate phase-specific are under the control of the RpoN-RpoS σ factor cascade and include, in addition to *ospC* and *dbpBA, bbk32, oppA5, bba64, bba66*, *ospF* and *mlp* (Fischer et al., 2003; Eggers et al., 2004; Grimm et al., 2004; Clifton et al., 2006; Fischer et al., 2006; Seshu et al., 2006; Gilmore et al., 2007; Medrano et al., 2007; Gautam et al., 2008; Maruskova et al., 2008; Shi et al., 2008). Many of these genes are differentially regulated under a variety of environmental conditions including temperature, pH, blood, and host adaptation (Revel et al., 2002; Brooks et al., 2003; Ojaimi et al., 2003; Tokarz et al., 2004; Caimano et al., 2007). These genes often encode surface lipoproteins, but many are hypothetical genes with no known function. Several chromosomal genes, including genes involved in chemotaxis and metabolism are also controlled by the RpoN-RpoS σ factor cascade (Fisher et al., 2005; Caimano et al., 2007; Ouyang et al., 2008). Recently, Caimano et al. (2019) compared the RpoS regulons of two *B. burgdorferi* strains and postulated that the RpoN-RpoS σ factor cascade controls two cohorts of genes: a core group that is required for maintenance of *B. burgdorferi* in the enzootic cycle and a group of plasmid-encoded genes encoding variable surface lipoproteins important for adaptation to diverse vertebrate reservoir hosts.

RpoS controls *B. burgdorferi* gene expression both directly and indirectly. Genetic evidence indicates that the −35/−10 promoters of *ospC*, *ospF* and several other RpoS-regulated genes are recognized exclusively by RpoS; in other words, RpoS directly governs *ospC* expression by binding to the *ospC* promoter (Eggers et al., 2004; Yang et al., 2005). RpoD (σ^{70}) and RpoS (σ^{S}) are closely related and recognize similar core promoter elements (Gaal et al., 2001; Hengge-Aronis, 2002b). Promoter selectivity by the two σ factors in *E. coli* is complicated, involving minor sequence differences, architectural DNA-binding proteins, or DNA supercoiling (Kusano et al., 1996; Colland et al., 2000; Becker and Hengge-Aronis, 2001; Gaal et al., 2001; Hengge-Aronis, 2002b; Typas et al., 2007). Alternative σ factor utilization by RNA polymerase is also influenced by 6S RNA (Wassarman and Storz, 2000; Cavanagh and Wassarman, 2014; Steuten et al., 2014a; Steuten et al., 2014b; Wassarman, 2018) (see section below on Small regulatory RNAs and RNA chaperones). In *B. burgdorferi*, the sequence within the extended −10 region of the promoter contributes to RpoS recognition and a point mutation of C to G at position −13 abolished RpoS-dependent expression of *ospC* (Eggers et al., 2004; Yang et al., 2005; Caimano et al., 2019). Replacing the −10 region of the RpoD-dependent promoter of the *ospE* gene with the −10 region of the RpoS-dependent promoter of *ospF* converted *ospE* to an RpoS-dependent gene (Eggers et al., 2006). A putative RpoS-dependent consensus extended −10 region sequence of TG(G/A)(G/A)ATA(T/A)ATT has been proposed based on analyzing the promoters of ten RpoS-

dependent genes of *B. burgdorferi* (Caimano et al., 2007). Although *ospC* transcription is chiefly controlled by RpoS, low levels of *ospC* mRNA have been detected in the absence of RpoS: He et al. (2008) reported that *ospC* expression was greatly reduced, but not completely eliminated in a *rpoS ospAB* double mutant. A recent transcriptome study detected *ospC* expression in the *rpoN* and *rpoS* mutants (Arnold et al., 2018). Given that RpoD and RpoS recognize similar promoter sequences, this *ospC* mRNA is likely due to leaky transcription mediated by RpoD.

RpoS appears also to regulate *B. burgdorferi* gene expression in an indirect manner. The *bba66* genes has a 29-bp inverted repeat (IR) element upstream of the promoter that is required for transcription, suggesting that RpoS may influence *bba66* indirectly via an unidentified factor that binds to the IR motif (Clifton et al., 2006). In addition, some genes are only partially controlled by RpoS, such as *gltP* (encoding a glutamate transporter) and *cdr* (encoding NAD-dependent coenzyme A disulphide reductase). These genes likely have a promoter that is recognized by both RpoS and RpoD (Caimano et al., 2007; Eggers et al., 2011).

In addition to activating transcription, RpoS represses expression of some *B. burgdorferi* genes (Caimano et al., 2005; Caimano et al., 2007; Grove et al., 2017; Arnold et al., 2018). Several of these RpoS-repressed genes are important for tick colonization and are downregulated following transmission, including *ospAB*, *lp6.6*, *bba74*, and *bb0365* (de Silva et al., 1996; Schwan and Piesman, 2000; Pal et al., 2008; Mulay et al., 2009). Some *bdr* paralogs, which encode members of the *Borrelia* direct repeat protein family with no known function (Zückert et al., 1999), are also repressed by RpoS (Roberts et al., 2002). While the mechanism of RpoS-dependent repression of this regulon remains unclear, the T-rich track upstream of the promoter was proposed to be involved (Sohaskey et al., 1999; Brooks et al., 2003; Caimano et al., 2007). Recently, using a GFP reporter and the dialysis membrane chambers (DMC) model, Grove et al. (2017) uncovered two distinct mechanisms of RpoN-dependent gene repression: one is direct repression (*ospA*, *glp*) in which RpoS competes with RpoD at the −35/−10 sequence for promoter binding and the other is indirect repression (*bba74*) via an unknown *trans*-acting repressor whose expression is controlled by RpoS. However, other data suggest that BosR, a transcriptional activator of *rpoS*, not RpoS, directly represses *ospAB* and other lipoprotein genes (Wang et al., 2013; Shi et al., 2014) (see next section on Transcription factors BosR and BadR).

Factors regulating the RpoN-RpoS σ factor cascade

Rrp2, encoded by *bb0763*, is the sole bEBP present in *B. burgdorferi* (Yang et al., 2003). Rrp2 belongs to the well-studied NtrC protein family (Studholme and Buck, 2000). Members of this family contain three putative functional domains: an N-terminal response regulator domain, a central RpoN-activation domain, and a C-terminal helix-turn-helix (HTH) DNA-binding domain. The central domain becomes activated upon phosphorylation at a conserved Asp residue within the N-terminal domain. The activated central domain then contacts the RpoN-holoenzyme through DNA looping, hydrolyzes ATP, and promotes open promoter complex formation for transcriptional initiation. Yang et al. (2003) first demonstrated that Rrp2 is the upstream activator for the RpoN-RpoS alternative σ factor

cascade (Figure 2): a single G239C point mutation within the central activation domain of Rrp2 abolished expression of *rpoS*, *ospC*, and other RpoN-RpoS pathway-dependent genes. The G239C mutation did not abolish the ATPase activity of Rrp2, suggesting that the mutation affects interaction of Rrp2 with RpoN necessary for initiation of open complex formation (Ouyang and Zhou, 2017). Artificial induction of an inducible *rrp2* construct also resulted in *rpoS* transcription (Ouyang et al., 2014c). Interestingly, Rrp2 activates *rpoS* transcription without binding to a specific enhancer binding site (Burtnick et al., 2007; Blevins et al., 2009), despite having a predicted HTH-containing C-terminal domain and being capable of non-specific binding to DNA (Burtnick et al., 2007; Blevins et al., 2009). Surprisingly, *rpoN* is not required for *B. burgdorferi* growth and is readily inactivated, but *rrp2* is essential (Yang et al., 2003; Burtnick et al., 2007; Blevins et al., 2009; Groshong et al., 2012; Yin et al., 2016). This suggests that Rrp2 has an additional role besides serving as the activator for the RpoN-RpoS σ factor cascade. Phosphorylation, ATPase activity, and the C-terminal-mediated DNA-binding are all required for the essential function of Rrp2, but the mechanistic details remain unclear (Yin et al., 2016; Ouyang and Zhou, 2017). Further work, such as chromatin immunoprecipitation (ChIP), will be required to identify Rrp2-dependent but RpoN-independent genes to gain insight the essential nature of Rrp2.

Given that Rrp2 is a two-component response regulator, its cognate histidine kinase Hk2 (BB0763) predicted in the genome was hypothesized to receive upstream signals to phosphorylate and activate Rrp2, which, in turn, activates the RpoN-RpoS σ factor cascade (Yang et al., 2003; Burtnick et al., 2007; Liu et al., 2020). However, unlike *rrp2*, *hk2* is not essential for cell survival and, unexpectedly, disruption of *hk2* does not affect the expression of *rpoS* and *ospC*, suggesting that Hk2 is not required for Rrp2 phosphorylation. One the other hand, Hk2 can function as a phosphatase to dephosphorylate Rrp2 and a strain that overexpresses Hk2 has reduced level of phosphorylated Rrp2 and impaired growth (Liu et al., 2020). Further study indicated that acetyl phosphate, a high-energy metabolic intermediate that can donate phosphoryl or acetyl groups, activates Rrp2 via phosphorylation (Xu et al., 2010); the hypothesis was compelling as *B. burgdorferi* senses the blood meal, begins to replicate and activates the RpoN-RpoS σ factor cascade upon tick feeding, so acetyl phosphate would serve to connect metabolic status and enzootic phase-specific gene expression. However, inactivation of the AckA-Pta pathway that produces acetyl phosphate proved to be lethal to spirochete survival but did not affect the expression of *rpoS* and *ospC* (Richards et al., 2015), arguing against a role for acetyl phosphate in Rrp2 phosphorylation. Therefore, the source of phosphate and the mechanism of Rrp2 phosphorylation are enigmatic, although acetyl phosphate, Hk2 and other small phosphate donors might have overlapping and complementary functions.

Another two-component system present in the *B. burgdorferi* genome, Hk1-Rrp1, appears to engage in cross-talk with the Rrp2-controlled RpoN-RpoS σ factor cascade (Rogers et al., 2009; He et al., 2011; Sze et al., 2013; He et al., 2014; Caimano et al., 2015) (see section below on Hk1/Rrp1 two-component system and c-di-GMP). The interplay between the Hk1-Rrp1 and Hk2-Rrp2 signaling systems has only begun to be revealed. Sze et al. (2013) reported that Rrp1 affects *rpoS* via regulation of *bosR*. c-di-GMP, synthesized by Rrp1, controls *rpoS* expression through the c-di-GMP binding protein PlzA and PlzA links the two sets of two-component systems (He et al., 2014; Caimano et al., 2015; Zhang et al., 2018).

Transcription factors BosR and BadR

BosR

BosR (BB0647; *Borrelia* oxidative stress regulator) is a transcriptional regulator that belongs to the ferric uptake regulator (Fur) family (Fraser et al., 1997; Boylan et al., 2003; Katona et al., 2004; Samuels and Radolf, 2009). It shares 22.5% and 21.8% identity, and 43% and 49% similarity, respectively, with the Fur protein in *Vibrio cholerae* and the stress-responsive Fur family protein PerR in *Bacillus subtilis*. *B. burgdorferi* does not rely on iron (Posey and Gherardini, 2000; Wang et al., 2012b), so BosR seemed unlikely to regulate iron homeostasis in the spirochete. Instead, BosR is analogous to PerR and was first reported to regulate the expression of *dps*/*napA*/*bicA* as well as other genes involved in oxidative stress response in *B. burgdorferi* (Boylan et al., 2003), but BosR was eventually shown to activate *rpoS* transcription and, thus, the RpoS-dependent regulon (Hyde et al., 2009; Ouyang et al., 2009b; Hyde et al., 2010; Ouyang et al., 2011; Ouyang et al., 2014a; Katona, 2015).

Expression and regulation of *bosR*—Transcription of *bosR* is driven by two promoters: a strong RpoD-dependent promoter located directly upstream of *bosR* (Katona et al., 2004; Hyde et al., 2010; Katona, 2015; Ouyang et al., 2016) and a second RpoD-dependent promoter upstream of an operon in which *bosR* is co-transcribed with *bb0648* (encoding a putative serine/threonine kinase) and *bb0646* (encoding a putative lipase) (Ouyang et al., 2009b; Ouyang et al., 2016). While *bosR* is expressed throughout the entire enzootic cycle (Medrano et al., 2007; Ouyang et al., 2016), environmental factors, such as temperature and pH, modulate its transcription (Katona et al., 2004; Ouyang et al., 2016). The intracellular second messengers c-di-GMP (Sze et al., 2013; He et al., 2014; Novak et al., 2014; Ye et al., 2014) and the alarmones (p)ppGpp (Bugrysheva et al., 2015; Drecktrah et al., 2015; Drecktrah et al., 2018) induce *bosR* expression at both transcriptional and post-transcriptional levels. Dissolved carbon dioxide (Hyde et al., 2007) and the transition metals manganese, zinc and copper (Troxell and Yang, 2013; Troxell et al., 2013; Wang et al., 2017) influence *bosR* expression at the post-transcriptional level. BadR, a ROK repressor, negatively affects *bosR* expression at both transcriptional and post-transcriptional levels (Miller et al., 2013; Ouyang and Zhou, 2015). Transcription of *bosR* also is activated by the BosR protein, indicating that BosR is an auto-activator (Ouyang et al., 2016), which is different from classical Fur family proteins as auto-repressors. The expression and regulatory patterns of *bosR* highlight the crucial role of BosR in the enzootic cycle of *B. burgdorferi*.

BosR is an atypical Fur family member—Fur family proteins consist of two structural domains including a N-terminal winged helix-turn-helix DNA-binding domain and a C-terminal dimerization domain, connected by a flexible hinge region. Structural analyses of numerous Fur proteins have revealed two sites involved in metal binding (Helmann, 2014; Pinochet-Barros and Helmann, 2018; Sarvan et al., 2018; Nader et al., 2019). The first site (S1), located in the dimerization domain, is usually occupied by a zinc ion and is crucial for protein dimerization, so it is called the structural site. The second metal binding site (S2) is located in the hinge region between the DNA-binding domain and the dimerization domain. The metal ion (e.g., iron, manganese, zinc, or nickel) occupying the S2 site determines the

specific regulatory function of the Fur protein; thus, it is called the regulatory site. Occasionally, Fur proteins such as those in *Helicobacter pylori* and *Campylobacter jejuni* contain a third metal binding site (S3) in the dimerization domain, which is also important for the regulatory function of the protein (Dian et al., 2011; Butcher et al., 2012). The regulatory functions of Fur family members are responsive to the availability of transition metal ions such as iron, manganese, zinc, and nickel (Hantke, 1981, 2001; Carpenter et al., 2009; Fillat, 2014). In the absence of metal cofactors, the DNA-binding domain of Fur typically has an "open" conformation that does not bind DNA. Occupancy of S1 with a zinc ion induces Fur dimerization, which brings the DNA-binding domains of two monomers into closer proximity. Binding of metal ions to S2 triggers additional conformational changes, which in turn stimulates the formation of a "closed" DNA-binding domain. As a result, the "closed" Fur binds to promoter DNA with high affinity and represses gene expression. In addition, metal-dependent Fur family proteins also sense non-metal signals. For example, PerR contains the zinc-binding structural S1 site and a manganese- or iron-coordinating regulatory S2 site (Traoré et al., 2006; Jacquamet et al., 2009; Traoré et al., 2009). Manganese or iron occupying S2 triggers the formation of a "closed" PerR (i.e., PerR-Zn-Mn and PerR-Zn-Fe) that binds to the Per box and blocks gene transcription. When iron in the S2 site detects peroxide, such as H_2O_2, histidine residues of the PerR-Zn-Fe are oxidized. This leads to the release of iron and changes PerR to the "open" conformation. The "open" PerR then dissociates from the Per box, which results in the derepression of genes involved in the peroxide stress response (Traoré et al., 2006; Jacquamet et al., 2009; Traoré et al., 2009).

Recombinant BosR contains zinc, but no other metal ions (including iron, copper, manganese, chromium, cadmium, nickel, magnesium, or lead) (Ouyang et al., 2011; Wang et al., 2012b; Mason et al., 2019). Two CXXC motifs were identified in the dimerization domain of BosR that constitute the zinc-coordination structural S1 site of BosR (Mason et al., 2019). This site is similar to the S1 site in many Fur family proteins including PerR (Figure 3). The ability of BosR to bind Zn was dramatically reduced when the CXXC motifs were mutated (Mason et al., 2019). The CXXC motifs also contribute to the ability of BosR to form dimers and are crucial for the regulatory function of BosR. However, BosR lacks a recognizable regulatory S2 site. Of the residues constituting the iron, manganese, zinc, or nickel-coordinating regulatory S2 site of Fur proteins, BosR retains only a single conserved residue (His111) in the hinge region (Figure 3). The vicinity surrounding His111 lacks metal coordinating residues other than the known CXXC motifs. Therefore, the DNA-binding conformation of BosR may not be induced by metal binding, other than at the S1 site. Instead, the His111 may be modified through an as yet unknown mechanism to facilitate DNA binding, which is consistent with the presence of only zinc in recombinant BosR (Ouyang et al., 2011; Wang et al., 2012b; Mason et al., 2019). Alternatively, BosR may use a noncanonical S2 site for signal sensing. Recombinant BosR is able to bind copper, zinc and iron when incubated with unusually high amounts of these metal ions, so perhaps BosR contains two regulatory sites coordinating zinc, iron, or copper in addition to the structural S1 site (Wang et al., 2017). Surprisingly, adding zinc or copper, but not iron, inhibited the binding of BosR to the *ospA* promoter region (Wang et al., 2017). However, we do not know which metal is bound to BosR *in vivo*.

There remain considerable knowledge gaps regarding BosR. The role of this regulatory protein in the oxidative stress response and transition metal homeostasis is, frankly, uncertain. In addition, the contributions of the BosR regulon to the enzootic cycle need to be addressed. The binding of Fur protein from other bacteria to DNA results in gene repression; however, the binding of BosR to the promoter region of *rpoS* and *bosR* is known to activate transcription, so the mechanistic basis for this unusual activity should be explored. Furthermore, whether BosR has a second regulatory metal-binding site like other Fur proteins is unknown. Dissecting the unusual DNA binding activity and regulatory function of BosR is fundamental toward determining the pathway by which BosR senses the host- and vector-specific signals to control gene expression during the enzootic cycle.

BosR function—Seshu et al. (2004b) constructed the first *bosR* mutant in a high-passage, non-infectious strain of *B. burgdorferi*. However, the *bosR* allele (*bosRR39K*) in the parental high-passage strain possessed a point mutation (R39K) relative to the wild-type *bosR* in infectious strains (Seshu et al., 2004a; Hyde et al., 2006); the R39 residue of BosR is essential for protein function (Seshu et al., 2004a; Katona, 2015). *bosR* deletion mutants were then generated in low-passage, infectious *B. burgdorferi* strains. Hyde et al. (2009, 2010) reported that *bosR* contributes to the *in vitro* growth of *B. burgdorferi*, and the levels of Dps and SodA were decreased in the *bosR* mutant. However, neither the growth defect nor the change in Dps synthesis were observed in the *bosR* mutant generated by Ouyang et al (2009b; 2011). Despite these and other discrepancies, the strategic *bosR* mutants yielded breakthroughs in our understanding of BosR function. First, BosR is essential for tick to vertebrate transmission and vertebrate infection by *B. burgdorferi*. Second, BosR is required for the transcriptional activation of *rpoS*. The expression of *rpoS* and RpoS-dependent OspC and DbpA was abolished in *bosR* mutants. BosR directly activates transcription of *rpoS* by binding to the *rpoS* promoter region (Ouyang et al., 2011; Ouyang et al., 2014a; Katona, 2015). Specifically, BosR binds to three sites in the *rpoS* promoter region as a homodimer, including a site overlapping the −24/−12 RpoN-dependent promoter (Ouyang et al., 2011). This suggests that BosR interacts with RpoN (σ^{54}) to facilitate the recruitment of RpoN-RNA polymerase holoenzyme to the *rpoS* promoter to activate gene expression. Further analyses revealed that BosR recognizes the *rpoS* promoter region via a core sequence called the BosR box, a novel DNA element consisting of a 13-bp palindromic sequence (ATTTAANTTAAAT) with dyad symmetry (Ouyang et al., 2014a). Different from the 19-bp Fur binding consensus (i.e., Fur box, GATAATGATAATCATTATC) and the 15-bp PerR binding consensus (i.e., Per box, TTATAATTATTATAA), the BosR box probably comprises a 6–1–6 inverted repeat composed of two hexamers (ATTTAA) in a head-to-tail orientation. The requirement of BosR in the activation of RpoN-dependent *rpoS* transcription was unexpected, as activation of bacterial RpoN (σ^{54}) systems generally only requires the bacterial enhancer binding protein (bEBP) (Bush and Dixon, 2012), and suggests that BosR and Rrp2 together mimic the bEBP.

The BosR regulon—Recombinant BosR binds to many DNA sequences including the Per box (Katona et al., 2004; Ouyang et al., 2014a) and the promoter regions of *dps* (Boylan et al., 2003; Katona et al., 2004; Ouyang et al., 2011), *sodA* (Seshu et al., 2004a), *cdr* (Boylan et al., 2006), *oppA4* (Medrano et al., 2007), *ospA* (Wang et al., 2013; Shi et al., 2014; Wang

et al., 2017), *ospD* (Wang et al., 2013), and *bosR* itself (Katona et al., 2004; Ouyang et al., 2011). Such versatile DNA recognition capacity is not typical of canonical Fur family proteins. Contradictory results were also obtained regarding the DNA binding activity of BosR. Boylan et al. (2003) reported that the binding of BosR to the *dps* promoter was inhibited by manganese and required zinc as well as the reduction with DTT. Katona et al. (2004), however, found that binding of BosR to the Per box and the *dps* promoter was not affected by manganese, but was inhibited by zinc. Wang et al. (2017) showed that the binding of BosR to the *ospA* promoter was not affected by manganese or iron but was inhibited by copper and zinc. These discrepancies may be due to proteins with different conformations used in the *in vitro* binding studies. Analytical ultracentrifugation analyses revealed a monomer-dimer equilibrium of recombinant BosR in free solution (Ouyang et al., 2014a). Metal ions and oxidizing reagents may induce conformational changes, and thus affect the DNA binding activity of recombinant protein. In an attempt to discern the global regulatory effects of BosR, Katona et al. (2004) searched the *B. burgdorferi* genome for the Per box by using the BlastN program and found 26 genes possibly regulated by BosR. Ouyang et al. (2014a) searched the genome (Fraser et al., 1997) for putative BosR boxes using the Regulatory Sequence Analysis Tools, and found 47 genes carrying one or more BosR box sequences in their promoter regions. However, identification of a BosR binding site in a promoter does not mean that BosR regulates the gene. Microarray-based transcriptomic analysis of the *bosR* mutant identified 137 and 62 genes that were upregulated or downregulated, respectively, by BosR, although the function of most them is unknown (Ouyang et al., 2009b). A comparison of the *bosR* and *rpoS* microarray data (Ouyang et al., 2008) found that 50 and 57 genes were upregulated or downregulated, respectively, only by BosR, which suggests that BosR is a dual-functional global regulator in *B. burgdorferi*.

BadR

BadR (BB0693; *Borrelia* host adaption regulator) is a DNA-binding transcriptional regulator that represses *rpoS* transcription in *B. burg*dorferi (Fraser et al., 1997; Miller et al., 2013; Ouyang and Zhou, 2015). BadR synthesis is induced at low temperature, mimicking the tick environment (Miller et al., 2013). *badR* is expressed throughout the entire enzootic cycle of *B. burgdorferi*, reaching a maximal level in intermolt larvae (Ouyang and Zhou, 2015). BadR is required by *B. burgdorferi* to infect vertebrates; *badR* mutants are incapable of infecting mice through needle injection (Miller et al., 2013; Ouyang and Zhou, 2015). In addition, BadR is crucial for optimal growth of *B. burgdorferi in vitro*; *badR* mutants display a marked growth defect in culture compared to the wild-type parental strain. Expression of *rpoS* is enhanced in *badR* mutants, indicating that BadR is a repressor of *rpoS* transcription. Recombinant BadR binds to multiple sites in the *rpoS* promoter region; several residues in the putative sugar-binding domain of BadR were crucial for binding. A merodiploid with *badR* expression controlled by an inducible promoter was constructed to overcome the inability to complement the *badR* null mutant: induction of *badR* inhibited transcription of *rpoS* (Ouyang and Zhou, 2015). Some differences exist between the two genetic studies of *badR* (Miller et al., 2013; Ouyang and Zhou, 2015) including whether expression of *rpoS* in the *badR* mutants is growth-phase dependent. In addition, Miller et al. (2013) reported that binding of recombinant BadR to the *rpoS* promoter region was inhibited by the addition of

phosphorylated sugars, while this inhibition was not observed by Ouyang et al (2015), which may be due to the presence of the maltose-binding protein tag on the recombinant BadR in the first study.

BadR inhibits expression of *bosR* at both transcriptional and post-transcriptional levels. Binding sites for BadR have been identified in the sequences upstream of *bosR* (Ouyang and Zhou, 2015). Both BadR and BosR bind to the promoter regions of *rpoS* and *bosR*, although they recognize different core sequences. BadR recognizes DNA at TAAAATAT motifs. Expression of *rpoS* is abolished in a *bosR-badR* double mutant (Ouyang and Zhou, 2015), which suggests that BosR functions to activate *rpoS* transcription rather than to derepress BadR. BadR represses or induces the expression of 127 or 80 genes, respectively (Miller et al., 2013). Subsequent RNA-seq analyses showed that expression of 134 and 100 genes were decreased and increased, respectively, in the *badR* mutant (Arnold et al., 2018). Many of these genes are associated with sugar transport and utilization.

How BadR modulates gene expression in *B. burgdorferi* remains elusive. BadR was originally annotated as a xylose operon regulatory protein XylR-1 involved in the utilization and degradation of D-xylose (Fraser et al., 1997). BadR, however, likely does not function as a xylose-responsive transcriptional regulator like XylR, as *B. burgdorferi* does not use xylose as a carbon source (von Lackum and Stevenson, 2005) and its genome does not encode proteins for xylose transport and catabolism (Fraser et al., 1997). Rather, BadR has ~21% amino acid sequence identity with the *E. coli* transcriptional regulator NagC. Three-dimensional protein modeling indicated that BadR had a structure similar to the Mlc protein in *V. cholerae* and *E. coli* (Ouyang and Zhou, 2015). Mlc is a homolog of NagC and both proteins belong to the ROK (Repressor, ORF, Kinase) protein family. ROK repressors contain an N-terminal helix-turn-helix (HTH) DNA-binding motif and a C-terminal sugar-binding domain. The DNA-binding activity and regulatory function of ROK repressors are responsive to the availability of specific sugars. Despite the homology to NagC and Mlc, BadR likely functions differently from other ROK repressors. Typically, ROK repressors bind a specific sugar or effector through specificity-determining residues (SDRs) (Titgemeyer et al., 1994; Kazanov et al., 2013). However, BadR lacks the SDRs found in NagC, Mlc, and other ROK repressors, consistent with the observation that the DNA-binding of recombinant BadR was not affected by phosphorylated sugars (Ouyang and Zhou, 2015), and the expression of genes involved in GlcNAc transport and utilization were not affected in the *badR* mutant (Miller et al., 2013), supporting that BadR does not function analogously to NagC or Mlc and is yet another example of an atypical transcriptional regulator influencing the expression of enzootic cycle-specific genes (Samuels and Radolf, 2009).

YebC

YebC (BB0025) is a transcription factor that belongs to a putative YebC/PmpR family of DNA-binding transcriptional regulators (TACO1 family, pfam PF01709) (Shin et al., 2002; Zhang et al., 2012) and controls *vlsE* expression (Zhang et al., 2020). VlsE undergoes antigenic variation in the vertebrate host and *vlsE* expression increases concomitantly with the decrease in *ospC* expression in response to the host adaptive immune response, but there

are few mechanistic details on the regulation of *vlsE* expression (Norris, 2014; Chaconas et al., 2020). Zhang et al. (2020) very recently demonstrated that deletion of *yebC* dramatically reduced *vlsE* mRNA levels *in vitro*. Expression of *yebC* and *vlsE* is co-regulated in response to culture temperature, and is inversely regulated with expression of *ospC* during vertebrate infection. The *yebC* mutant has a similar phenotype as the *vlsE* mutant: both are infectious in immunocompromised mice but unable to infect immunocompetent mice. YebC binds to the *vlsE* promoter, suggesting that YebC directly regulates *vlsE* transcription; however, how YebC is activated during the vertebrate phase remains unknown.

Hk1/Rrp1 two-component system and c-di-GMP

Since its discovery as a novel allosteric activator of cellulose synthase in *Komagataeibacter xylinus* (formerly *Acetobacter xylinus*) more than 30 years ago (Ross et al., 1987), bis-(3′−5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) has emerged as a ubiquitous global secondary messenger associated with a wide range of lifestyle control networks, including the transition from planktonic to sessile states, biofilm formation, cell cycle progression, and virulence (Cotter and Stibitz, 2007; Wolfe and Visick, 2008; Hengge, 2009; Römling et al., 2013; Valentini and Filloux, 2019). Diguanylate cyclases (DGCs), the enzymes responsible for synthesis of c-di-GMP from two GTP molecules, are readily identified by the presence of an ~170 amino acid GGDEF (Gly-Gly-Asp-Glu-Phe) conserved domain (Pfam PF00990) (Pei and Grishin, 2001; Schirmer and Jenal, 2009). GGDEF domain-containing proteins are omnipresent in bacteria (Galperin, 2004; Ryjenkov et al., 2005), with most bacterial genomes encoding multiple DGCs. The potential relevance of c-di-GMP for environmental signaling in Lyme disease spirochetes first came to light in a bioinformatics study by Galperin et al. (2001). Using purified recombinant protein, Ryjenkov et al. (2005) provided direct biochemical evidence that Rrp1 (BB0419), the only identifiable GGDEF domain-containing protein encoded by *Borrelia* spp. (Galperin et al., 2001), functions as a diguanylate cyclase. Intracellular concentrations of c-di-GMP in *B. burgdorferi* are controlled by the activity of two evolutionarily distinct phosphodiesterases (PDEs), PdeA and PdeB, belonging to the EAL and HD-GYP families of non-specific PDEs, respectively (Sultan et al., 2010; Sultan et al., 2011; Novak et al., 2014). While both borrelial PDEs hydrolyze c-di-GMP with high affinity (Sultan et al., 2010; Sultan et al., 2011; Novak et al., 2014), PdeA- and PdeB-deficient spirochetes display different motility- and virulence-related phenotypes (Sultan et al., 2010; Sultan et al., 2011), suggesting that these PDEs are part of a complex regulatory system that involves multiple effectors and control mechanisms.

Rrp1 along with Hk1 form a prototypical bacterial sensory two-component signal transduction system (TCS). Hk1, the sensory component, is composed of four modular domains: a periplasmic sensor domain of three ligand-binding subdomains, each with homology to bacterial periplasmic solute-binding proteins (PBPs), a histidine kinase domain, a phosphoacceptor receiver (REC) domain, and a histidine phosphotransferase (Hpt) domain (Bauer et al., 2015). Ligand binding by Hk1 initiates a signal transduction cascade that results in phosphorylation of the Rrp1 REC domain and activation of c-di-GMP synthesis (Caimano et al., 2011; Bauer et al., 2015). Although the small molecule(s) responsible for activating Hk1 in nature have yet to be identified, bioinformatics and

structural analyses of the Hk1 sensory PBP domains point to exogenously-derived amino acids and/or their derivatives as potential activating ligands (Caimano et al., 2011; Caimano et al., 2015). Consistent with this prediction, the Hk1/Rrp1 TCS is active when spirochetes are grown in BSK II (Rogers et al., 2009; Caimano et al., 2011), a semi-defined medium that contains abundant free amino acids (Barbour, 1984). The genes encoding Hk1 and Rrp1 (*bb0420* and *bb0419*, respectively) are co-transcribed as a bicistronic message (Caimano et al., 2011). *hk1/rrp1* is expressed more or less constitutively *in vitro* and throughout the enzootic cycle (Rogers et al., 2009; Caimano et al., 2011).

Phenotypic characterization of *hk1* and *rrp1* mutants demonstrated that this TCS functions exclusively during the tick phase of the enzootic cycle (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). Hk1- and Rrp1-deficient organisms display wild-type burdens in murine tissues following needle inoculation but are rapidly destroyed in the midguts of feeding larvae and nymphs as early as 36 hours into the acquisition blood meal (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). The phenotypic similarities between *hk1* and *rrp1* mutants in larvae and nymphs suggest that the ligand(s) responsible for activating c-di-GMP signaling are not tick life-stage-specific (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011; Caimano et al., 2015). Little to no killing was observed in the absence of tick feeding, indicating that the destruction of both mutants is mediated by the feeding process. An important clue regarding this unusual tick phenotype was provided by Bontemps-Gallo et al. (2016), who found that *hk1* and *rrp1* mutant *B. burgdorferi* grown at 34°C in BSK II were substantially more osmo-sensitive than their wild-type isogenic parent under conditions that mimic the high osmolality (~550–650 mOsm) within fed midguts. Additional insight into the contribution of the Hk1/Rrp1 TCS to tick adaptation comes from genome-wide transcriptomics analyses by microarray (Rogers et al., 2009; He et al., 2011) and RNA-seq (Caimano et al., 2015). Results from these analyses revealed two broad categories of c-di-GMP-regulated genes that could contribute to survival within tick midguts during the blood meal. The first includes genes involved in utilization of alternate carbon sources for energy generation (glycolysis) and biosynthesis of phospholipids and/or peptidoglycan. In addition to the glycerol utilization (Glp) system, c-di-GMP upregulates expression of PTS transporters (*malX2* and *chbC*) for *N*-acetylglucosamine (GlcNAc) and chitobiose (a GlcNAc dimer derived from chitin, the major component of tick cuticle), respectively (Tilly et al., 2001; Tilly et al., 2004; Rhodes et al., 2009; Gherardini et al., 2010; Pappas et al., 2011; Sze et al., 2013; Caimano et al., 2015; Corona and Schwartz, 2015; Caimano et al., 2016; Zhang et al., 2018). The phenotypes for *hk1* and *rrp1* mutants in feeding ticks, however, are more pronounced than those of either *glp* or *chbC* mutants. Presumably, utilization of multiple alternative carbon sources enables spirochetes to expand exponentially and maintain cell envelope homeostasis as blood meal-derived glucose becomes increasingly limited due to competition from midgut epithelial cells. Additional c-di-GMP-regulated genes contribute to maintenance of the cell envelope: the Rrp1 regulon includes *ackA*, encoding the acetate kinase required for generation of acetyl phosphate, which in turn gives rise to acetyl-CoA, the starting point for synthesis of peptidoglycan via the mevalonate pathway (Xu et al., 2010; Van Laar et al., 2012). The second category of c-di-GMP regulated genes includes differentially-expressed lipoproteins, including the cp32-encoded OspE/BbCRASP lipoproteins, which have been shown to inhibit complement-

mediated lysis by binding complement factor H (CFH) and CFH-related proteins (Radolf et al., 2012; Bhattacharjee et al., 2013; Kraiczy and Stevenson, 2013). Other lipoproteins regulated by c-di-GMP (e.g., Mlps) also may protect *B. burgdorferi* from hazards encountered in the fed midgut (e.g., antimicrobial peptides and reactive oxygen species).

c-di-GMP modulates cellular processes in bacteria by a variety of mechanisms, including transcriptional, translational, allosteric inhibition of enzymatic activity (I-sites), protein-protein interactions, protein secretion, and protein stability (Cotter and Stibitz, 2007; Wolfe and Visick, 2008; Römling et al., 2013; Valentini and Filloux, 2019). A wide range of effector molecules involved in c-di-GMP signaling have been identified, including two types of riboswitches (Sudarsan et al., 2008; Lee et al., 2010; Smith et al., 2011) and numerous c-di-GMP receptor proteins (Schirmer and Jenal, 2009; Dahlstrom and O'Toole, 2017; Valentini and Filloux, 2019). One of the most well-studied group of c-di-GMP effector proteins are those containing an ~110 amino acid PilZ domain (PF07238) (Amikam and Galperin, 2006; Schirmer and Jenal, 2009), first identified at the C-terminus of the cellulose synthase catalytic subunit of BcsA from *K. xylinum* (Ross et al., 1987). To date, two c-di-GMP effector PilZ domain proteins, PlzA (BB0733) and PlzB, have been described in *B. burgdorferi* (Freedman et al., 2010; Pitzer et al., 2011; He et al., 2014; Mallory et al., 2016). A third PilZ-domain protein, PlzC, is found only in relapsing fever spirochetes (Mallory et al., 2016). PlzA, encoded on the chromosome, and PlzB, encoded on a linear plasmid, share only 63% amino acid identity and appear to carry out distinct functions (Mallory et al., 2016). The majority of Lyme disease spirochetes encode only PlzA. c-di-GMP binding by borrelial Plz proteins is mediated by a signature PilZ domain motif: RIHER and DzSYGG (z=hydrophobic residue) (Amikam and Galperin, 2006; Ryjenkov et al., 2006; Freedman et al., 2010; Pitzer et al., 2011; Mallory et al., 2016). In independent studies, Motaleb, Marconi and colleagues demonstrated that recombinant PlzA binds c-di-GMP at 1:1 stoichiometry with relatively high affinity (~1 – 6 μM) (Freedman et al., 2010; Pitzer et al., 2011; Mallory et al., 2016). Binding of c-di-GMP by PlzA is thought to induce a conformational change enabling a 'switch mechanism' (Mallory et al., 2016). Interestingly, the PilZ motifs in borrelial Plz proteins are separated by a variable 32 amino acid spacer that is significantly longer than the 19 to 23 residues typically found in prototypical PilZ domains, perhaps allowing spirochetal Plz proteins to engage a more extensive collection of downstream effector molecules (Mallory et al., 2016). Consistent with its c-di-GMP effector function, expression of *plzA* is increased during tick feeding (Freedman et al., 2010; Pitzer et al., 2011). However, there are conflicting reports regarding the extent to which PlzA is required for survival during tick feeding. Pitzer et al. (2011) reported that *plzA* mutants survive poorly in naturally-infected ticks during the acquisition and transmission blood meals, while Kostick-Dunn et al. (2018) found that PlzA was not strictly required to establish infection in ticks. Nonetheless, both groups demonstrated that *plzA*-infected ticks are unable to transmit spirochetes to naïve mice. Moreover, overexpression of PlzA is able to partially compensate for the loss of Rrp1 in feeding ticks (Zhang et al., 2018). This phenotype stems, in part, from the requirement for both c-di-GMP and PlzA for transcription of the *glp* operon (*bb0240-bb0243*) (Caimano et al., 2015; Zhang et al., 2018), which encodes a system for the uptake and utilization of glycerol (Pappas et al., 2011). However, unlike *rrp1* mutants, which are infectious in mice, spirochetes lacking PlzA are substantially less virulent by needle-

inoculation (Pitzer et al., 2011; He et al., 2014; Kostick-Dunn et al., 2018; Zhang et al., 2018). Decreased virulence of *plzA* mutants has been attributed, at least in part, to aberrant motility (i.e., decreased swarming, reduced translational motility and flex frequency) (Pitzer et al., 2011; Kostick-Dunn et al., 2018) and/or dysregulation of RpoS-dependent gene regulation (Sze et al., 2013; He et al., 2014). He et al. (2014) reported that PlzA influences the RpoS pathway via its effects on BosR, a Fur family protein required for expression of *rpoS* (see the section above on BosR and BadR transcription factors) (Samuels and Radolf, 2009); *plzA* mutants express two- to threefold lower levels of *bosR* mRNA compared to wild type and little to no BosR protein *in vitro* (He et al., 2014). Given that the Hk1/Rrp1 TCS is not required and presumably 'OFF' in vertebrates, any virulence-related aspects of the *plzA* mutant phenotype, including dysregulation of BosR and/or RpoS, are likely mediated by apo-PlzA (Pitzer et al., 2011; Zhang et al., 2018).

Genome-wide comparison of the RpoS and Hk1/Rrp1 regulons identified only a handful of genes that are regulated by both RpoS and c-di-GMP (Caimano et al., 2015). The expression profiles of these co-regulated genes also were strikingly different from those of the prototypical RpoS-regulated genes *ospC* and *dbpA* in fed nymphs (i.e., when both pathways are active) (Dunham-Ems et al., 2012; Caimano et al., 2019). Thus, while c-di-GMP may enhance expression of some RpoS-upregulated genes, these two pathways likely function in parallel rather than cooperatively. As part of its gatekeeper function, RpoS also represses the expression of RpoD (σ^{70})-dependent tick-phase genes, including *ospA*, *bba62*, and the *glp* operon, within the vertebrate (Caimano et al., 2005; Caimano et al., 2007; Dunham-Ems et al., 2012; Grove et al., 2017; Caimano et al., 2019) (see the section above on the RpoN-RpoS σ factor cascade). Although transcription of RpoS-upregulated genes begins during the transmission blood meal (Schwan et al., 1995; Dunham-Ems et al., 2012; Ouyang et al., 2012; Caimano et al., 2019), RpoS-mediated repression does not occur to any appreciable extent until spirochetes have entered the vertebrate host (Dunham-Ems et al., 2012; Caimano et al., 2019). Overlap between the Hk1/Rrp1 and RpoS regulons includes a number of tick phase-specific genes that are repressed by RpoS during infection. These data led Caimano et al. (2015) to propose that c-di-GMP antagonizes RpoS-mediated repression either directly or indirectly in feeding ticks. Once *B. burgdorferi* is in a vertebrate, the absence of c-di-GMP would allow unfettered repression of these tick phase-specific genes.

Stringent response and (p)ppGpp

Rel_{Bbu} and DksA mediate adaptation to nutrient stress

B. burgdorferi adapts to environmental nutritional changes encountered during its enzootic cycle by globally altering gene expression through the stringent response, which is particularly pivotal during persistence in the tick vector between blood meals. The stringent response steels the spirochete by regulating the expression of numerous pathways, including replication, motility, morphology, stress responses, and virulence factors. These changes are driven by modulating the levels of two intracellular alarmones, guanosine pentaphosphate and guanosine tetraphosphate, collectively known as (p)ppGpp, which directly bind RNA polymerase and indirectly affect numerous regulatory systems to alter transcription (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Gaca et al., 2015; Hauryliuk et

al., 2015; Liu et al., 2015; Steinchen and Bange, 2016; Gourse et al., 2018). In *B. burgdorferi*, (p)ppGpp levels are regulated by a single gene product, Rel_{Bbu}, containing both a canonical synthetase (RelA) domain induced by amino acid starvation and a bifunctional synthetase/hydrolase (SpoT) domain (Concepcion and Nelson, 2003; Bugrysheva et al., 2005; Drecktrah et al., 2015). In addition to the catalytic domains, Rel_{Bbu} also contains the regulatory TGS (threonyl-tRNA synthetases, GTPases and SpoT) and DC (aspartate-cysteine) oligomerization domains, but lacks the ACT regulatory domain in the C-terminal region (Grant, 2006; Hauryliuk et al., 2015; Ronneau and Hallez, 2019). (p)ppGpp levels rise during nutrient deprivation and fall upon return to nutrient-rich conditions in a Rel_{Bbu}-dependent manner (Concepcion and Nelson, 2003; Bugrysheva et al., 2005; Drecktrah et al., 2015). The specific triggers of the stringent response in *B. burgdorferi* remain unknown, but amino acid starvation may not serve as the classic signal since the *B. burgdorferi* genome is predicted to contain few amino acid transporters and may rely more on peptide transport and proteolysis (Fraser et al., 1997; Groshong et al., 2017); instead, the spirochete may sense changes in levels of lipids or phosphate.

The Rel_{Bbu}-mediated transcriptional changes of the stringent response play an important role in survival during nutrient stress both in culture and within the nymphal tick midgut during the transmission blood meal (Drecktrah et al., 2015). Survival in the midgut and transmission to mice via tick bite are not abolished in a rel_{Bbu} mutant incapable of synthesizing (p)ppGpp, but are significantly curtailed (Drecktrah et al., 2015). Growth in nutrient-rich media at 23°C, the temperature used to represent the tick environment, is also Rel_{Bbu}-dependent (Bugrysheva et al., 2015). The stringent response also regulates *B. burgdorferi* morphology as the rel_{Bbu} mutant is more likely to form round bodies, a spherical condensed form within an outer membrane observed *in vitro* and within ticks between blood meals, in response to nutrient stress (Brorson and Brorson, 1997, 1998; Alban et al., 2000; Dunham-Ems et al., 2012; Drecktrah et al., 2015). These studies illustrate an important role for Rel_{Bbu} and the stringent response in persistence in the tick and subsequent transmission to the host.

The transcription factor DksA (DnaK suppressor), which potentiates the transcriptional effects of (p)ppGpp on RNA polymerase activity (Gourse et al., 2018), also globally reprograms the *B. burgdorferi* transcriptome in response to nutrient stress (Boyle et al., 2019; Mason et al., 2020). There is considerable overlap between the DksA and Rel_{Bbu} regulons, but differences exist (see below), illustrating that DksA is a cooperating partner in the stringent response but also exerts an independent influence. Recent studies have found that, similar to Rel_{Bbu}, DksA regulates genes that affect *B. burgdorferi* survival under nutrient and osmotic stresses (Boyle et al., 2019). Surprisingly, DksA is essential for *B. burgdorferi* infectivity in the murine model of Lyme disease, at least by needle inoculation (Mason et al., 2020), while Rel_{Bbu} is dispensable for murine infectivity (Drecktrah et al., 2015). Tick transmission studies await further investigation as do experiments examining the role of DksA in persistence in the tick.

The Rel_{Bbu} regulon

The Rel_{Bbu}-dependent regulon is characterized by broad patterns of gene regulation typical of bacteria adapting to nutrient stress by reducing growth, including repression of many genes involved in metabolism (glycolysis and mevalonate biosynthesis), translation (ribosomal proteins) and transcription (RNA polymerase subunits), as revealed by RNA-seq and microarray analyses (Bugrysheva et al., 2015; Drecktrah et al., 2015). Rel_{Bbu}-dependent regulation of gene products whose function is not fully understood may provide clues to their role in the enzootic cycle. For example, the expression of the genes encoding the oligopeptide binding proteins (*oppA1*, *oppA2*, *oppA3* and *oppA5*) are Rel_{Bbu}-upregulated suggesting that peptide uptake functions during persistence of the spirochete in the tick, as this is the phase where survival of the rel_{Bbu} mutant is compromised (Drecktrah et al., 2015). Rel_{Bbu} also regulates the glycerol metabolic (*glp*) operon (Bugrysheva et al., 2015; Drecktrah et al., 2015). The *glp* operon contributes to persistence of *B. burgdorferi* in the tick where the spirochete is thought to utilize glycerol as a carbon source and environmental signal, as has been demonstrated *in vitro* (He et al., 2011; Pappas et al., 2011; Bugrysheva et al., 2015). The defect in persistence in the tick of the rel_{Bbu} mutant is likely due, at least in part, to compromised glycerol metabolism. The metabolic fate of glycerol may be directed by the stringent response as well: Rel_{Bbu} upregulates expression of genes encoding the glycerol uptake facilitator (*glpF*) and glycerol kinase (*glpK*), but studies disagree on the effect on the last gene in the operon, glycerol 3-phosphate dehydrogenase (*glpD*) (Bugrysheva et al., 2015; Drecktrah et al., 2015). If GlpD is upregulated then glycerol 3-phosphate would be trafficked to the glycolytic pathway, while downregulation of GlpD would instead shuttle phosphorylated glycerol to membrane and lipoprotein biosynthesis. This suggests that the stringent response coordinates transcriptional changes to affect metabolism to adapt the spirochete to nutritional flux in the enzootic cycle.

These transcriptomic studies also illuminate potential functions for Rel_{Bbu} in processes not traditionally thought to be regulated by the stringent response. Expression of *vlsE*, a Vmp-like surface lipoprotein used to evade the adaptive host response (Norris, 2014; Chaconas et al., 2020) (see Radolf and Samuels, 2021), is Rel_{Bbu}-upregulated (Drecktrah et al., 2015). Although *vlsE* recombination only occurs in the vertebrate host, the stringent response is the first signaling system identified that regulates *vlsE* expression. In addition, Rel_{Bbu} regulates expression of the *bosR* gene (Drecktrah et al., 2015) (see section above on Transcription factors BosR and BadR). The reduction in *bosR* expression is not enough to render the rel_{Bbu} mutant non-infectious by needle inoculation, but could play a role in the tick transmission defect. Rel_{Bbu} also upregulates a number of genes encoding extracellular matrix-binding proteins important in the vertebrate host, such as *dbpBA* and *bbk32* (Drecktrah et al., 2015). These findings suggest that *B. burgdorferi* may encounter nutrient-limited niches in the vertebrate, such as the synovial regions in joints, that trigger the stringent response or there may be subtler, as of yet unidentified, signals inducing (p)ppGpp production in the spirochete to modulate gene expression in the vertebrate host.

Rel_{Bbu} also seems to coordinate the life cycle of *B. burgdorferi* bacteriophages by controlling numerous genes located on the cp32 prophages (Eggers and Samuels, 1999; Zhang and Marconi, 2005). Rel_{Bbu} represses expression of many more cp32 genes than it

activates, with most located in the putative late operon (Drecktrah et al., 2015). Furthermore, expression of the essential gene *ftsH* (Chu et al., 2016) was also Rel_{Bbu}-downregulated (Bugrysheva et al., 2015; Drecktrah et al., 2015); the *ftsH* homolog in *E. coli* encodes a protease that regulates the λ phage life cycle (Kihara et al., 1997; Shotland et al., 1997). Thus, changes in nutrient availability in the tick midgut or vertebrate may signal, through the stringent response, conditions for prophage induction.

Rel_{Bbu} regulates sRNAs

The stringent response regulates expression of a third of all identified small RNAs (sRNAs) in *B. burgdorferi*, including intragenic sRNAs, antisense sRNAs and 5′ UTR sRNAs (Popitsch et al., 2017; Drecktrah et al., 2018) (see the section below on Small regulatory RNAs and RNA chaperones). Threefold more sRNAs were Rel_{Bbu}-upregulated than downregulated and were mapped throughout the genome (Drecktrah et al., 2015). Antisense sRNAs and 5′ UTR sRNAs associated with genes of known or predicted function mostly targeted genes encoding translation, transporter and cell envelope functions. One intriguing example is that Rel_{Bbu} downregulates an antisense sRNA within the *glpF* ORF (SR0186) while concurrently upregulating the *glpF* mRNA (Drecktrah et al., 2018). The sense:antisense pairing may favor degradation of the *glpF* transcript in response to vertebrate-specific signals (Drecktrah et al., 2015). Thus, one mechanism *B. burgdorferi* may employ to survive in the tick involves sensing nutrient stress between blood meals to induce the stringent response that represses the *glpF* antisense sRNA, which, in turn, stabilizes the *glpF* mRNA and increases GlpF production to enhance transport and utilization of glycerol.

Rel_{Bbu} may also indirectly modulate RNA polymerase activity to alter the global transcriptome through the Bb6S RNA (see the section below on Small regulatory RNAs and RNA chaperones). Bb6S RNA is a highly expressed sRNA that binds directly to the RpoD (σ^{70})-RNA polymerase (Drecktrah et al., 2020). Rel_{Bbu} upregulates Bb6S RNA, potentially providing another mechanism to shape the transcriptional landscape in addition to the direct interaction of (p)ppGpp with RNA polymerase (Drecktrah et al., 2018). The vast contingent of sRNAs controlled by the stringent response represent multiple pathways for regulating gene expression in response to nutrient stress.

The DksA regulon

The transcription factor DksA collaborates with (p)ppGpp to globally regulate gene expression (Gourse et al., 2018). DksA targets many of the same genes as Rel_{Bbu}, but also affects a distinct set (Boyle et al., 2019), a phenomenon that has been reported in other bacteria (Potrykus and Cashel, 2008; Dalebroux et al., 2010). Downregulation of genes encoding ribosomal proteins, DNA replication proteins, and transcriptional and translational machinery was DksA-dependent in addition to Rel_{Bbu}-dependent (Bugrysheva et al., 2015; Drecktrah et al., 2015; Boyle et al., 2019). DksA-dependent genes were similarly found throughout the genome and spanned all functional categories, reinforcing the extensive genomic reach of this stringent response-associated transcription factor. As with Rel_{Bbu}, DksA upregulated expression of factors important for persistence in the tick, such as the glycerol metabolic genes *glpF* and *glpK*, the bacterioferritin ortholog gene *dps/napA/bicA*, as well as the tick phase-specific lipoprotein-encoding genes *ospA* and *lp6.6* (Boyle et al.,

2019). Further transcriptional overlap with the Rel_{Bbu} regulon includes the repression of many cp32 genes in the late phage operon and upregulation of a number of genes encoding lipoproteins associated with the vertebrate phase of the enzootic cycle, including *dbpA* and *ospC* (Drecktrah et al., 2015; Boyle et al., 2019).

Interplay between (p)ppGpp and DksA is evident as levels of *dksA* transcript increase in the rel_{Bbu} null mutant (Bugrysheva et al., 2015; Drecktrah et al., 2015) while (p)ppGpp levels are increased in a *dksA* null mutant (Boyle et al., 2019). Thus, the spirochete may be compensating for the lack of one component of the stringent response by increasing the levels of its partner, illustrating the cooperative and complex relationship of (p)ppGpp and DksA in remodeling the transcriptome to adapt to nutrient flux in the dynamic environments of the enzootic cycle.

That both Rel_{Bbu} and DksA upregulate expression of gene products controlled by the RpoN-RpoS σ factor cascade mediating vertebrate phase-specific gene expression and host infectivity was unexpected, and suggests a more intricate role for these factors than merely mediating the response to nutrient limitations. DksA, as well as Rel_{Bbu}, does not alter the level of *rpoS* transcript during nutrient stress, but rather appears to affect RpoS levels post-transcriptionally (Drecktrah et al., 2015; Boyle et al., 2019; Mason et al., 2020), a function that has been previously reported in *E. coli* (Brown et al., 2002). The mechanism for post-transcriptional RpoS regulation in these mutants has not been determined but may be driven, at least in part, by Rel_{Bbu}-upregulation of $DsrA_{Bb}$, an sRNA known to post-transcriptionally increase RpoS levels (Lybecker and Samuels, 2007; Drecktrah et al., 2018). Thus, reduction of *ospC* and *dbpA* transcripts is likely due to the lack of RpoS protein in the DksA mutant and may account for the non-infectious phenotype observed when mice are challenged by needle inoculation (Mason et al., 2020). The finding that the Rel_{Bbu} mutant is infectious indicates a marked divergence of the two regulators that have similar regulons and are thought to work hand-in-hand. Future studies are required to discern the subtle functional differences between Rel_{Bbu} and DksA, and elucidate the induction mechanisms of genes encoding lipoproteins involved in *B. burgdorferi* infectivity as well as the complexities of the stringent response.

Small regulatory RNAs and RNA chaperones

Small non-coding RNAs have been described as the “dark matter of the cell” (Riddihough, 2005) because, despite their plethora, they remained mostly undiscovered in the pre-genomic world. Of course, many of these small non-coding RNAs were known since the early days of molecular biology, such as 6S RNA, the ubiquitous and, in certain situations, predominant global regulator of bacterial transcription whose function was outright mysterious for over 30 years (Wassarman and Storz, 2000). Aside from a few functional small RNAs (sRNAs), such as tmRNA (SsrA), annotated in the genome sequence (Fraser et al., 1997), the first small RNA identified in *B. burgdorferi* was $DsrA_{Bb}$, a post-transcriptional regulator of the RpoN-RpoS alternative σ factor cascade (Lybecker and Samuels, 2007). The constellation of non-coding sRNAs in *B. burgdorferi* exponentially expanded a decade later via high-throughput transcriptome studies (Arnold et al., 2016; Adams et al., 2017; Popitsch et al., 2017; Drecktrah et al., 2018). The bevy of sRNAs was classified based on their genomic

location: intergenic sRNAs, intragenic sRNAs, antisense sRNAs, and 5′ UTR-associated sRNAs (Figure 4) (Popitsch et al., 2017).

Several mechanisms of riboregulation by sRNAs have been defined, including those employed by pathogenic bacteria to modulate virulence strategies (Fröhlich and Vogel, 2009; Papenfort and Vanderpool, 2015; Svensson and Sharma, 2016; Hör et al., 2018; Lejars et al., 2019; Adams and Storz, 2020; González Plaza, 2020). Many sRNAs regulate gene expression via base-pairing with target mRNAs, affecting their stability, translation, transcription, or processing: *trans*-acting sRNAs base-pair with their target mRNAs and *cis*-acting antisense sRNAs are completely complementarity to their cognate mRNA (Waters and Storz, 2009; Storz et al., 2011; Caldelari et al., 2013). Other sRNAs interact with proteins, titrating their levels or otherwise affecting their activities. As of 2020, only three sRNAs have been functionally characterized in *B. burgdorferi*. (i) $DsrA_{Bb}$ is a *trans*-acting sRNA that base-pairs with *rpoS* mRNA regulating its translation (Lybecker and Samuels, 2007). (ii) Bb6S RNA is a protein-interacting sRNA that directly influences σ factor selectivity by RNA polymerase and globally regulates gene expression (Drecktrah et al., 2020). (iii) *ittA* is an sRNA that affects expression of several genes and is required for dissemination in the vertebrate host via an unknown molecular mechanism (Medina-Pérez et al., 2020). To date, there is no experimental evidence in *B. burgdorferi* for riboswitches, *cis*-acting regulatory RNAs usually found in the 5′ UTR of the mRNA they regulate that function by altering their secondary structure in response to binding intracellular metabolites (Roth and Breaker, 2009; Serganov and Nudler, 2013; Sherwood and Henkin, 2016; McCown et al., 2017); however, the class of 5′ UTR-associated sRNAs (Popitsch et al., 2017) may represent heretofore unrecognized riboswitches.

sRNAs

$DsrA_{Bb}$—In *Escherichia coli*, DsrA is one of three regulatory sRNAs that affect levels of the σ factor RpoS (Hengge-Aronis, 2002a; Repoila et al., 2003; Majdalani et al., 2005; Narberhaus et al., 2006; Waters and Storz, 2009). The sRNA disrupts a hairpin stem-loop that blocks access of the ribosome to the Shine-Dalgarno sequence on the *rpoS* mRNA and enhances its translation at low temperature (Hengge-Aronis, 2002a; Repoila et al., 2003). DsrA is considered to be “a thermometer for the RpoS regulon” in *E. coli* (Repoila et al., 2003) because both its transcription and stability increase at low temperature, causing an accumulation of RpoS protein during the cold stress response (Repoila and Gottesman, 2001).

An sRNA was discovered in *B. burgdorferi* that has extensive complementarity to the upstream region of the *rpoS* mRNA, which appears to assume a hairpin stem-loop structure akin to that of *E. coli*, implying a similar mode of regulation (Lybecker and Samuels, 2007). Several experiments suggested that the sRNA functioned in post-transcriptional temperature-mediated regulation of RpoS, so it was called $DsrA_{Bb}$; however, there are substantial differences between the *E. coli* and *B. burgdorferi* DsrA analogs (Lybecker and Samuels, 2007). Notably, $DsrA_{Bb}$ senses an increase in temperature, presumably when warm vertebrate blood enters the ambient feeding tick, while DsrA in *E. coli* responds to a decrease in temperature. In addition, $DsrA_{Bb}$, which is about four times longer than DsrA

from *E. coli*, has more extensive base-pairing potential (31 of 34 nucleotides, or up to 51 of 68 nucleotides, compared to 26 of 38 nucleotides) with its target, the *rpoS* mRNA in *B. burgdorferi* (Lybecker and Samuels, 2007). Furthermore, via an as yet unknown mechanism, $DsrA_{Bb}$ requires translation of an upstream and overlapping open reading frame encoded by *bb0577* (Lybecker and Samuels, 2007). Lastly, the steady-state levels of DsrA in *E. coli* are influenced by temperature, while $DsrA_{Bb}$ is unaffected by temperature (Lybecker and Samuels, 2007) and instead is upregulated by the stringent response (Drecktrah et al., 2018) (see the section above on the Stringent response and (p)ppGpp). Other than the potential crosstalk between the stringent response (which regulates gene expression in response to starvation in the unfed tick midgut) and the RpoN-RpoS alternative σ factor cascade (which activates gene expression required in the vertebrate host and represses gene expression required in the tick vector), almost nothing is known about the temperature-sensing modus operandi of $DsrA_{Bb}$.

$DsrA_{Bb}$ is hypothesized to serve as a molecular thermometer regulating gene expression during the enzootic cycle of *B. burgdorferi* by modulating the RpoN-RpoS σ factor cascade. Yet, the increase in *rpoS* mRNA levels (Caimano et al., 2004) does not require $DsrA_{Bb}$ (Lybecker and Samuels, 2007), which suggests the presence of two independent mechanisms that regulate RpoS. Two *rpoS* mRNAs have been identified (Lybecker and Samuels, 2007; Smith et al., 2007): a short *rpoS* mRNA that is transcribed from an RpoN-dependent promoter (Smith et al., 2007) at high cell density (Lybecker and Samuels, 2007) and a long *rpoS* mRNA that is transcribed from an RpoD-dependent promoter at low cell density (Lybecker and Samuels, 2007). Of note, $DsrA_{Bb}$ stimulates the translation of *rpoS* mRNA exclusively at low cell density in culture (Lybecker and Samuels, 2007). Only the long *rpoS* mRNA contains the sequence complementary to $DsrA_{Bb}$ (Lybecker and Samuels, 2007) and, therefore, $DsrA_{Bb}$ regulates translation of the RpoD-dependent long *rpoS* mRNA, but not translation of the RpoN-dependent short *rpoS* mRNA. The seminal studies (Hübner et al., 2001; Smith et al., 2007) on *rpoS* regulation missed the long *rpoS* mRNA presumably because the spirochetes were grown to a high cell density, culture conditions in which the short *rpoS* mRNA is transcribed (Lybecker and Samuels, 2007). The long *rpoS* transcript has been observed in both an *rpoN* null mutant and in a strain with the RpoN-dependent *rpoS* promoter mutated (Hall et al., 2021). What, then, is the regulatory role of the two *rpoS* mRNA species, and the $DsrA_{Bb}$ sRNA, in the enzootic cycle? The proposed model is that the pathway controlling RpoS and, subsequently, enzootic cycle phase-specific gene expression, bifurcates: the $DsrA_{Bb}$-RpoS branch activates RpoS during transmission from the tick to the vertebrate and the RpoN-RpoS branch maintains RpoS levels, and RpoS-dependent gene expression, in the vertebrate (Hall et al., 2021). This accounts for the apparently discrepant observations that *rpoN* mutants migrate from the midgut to the salivary glands (Fisher et al., 2005), but *rpoS* mutants cannot exit the midgut (Dunham-Ems et al., 2012).

Bb6S RNA—6S RNA, encoded by *ssrS*, directly interacts with RpoD-holoenzyme (E), the RNA polymerase carrying the RpoD ($E\sigma^{70}$) σ factor (Wassarman and Storz, 2000; Cavanagh and Wassarman, 2014; Steuten et al., 2014a; Steuten et al., 2014b; Burenina et al., 2015; Wassarman, 2018), and serves as a global regulator of transcription (Waters and Storz, 2009;

Storz et al., 2011). The primary sequences of 6S RNAs from different bacterial species are not conserved, but rather share a conserved secondary structure that imitates the open promoter complex (Wassarman and Storz, 2000; Barrick et al., 2005; Trotochaud and Wassarman, 2005; Chen et al., 2017), and 6S RNA prevents binding of $E\sigma^{70}$ to RpoD-dependent promoters, resulting in decreased transcription of RpoD-dependent genes and increased transcription of many RpoS-dependent genes (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004, 2006; Cavanagh et al., 2008; Cavanagh et al., 2010; Neusser et al., 2010). In general, the function of 6S RNA is to shift expression from the RpoD-dependent regulon to the RpoS-dependent regulon (Cavanagh et al., 2008). In *E. coli* and other bacteria, 6S RNA has been associated with adaptation to environmental stresses, but 6S RNA also affects bacterial virulence and infectivity (Faucher et al., 2010; Yan et al., 2013; Warrier et al., 2014; Ren et al., 2017).

Bb6S RNA, the canonical 6S RNA in *B. burgdorferi*, was first predicted by computational genome mining (Barrick et al., 2005) and was then found in the noncoding sRNA transcriptomes of *B. burgdorferi* (Arnold et al., 2016; Popitsch et al., 2017; Drecktrah et al., 2018). Recently, Bb6S RNA was shown to bind RNA polymerase *in vivo*, be processed by RNase Y, regulate expression of lipoprotein genes, and play a role in infectivity in the murine model (Drecktrah et al., 2020). Mapping and modeling of Bb6S RNA suggest that the secondary structure is conserved with a single-stranded central bubble surrounded by double-stranded stem regions (Barrick et al., 2005; Trotochaud and Wassarman, 2005; Chen et al., 2017; Drecktrah et al., 2020).

Unlike 6S RNA from *E. coli* and some other bacteria (Wassarman and Storz, 2000; Cavanagh and Wassarman, 2014; Steuten et al., 2014b), steady-state levels of Bb6S RNA were not affected in cultured *B. burgdorferi* by nutrient stress or growth phase (Drecktrah et al., 2020), which is consistent with RNA-seq studies showing temperature and growth phase had no effect on Bb6S RNA levels (Arnold et al., 2016; Popitsch et al., 2017). However, the amount of Bb6S RNA increased as fed larvae molted into nymphs and decreased after nymphs fed, indicating a function for Bb6S RNA during spirochete persistence in the tick vector (Drecktrah et al., 2020). Bb6S RNA also is upregulated by the stringent response (Drecktrah et al., 2018), which facilitates survival of *B. burgdorferi* between the larval and nymph blood meals (Drecktrah et al., 2015) (see section above on the Stringent response and (p)ppGpp). However, Bb6S RNA was not required for spirochetes to persist through the larval and nymph stages (Drecktrah et al., 2020). Note, though, that in other bacteria, the phenotypes of 6S RNA mutants are typically subtle (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004, 2006; Cavanagh et al., 2012; Cavanagh and Wassarman, 2013) and Bb6S RNA was required for full infectivity of mice by needle inoculation, although, the infectivity defect of the Bb6S RNA mutant was overcome by either an increased inoculum or tick transmission (Drecktrah et al., 2020). Bb6S RNA regulates expression of the RpoS-dependent genes *ospC* and *dbpBA* without significantly changing RpoS protein levels, results consistent with Bb6S RNA sequestering $E\sigma^{70}$ and promoting transcription by the RpoS-holoenzyme ($E\sigma^{S}$) as found in model microorganisms (Drecktrah et al., 2020).

ittA

The intergenic sRNA SR0736, encoded between *bbd18* and *bbd21* on lp17, was recently identified as attenuating vertebrate infectivity and dissemination of *B. burgdorferi* by filtering data from a global transposon insertion (Lin et al., 2012) sequencing screen with the output of an sRNA transcriptome (Popitsch et al., 2017). This sRNA was renamed *ittA*, for infectivity-associated and tissue-tropic sRNA locus A (Medina-Pérez et al., 2020). An *ittA* mutant strain differentially expresses 19 genes compared to the wild-type parental strain, including increased levels of *bba66* and *vra*, as well as 11 other mRNAs, and decreased levels of *ospD*, *ospA* and *bba74*/*oms28*, as well as three other mRNAs (Medina-Pérez et al., 2020). The molecular mechanism(s) *ittA* employs to regulate gene expression are currently unknown. Curiously, despite restoring the *in vivo* infectivity phenotype, the *ittA* complement did not rescue the gene expression defect of many of the validated differentially regulated genes.

RNA chaperones

Hfq—Hfq is an RNA chaperone that facilitates the interaction between sRNAs and their targets (Gottesman, 2004; Valentin-Hansen et al., 2004; Majdalani et al., 2005; Brennan and Link, 2007; Waters and Storz, 2009). The Hfq of *B. burgdorferi* was difficult to identify because it has limited homology with the Hfq from *E. coli* and other bacteria, but it is able to heterologously complement an *hfq* mutant of *E. coli* (Lybecker et al., 2010). Conversely, the *E. coli hfq* gene heterologously complements several phenotypes of the pleiotropic *B. burgdorferi hfq* mutant *in vitro*, albeit not the vertebrate infectivity defect (Lybecker et al., 2010). *B. burgdorferi* Hfq is required for infectivity in mice, as well as induction of RpoS and its regulon (Lybecker et al., 2010); therefore, the phenotype is not due solely to a defect in facilitating the $DsrA_{Bb}$ interaction with *rpoS* mRNA, as RpoS can be induced at high cell density in the $dsrA_{Bb}$ mutant (Lybecker and Samuels, 2007), suggesting an Hfq-dependent sRNA network in the spirochete that regulates infectivity (Lybecker et al., 2010).

CsrA—CsrA is an RNA-binding protein that inhibits translation of several genes by binding the ribosome-binding sites of their mRNAs and blocking the ribosome; CsrB and CsrC are sRNAs that bind to and sequester CsrA, inhibiting its activity on mRNAs (Romeo, 1998; Majdalani et al., 2005; Hör et al., 2018; Romeo and Babitzke, 2018). CsrA regulates a variety of physiological processes in *E. coli* and other bacteria, including carbon metabolism and motility (Romeo, 1998; Majdalani et al., 2005; Hör et al., 2018; Romeo and Babitzke, 2018), as well as bacterial infectivity (Lucchetti-Miganeh et al., 2008; Vakulskas et al., 2015). Levels of CsrA in *B. burgdorferi* increase in response to elevated temperature and decreased pH (Sanjuan et al., 2009). Initial reports suggested that CsrA affects the expression of *ospC* as well as other RpoS-dependent genes and is required for murine infectivity in *B. burgdorferi* (Sanjuan et al., 2009; Karna et al., 2011; Sze and Li, 2011; Sze et al., 2011; Karna et al., 2013; Arnold et al., 2018; Lin et al., 2018); however, a subsequent study reported that CsrA does not activate RpoS or its regulon and that *csrA* mutants are infectious in the murine model (Ouyang et al., 2014b). As of 2020, the role of CsrA in the physiology of *B. burgdorferi* remains enigmatic and its RNA targets remain undefined.

RNA turnover and ribonucleases

The steady-state levels of RNAs are controlled not just by the rate of their transcription, but also by the rate of their degradation, which is catalyzed by a variety of ribonucleases (Mohanty and Kushner, 2016; Bechhofer and Deutscher, 2019; Trinquier et al., 2020). Compared to other bacteria, *B. burgdorferi* encodes a sparse quiver of ribonucleases, which includes the endoribonucleases RNase III (BB0705), RNase P (BB0441), RNase Z (BB0755), YbeY (BB0060), RNase Y (BB0504), RNase M5 (BB0626), and RNase HII (BB0046) and the exoribonuclease PNPase (BB0805) (Fraser et al., 1997; Archambault et al., 2013; Anacker et al., 2018; Drecktrah et al., 2020). Lyme disease spirochetes lack detectable orthologs of RNase E/G and RNase J1/J2; RNase Y functions in RNA processing and decay in *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* in an analogous fashion as RNase E or G in *E. coli*, so *B. burgdorferi* appears to be more similar in its suite of ribonucleases to the gram-positive bacteria (Shahbabian et al., 2009; Yao and Bechhofer, 2010; Bechhofer, 2011; Lehnik-Habrink et al., 2011; Marincola et al., 2012; Chen et al., 2013; Bechhofer and Deutscher, 2019). Two ribonucleases have been genetically characterized in *B. burgdorferi*: RNase III, encoded by the *rnc* gene (Anacker et al., 2018) and RNase Y, encoded by the essential *rny* gene (Drecktrah et al., 2020). RNase III, which recognizes and cleaves double-stranded RNA, is required for proper rRNA processing (Anacker et al., 2018) and for mRNA decay, at least for several transcripts (Snow et al., 2020), in the spirochete. RNase Y is involved in the biogenesis of 6S RNA in *B. burgdorferi* (Drecktrah et al., 2020), which has not been shown in any other bacterium, but the ribonuclease surely has additional roles in the cell, likely in the turnover of mRNA based on precedence in other bacteria (Lehnik-Habrink et al., 2011; Durand et al., 2012; Chen et al., 2013), as Bb6S RNA null mutants are viable but *rny* is essential for growth of the spirochete (Drecktrah et al., 2020).

Schlax and colleagues have assayed mRNA turnover kinetics *in vitro* and demonstrated that half-lives vary from a minute to almost an hour, which is considerably longer than the half-lives measured for the majority of mRNAs in most bacteria (Archambault et al., 2013). Note that these studies are more complicated in *B. burgdorferi* because mRNA turnover in bacteria is generally quantified following inhibition of transcription with rifampicin, but the unusual RNA polymerase of *B. burgdorferi* is resistant to this antibiotic, so, instead, actinomycin D is used to arrest transcription (Archambault et al., 2013). The half-lives and steady-state levels of several mRNAs, as well as the steady-state levels of the stable rRNAs, were significantly increased in an *rnc* mutant, indicating a role for RNase III in RNA degradation (Snow et al., 2020). However, the steady-state levels of a couple mRNAs decreased in the *rnc* mutant, suggesting a more nuanced regulatory scheme involving RNase III. At this point, the mechanism by which RNA degradation and RNase activity is regulated to effect post-transcriptional levels of gene expression in *B. burgdorferi* is unknown; however, RNase III is known to regulate the interaction between antisense sRNAs and their target mRNAs in several bacteria, including *E. coli*, *B. subtilis*, *S. aureus*, *S. pyogenes* and *Streptomyces antibioticus* (Lasa et al., 2011; Lasa et al., 2012; Lioliou et al., 2012; Lee et al., 2013; Lybecker et al., 2014a; Lybecker et al., 2014b; Le Rhun et al., 2016), and a similar mechanism may be employed by the spirochete.

Nucleic acid-binding proteins

The genome of *B. burgdorferi* encodes several proteins that resemble known nucleic acid-binding proteins of other bacteria, such as BosR and BadR (see the section above). However, most of those predicted nucleic acid-binding proteins have yet to be characterized. In addition, novel proteins have been identified that do not possess structures previously associated with nucleic acid interactions. Two of the most notable of these borrelial proteins, EbfC and SpoVG, are also encoded by a wide spectrum of other bacterial species. Thus, studies of *B. burgdorferi* are providing insights on the physiology of bacteria ranging from *E. coli* to *S. aureus*.

At least two novel *B. burgdorferi* proteins, SpoVG and BpuR, initially discovered due to their DNA-binding activities, have since been found to have greater affinities for RNA than for DNA. Combined with data from other species, this finding raises the possibility that many other “DNA-binding” proteins might also preferentially bind to RNAs. Additionally, the known activities of BpuR and SpoVG support a paradigm that RNA-binding proteins can have functions beyond serving as RNA chaperones, such as operating solo to directly affect mRNA translation.

EbfC

This 99-amino acid protein forms a homodimer in solution; the alpha helical N- and C-termini of both subunits form a “pincer” that fits over the DNA double helix (Lim et al., 2003; Riley et al., 2009). Consistent with binding by a homodimer, EbfC preferentially interacts with a palindromic sequence, 5′-GTnAC-3′. This sequence occurs approximately every 1.1 kb throughout the *B. burgdorferi* genome. EbfC also forms higher-order oligomers, including tetramers and octamers, an activity that could serve to bridge separate DNA strands (Riley et al., 2009). Microscopic analyses of GFP-tagged EbfC revealed that it associates with the borrelial nucleoid (Jutras et al., 2012a). These are all characteristics of nucleoid-associated proteins, leading to a model that a major function of EbfC is to organize the structure of borrelial DNA. Moreover, the *ebfC* gene is coexpressed with *dnaX*, which encodes a subunit of DNA polymerase, suggesting that DNA-binding by EbfC plays an indirect role in replication (Jutras et al., 2012a). Almost all known species of bacteria also encode an EbfC homolog (variously named YbaB, ORF12, and other terms.), implying a conserved function (Cooley et al., 2009; Wang et al., 2012a).

EbfC initially was identified due to its high affinity binding to the upstream region of *B. burgdorferi erp* operons (Babb et al., 2006). The majority of *erp* operators contain two to three complete EbfC-binding sites, a density that has not been identified elsewhere in the *B. burgdorferi* genome. EbfC acts as an anti-repressor of *erp* transcription by competing with the BpaB repressor for binding to the operator (Jutras et al., 2012b). Erp protein expression is repressed during colonization of an unfed tick, but is induced when the tick begins to feed, and those outer surface lipoproteins are expressed during transmission and throughout the vertebrate phase of the enzootic cycle. Studies of *B. burgdorferi* cultivated *in vitro* found that *erp* transcript levels increase as bacterial replication rates increase, a phenomenon that also occurs as a tick begins to feed and the spirochetes rapidly multiply due to nutrients in the blood (Jutras et al., 2013c). The current model for *erp* regulation posits that the rapid

bacterial replication rate in feeding ticks necessitates DnaX and EbfC for DNA replication, and that *erp* operons have evolved to recognize elevated EbfC levels as a signal that the tick is feeding and the time is appropriate to produce the Erp proteins (Jutras et al., 2012a; Jutras et al., 2013c).

BpaB

All of the plasmids in the segmented genome of *B. burgdorferi* encode a unique BpaB protein. BpaB and two other ORFs are essential for plasmid maintenance and partitioning, with BpaB apparently performing a function comparable to the ParB proteins of other bacterial plasmids. The unique sequences of BpaB from each plasmid appear to be involved with compatibility (Zückert and Meyer, 1996; Casjens et al., 1997; Fraser et al., 1997; Casjens et al., 2000; Stewart et al., 2001; Eggers et al., 2002; Stewart et al., 2003; Brisson et al., 2013). The BpaB proteins of the cp32 family of prophages are the best studied to date. While BpaB presumably binds DNA near the origin of replication, as do ParB proteins from other species, three other binding sites have been identified on cp32s: one each in the *erp* operator, adjacent to the *nucP* promoter, and adjacent to the *ssbP* promoter (Burns et al., 2010; Chenail et al., 2012). In *erp* operators, BpaB binds to a specific sequence 5′ of the promoter, then oligomerizes along the DNA, stabilized by protein-protein interactions (Burns et al., 2010). Occlusion of the promoter by BpaB prevents RNA polymerase binding, thereby inhibiting transcription (Jutras et al., 2012b). BpaB appears to enhance transcription of *nucP* and *ssbP*, through unknown mechanisms (Chenail et al., 2012).

BpuR

BpuR is a homodimer of two 122-amino acid subunits, which fold together into a “PUR” domain (Graebsch et al., 2010; Jutras et al., 2013a). This domain is named because it preferentially binds to purine-rich stretches of nucleic acids. PUR domains are found in a few other bacterial genera, such as *Treponema* and *Bacteroides*, and in all multicellular eukaryotes. This curious distribution suggests that there was an exchange of a PUR motif between Domains over the course of evolution.

PUR domain proteins bind to double-stranded and single-stranded DNAs and to RNA, with BpuR exhibiting its greatest affinity for RNA. A specific nucleotide sequence has yet to be identified for BpuR binding, a feature shared with eukaryotic PUR domain proteins. This suggests that PUR domain proteins interact with higher-order structural determinants.

BpuR was first identified due to its ability to bind DNA, as it has a high affinity for sequences in *erp* operator sites. It acts as a co-repressor of *erp* transcription, enhancing the repressor activity of BpaB through an undefined mechanism. BpuR has also been found to bind several borrelial mRNAs, including *bpuR* itself, where binding to the 5′ end inhibits translation (Jutras et al., 2013d). Comparisons of transcriptomic and proteomic data indicate that BpuR is also likely to affect translation of numerous mRNAs (Jutras et al., 2019).

SpoVG

Homologs of SpoVG are produced by many species of spirochetes and firmicutes. Its name comes from early studies of *Bacillus subtilis*, where *spoVG* mutants are defective in stage V

of sporulation (Rosenbluh et al., 1981). SpoVG proteins do not contain previously known nucleic acid-binding motifs, and their functions were a mystery until investigations of a DNA-binding protein of *B. burgdorferi* resulted in fishing out SpoVG (Jutras et al., 2013b). That initial study further demonstrated that the SpoVG proteins of *Listeria monocytogenes* and *Staphylococcus aureus* also bind to DNA, and mapped nucleic acid-binding specificity to the C-terminal alpha helix. Subsequent studies found that the *L. monocytogenes* and *B. burgdorferi* SpoVG proteins exhibit higher affinities for RNA than for DNA (Burke and Portnoy, 2016; Savage et al., 2018). A defined nucleotide sequence for SpoVG-binding has yet to be identified, suggesting that it interacts with conformational aspects of the nucleic acid sugar-phosphate backbone. *B. burgdorferi spoVG* mutants exhibit pleotropic defects, evidently due to both transcriptional and post-transcriptional regulatory activities (Savage et al., 2018).

Hbb

Integration host factor (IHF) was discovered on the basis of its role in the integration of bacteriophage λ into the *E. coli* chromosome (Drlica and Rouviere-Yaniv, 1987; Friedman, 1988; Nash, 1996). IHF binds to DNA at specific sites and induces bending. The origin of replication on the *E. coli* chromosome contains an IHF-binding site, which is conserved in several bacteria, and IHF is essential for replication (Nash, 1996). Another small DNA-binding protein in *E. coli* is HU, which is homologous to IHF (Drlica and Rouviere-Yaniv, 1987; Schmid, 1990; Nash, 1996). HU also bends DNA, but it lacks sequence specificity. Many bacteria have homologs of both IHF and HU, although several species have only a single homolog. Tilly et al. (1996) isolated a gene encoding an IHF/HU homolog, termed Hbb, from *B. burgdorferi* in a genetic screen based on complementation of the λ DNA packaging defect of *E. coli* IHF/HU mutants. Hbb is most similar to homologs from bacteria that encode a single IHF/HU homolog and is 27 to 30% identical to IHF and HU from *E. coli* (Tilly et al., 1996). Hbb is unable to complement an *E. coli* HU mutant for the defect in bacteriophage Mu growth (Tilly et al., 1996) and it binds DNA in a sequence-specific fashion (Kobryn et al., 2000; Mouw and Rice, 2007), suggesting that Hbb is more like IHF than HU. Kobryn et al. (2000) demonstrated that Hbb binds to a specific sequence that is related to the IHF consensus binding site. In addition, IHF binds to this Hbb-binding site with high affinity and Hbb will bind to an IHF consensus site (Mouw and Rice, 2007). However, Hbb affects DNA topology, which is a property of HU, so Hbb has a substantial non-specific DNA-binding activity and is not a straightforward IHF homolog (Kobryn et al., 2000).

Mouw and Rice (2007) solved the structure of an Hbb-DNA complex. The structure and mechanism of DNA bending is similar to that of IHF and HU proteins. Hbb binds DNA as a homodimer with two β-ribbon “arms” that wrap in the minor groove, with a pair of conserved prolines intercalating between adjacent base pairs (Mouw and Rice, 2007), which results in a DNA bend of more than 180° (Kobryn et al., 2000; Mouw and Rice, 2007). The binding site identified by Kobryn et al. (2000) is near the origin of replication on the chromosome (between *dnaA* and *dnaN*). Thus, Hbb may be involved in replication initiation (Chaconas, 2005), although results in an *E. coli* surrogate system show that recombinant Hbb increases transcription of *oppA2* and *oppA4* promoter fusions (Medrano et al., 2007).

Gac

Knight and Samuels (1999) identified Gac in a biochemical screen for proteins that bound to a sub-telomeric sequence from linear plasmid lp17. Gac has HU-like non-specific DNA-binding activity (Knight and Samuels, 1999; Corbett et al., 2004), but it is a novel protein that completely lacks sequence similarity to HU or other members of the architectural DNA-binding protein family: it has only been identified to date in *B. burgdorferi*. Gac is the C-terminal domain of GyrA, the A subunit of DNA gyrase, and it is produced as an independent protein by a mechanism unprecedented in bacteria. *B. burgdorferi* transcribes two mRNAs carrying *gac* sequences: the larger one encodes the entire *gyrBA* operon that presumably generates DNA gyrase, the essential type II DNA topoisomerase in bacteria that introduces negative DNA supercoiling (Reece and Maxwell, 1991; Corbett and Berger, 2004), and the smaller, more abundant one, encodes Gac. The *gac* mRNA is predicted to be transcribed from its own promoter embedded in the *gyrA* gene (Knight and Samuels, 1999). This unusual architectural DNA-binding protein was termed Gac (for GyrA C-terminal domain), and its gene *gac*, because, although it is translated from a subset of the GyrA ORF, it is a different gene product of a unique genetic locus (Knight et al., 2000). In other words, mutations can disrupt the synthesis of Gac, but have no phenotypic effect on GyrA. In fact, genetic disruption of Gac was accomplished by mutating the start codon and Met 5 codon (to Leu and Ile, respectively) (Knight et al., 2000). Disappointingly, the *gac* mutant had no detectable phenotype. The phenotype was hypothesized to be suppressed by the coumermycin A_1 resistance marker in the upstream *gyrB* gene used to introduce the site-specific mutations (Samuels, 2006). Although reverse genetics has so far failed to reveal the function of Gac in *B. burgdorferi*, other experiments suggest that Gac is an architectural DNA-binding protein with HU activity. HU is essential for replicative transposition of bacteriophage Mu in *E. coli*: generating the Type I complex, an early intermediate, requires HU (Craigie et al., 1985). Gac was able to substitute for HU, albeit not as efficiently, in promoting formation of the Type I complex in an *in vitro* donor-cleavage reaction (Knight and Samuels, 1999). Notably, Gac was not able to substitute in the assay for IHF. In addition, Gac, expressed from its own promoter, was able to complement the defect in Mu growth, as assayed by efficiency of plating, in an *E. coli* HU mutant (Knight and Samuels, 1999). Like HU, IHF, and Hbb, Gac bends DNA, which was demonstrated by FRET measurements (Corbett et al., 2004). However, the mechanism by which Gac bends DNA is completely different than the other architectural DNA-binding proteins that involve a β-sheet in the minor groove. Instead, Gac has a novel structure, determined by Corbett et al. (2004) and termed a β-pinwheel, which is similar to a β-propeller; the DNA wraps around the perimeter of four of the six blades of the pinwheel, resulting in a profound bend. This structure also provided insight into the enzymatic mechanism of DNA gyrase (Corbett et al., 2004), as Gac, and the C-terminal domains of DNA gyrases from other bacteria, impart positive supercoiling on the wrapped DNA, which is converted to negative supercoiling following the strand-passage reaction (Ruthenburg et al., 2005; Huang et al., 2006; Kramlinger and Hiasa, 2006).

DNA supercoiling and *cis*-acting sequences

DNA supercoiling, which is known to regulate gene expression in other bacteria (López-García and Forterre, 2000; Cheung et al., 2003; Rui and Tse-Dinh, 2003; Peter et al., 2004), has been hypothesized to serve as a molecular thermometer for *ospC* expression (Alverson et al., 2003). There are several mechanisms by which DNA supercoiling affects gene expression, including the regulation of promoter recognition by RpoS. *E. coli* RpoS preferentially recognizes promoters on a relaxed template (Kusano et al., 1996) and DNA relaxation in *E. coli* is required for transcription by RNA polymerase holoenzyme containing RpoS during the cellular response to osmotic stress (Bordes et al., 2003). In fact, DNA supercoiling has been proposed to be a post-translational regulator of RpoS activity (Hengge-Aronis, 2002b; Bordes et al., 2003).

In *B. burgdorferi*, *ospC* gene is transcribed from an RpoS-dependent promoter (see the section above on the RpoN-RpoS alternative σ factor cascade), but transcription is repressed early during the infection because OspC elicits a potent immune response (Liang et al., 2002a; Liang et al., 2002b; Crother et al., 2004; Liang et al., 2004; Tilly et al., 2006). Two overlapping inverted repeats (IRs) are present upstream of the promoter (Margolis et al., 1994) that serve as an operator to mediate repression (Xu et al., 2007). The inverted repeats probably either bind a *trans*-acting repressor or alter secondary structure in response to changes in DNA supercoiling, or both. Surprisingly, mutating the inverted repeats has little effect on *ospC* transcription in *trans* from a shuttle vector (Eggers et al., 2004; Yang et al., 2005). Drecktrah et al. (2013) obtained different results using site-directed mutagenesis of the inverted repeats in *cis* on cp26: induction of *ospC* by DNA supercoiling, temperature, and pH required the distal inverted repeat, but not the proximal inverted repeat. In addition, the sequence of the IRs was less important for the regulation of *ospC* expression than the base-pairing potential of the two halves of the IR element (Drecktrah et al., 2013).

Cyclic-di-AMP

Cyclic-di-AMP is a recently identified second messenger in bacteria (Römling, 2008; Corrigan and Gründling, 2013; Commichau et al., 2019). It is synthesized from two molecules of ATP by diadenylate cyclase (DAC), and is hydrolyzed by phosphodiesterases (PDEs). Virtually all organisms identified to date that are endowed with c-di-AMP are gram-positive bacteria. *B. burgdorferi* is one of a few exceptions (Barker et al., 2013; Ye et al., 2014; Savage et al., 2015; Rubin et al., 2018). *B. burgdorferi* encodes a diadenylate cyclase, CdaA (BB0008) (Savage et al., 2015), and a phosphodiesterase, DhhP (BB0619) (Ye et al., 2014). In gram-positive bacteria, genes encoding c-di-AMP phosphodiesterase are often non-essential: mutants lacking phosphodiesterase were readily obtained and those mutants have been key to our understanding of c-di-AMP signaling (Römling, 2008; Corrigan and Gründling, 2013; Commichau et al., 2019). On the other hand, genes encoding c-di-AMP cyclase appear to be essential in most bacteria. In *B. burgdorferi*, the phenotypes of c-di-AMP mutants are unique. Inactivation of *dhhP* in *B. burgdorferi* could only be achieved in the presence of a plasmid encoded inducible *dhhP* gene (Ye et al., 2014). The conditional *dhhP* mutant failed to grow in the absence of the inducer. This mutant had a decreased, not

increased, resistance to β-lactamase antibiotics, which also differs from gram-positive bacteria.

Wild-type *B. burgdorferi* cultured *in vitro* has very low levels of intracellular c-di-AMP (~9 nM), and overproduction of CdaA does not increase c-di-AMP levels (Ye et al., 2014; Savage et al., 2015), suggesting that CdaA may not be active under standard *in vitro* culture conditions. However, this may not be true as inactivation of *dhhP* resulted in over fiftyfold increase of intracellular c-di-AMP levels, suggesting that CdaA is active to produce c-di-AMP in cultured spirochetes. c-di-AMP appears to be involved in several cellular processes in other bacteria, including potassium transport, cell wall and membrane homeostasis, DNA damage repair, sporulation, and osmoregulation (Römling, 2008; Corrigan and Gründling, 2013; Commichau et al., 2019). In *B. burgdorferi*, the essential nature of the *dhhP* mutant strongly indicates that c-di-AMP plays an important role that is yet to be elucidated.

Transcriptomics

Transcriptomics, or genome-wide expression profiling, is a powerful tool that comparatively quantifies RNAs produced from the genome. Methods of transcriptome analyses are continuously evolving, but two of the most commonly used approaches to date are microarrays and RNA-sequencing (RNA-seq) technologies. Microarray-based studies quantify transcript levels of predetermined RNAs, while RNA-seq can identify all expressed RNAs. Microarray-based transcriptomic data from multiple *B. burgdorferi* studies was recently reviewed by Iyer et al. (2016). RNA-seq has more recently been utilized for differential gene expression analysis, and to globally identify transcription start sites (TSSs) and small RNAs (sRNAs).

The vast majority of transcriptomic studies have been performed on spirochetes cultivated *in vitro* under conditions attempting, perhaps not successfully, to mimic either the vertebrate host or tick vector (Table 1). Performing transcriptomics on spirochetes within infected ticks or vertebrate tissues is challenging due to the paucity of organisms present within these host environments. Using an enrichment-based microarray approach, Iyer et al. (2015) identified an *in vivo* transcriptome during acquisition (fed larvae) and transmission (fed nymphs), while Narasimhan et al. (2003) examined *in vivo* gene expression in the central nervous system (CNS) and heart tissues of non-human primates (NHP). Notably, the genome coverage is low in both the tick and NHP transcriptomes, perhaps because the transcriptome is indeed reduced *in vivo* compared to *in vitro*, or because of limitations of the microarray technology to detect low-level transcripts. In addition, differentially-expressed genes may have been missed due to the limited dynamic range of microarray technology. Several groups have taken advantage of a model developed by Akins et al. (1998) in which spirochetes are cultivated within dialysis membrane chambers (DMCs) implanted into the peritoneal cavity of a rat or rabbit to generated large numbers of spirochetes in a mammalian ‘host-adapted’ state. These ‘host-adapted’ organisms undergo many, but not all, of the transcriptomic and proteomic changes associated with vertebrate infection, including repression of tick-phase genes, such as *ospA* and the *glp* operon.

Comparative transcriptome analyses using either microarray or RNA-seq have identified cohorts of genes regulated by specific environmental conditions as well as important regulatory networks in *B. burgdorferi*. Strikingly, there is often limited overlap between published data sets for seemingly identical strains (i.e., wild type) grown under ostensibly the same environmental condition(s). The lack of concordance between different data sets is likely due to a combination of biological and technical factors, including variations in growth media components, phase of growth at the time of harvest, methods used for RNA extraction, cDNA synthesis and library preparation, approach used to identify differentially expressed genes (i.e., microarray or RNA-seq), and data analyses. Population and cell-to-cell heterogeneity likely account for some of the differential gene expression observed in transcriptome studies, which is further confounded by the complex plasmid profiles of *B. burgdorferi*. Plasmid content and the repertoires of plasmid-encoded genes vary between strains. In addition, plasmids frequently are lost during *in vitro* cultivation. Several studies have compared gene expression profiles of different strains of *B. burgdorferi*, revealing strain-specific gene regulation (Ojaimi et al., 2005; Caimano et al., 2019).

Despite the lack of concordance among transcriptome studies in *B. burgdorferi*, several trends have emerged. Genes encoding outer surface proteins induced during transmission and vertebrate infection (OspC-like) often are induced by a temperature upshift, cultivation in DMCs and the RpoN/RpoS alternative σ factor cascade (Revel et al., 2002; Brooks et al., 2003; Ojaimi et al., 2003; Tokarz et al., 2004; Fisher et al., 2005; Hyde et al., 2006; Caimano et al., 2007; Boardman et al., 2008; Livengood et al., 2008; Ouyang et al., 2008; Ouyang et al., 2011; Caimano et al., 2019). Starvation, and the signaling molecules (p)ppGpp and c-di-GMP regulate genes encoding outer surface proteins, chemotactic proteins and transporters and enzymes required to utilize different carbon sources. Strikingly, differential expression of the *glp* operon in many of the transcriptome studies substantiate its role in the tick. This operon encodes three genes, *glpF*, *glpK*, and *glpD*, required for uptake and utilization of glycerol as an alternative carbon source in ticks; *glp*-deficient spirochetes survive at much lower levels during the tick blood meal and intermolt (He et al., 2011; Pappas et al., 2011). The *glp* operon or specific genes in the operon are upregulated by tick-associated regulators Rrp1, $RelA_{Bbu}$, and DksA as well as starvation, while it is repressed by a vertebrate phase-specific environmental cues (temperature upshift, recovery from starvation, co-cultivation with neuroglial cells, and cultivation within DMCs) along with vertebrate phase regulators BosR, BadR and CsrA (Revel et al., 2002; Fisher et al., 2005; Hyde et al., 2006; Livengood et al., 2008; Ouyang et al., 2009b; Rogers et al., 2009; He et al., 2011; Miller et al., 2013; Ouyang et al., 2014b; Bugrysheva et al., 2015; Caimano et al., 2015; Drecktrah et al., 2015; Iyer et al., 2015; Ouyang and Zhou, 2015; Iyer and Schwartz, 2016; Schneider and Rhodes, 2018; Boyle et al., 2019).

Temperature response

Growth at different temperatures is often employed to mimic the environmental change spirochetes undergo as *B. burgdorferi* transitions between the tick vector and vertebrate host (23°C and 34–37°C, respectively). Revel et al. (2002) altered both the pH and temperature of *in vitro B. burgdorferi* cultures to simulate the unfed and fed tick environments (23°C/pH 7.5 and 37°C/pH 6.8, respectively). Ojaimi et al. (2003) and Iyer et al. (2015) also compared

differential gene expression of cells grown at 23°C and 35°C, but did not alter the pH. To simulate nymphal transmission, Tokarz et al. (2004) compared the transcriptomes of spirochetes grown at 35°C in BSK II medium with and without the addition of 6% human blood.

Starvation response

In order to persist in the tick midgut in between blood meals, *B. burgdorferi* must adapt to and survive nutrient deprivation. Three transcriptomic analyses of *B. burgdorferi* cultured in nutrient-limited media provide insight into gene expression during spirochete persistence (Drecktrah et al., 2015; Schneider and Rhodes, 2018; Boyle et al., 2019). Drecktrah et al. (2015) shifted *B. burgdorferi* cultures growing in BSK II with rabbit serum (BSK II + RS) at stationary phase to RPMI without rabbit serum for 6 hours to represent nutrient limitation and starvation conditions in the tick. RPMI lacks many of the nutrients present in BSK II including neopeptone, yeastolate, *N*-acetylglucosamine and bovine serum albumin. To mimic the influx of nutrients that accompanies an incoming blood meal during transmission, cultures were subsequently shifted back to BSK II + RS for 2 hours. Boyle et al. (2019) also examined differential gene expression in response to starvation in RPMI media using microarray analyses instead of RNA-seq.

N-acetylglucosamine (GlcNAc) starvation also has been used to imitate tick overwintering *in vitro* (Schneider and Rhodes, 2018). GlcNAc is required for cell wall synthesis and, once phosphorylated, the sugar can be shuttled into the glycolytic pathway. *B. burgdorferi* is not able to synthesize GlcNAc from glucose but acquires it from the breakdown of chitobiose, a major component of tick cuticle, via the phosphoenolpyruvate-phosphotransferase systems (PEP-PTS) (Tilly et al., 2001; Corona and Schwartz, 2015; Caimano et al., 2016; Troy et al., 2016). Schneider and Rhodes (2018) used RNA-seq to compare the gene expression profiles of wild-type *B. burgdorferi* cultivated in the presence or absence of GlcNAc.

In vivo tick transcriptomics

As previously discussed, *in vivo* transcriptomic analyses are challenging due to the low numbers of spirochetes that can be recovered from the tick vector or infected host tissues. To date, the only genome-wide transcriptomic study of *B. burgdorferi* within ticks compared fed larvae (acquisition) and fed nymphs (transmission). Using an RNA enrichment pre-amplification step prior to microarray analysis, Iyer et al. (2015) detected ~500 transcripts in the fed nymphs and larvae. The transcriptomes of *B. burgdorferi* in both tick life stages were also compared to *B. burgdorferi* grown *in vitro* following temperature-shift or cultivated within DMCs. Hierarchal clustering revealed that the expression profile of temperature-shifted *B. burgdorferi* was more similar to that of fed larvae than fed nymphs, although the *in vivo* transcriptomes were very different than the *in vitro* transcriptome.

Host-adapted transcriptomics

Several studies also sought to demonstrate the transcriptomic expression profile of mammalian host-adapted organisms. Narasimhan et al. (2003) examined *in vivo* gene expression in the central nervous system (CNS) and heart tissues of non-human primates (NHP), which included a comparison between steroid treated and immunocompromised

NHPs. An enrichment technique to selectively amplify specific bacterial transcriptomes was used to enhance detection of spirochetal mRNA in NHP tissues. While the detectable transcriptome was limited (~500 transcripts), the authors identified over 150 differentially expressed genes in the heart compared to CNS tissue. To date, this is the only *in vivo* transcriptome analysis performed using infected vertebrate tissues. However, multiple groups have attempted to recapitulate the tissue environment by cultivating spirochetes within DMCs or by co-cultivating spirochetes with human neuroglial cells (Revel et al., 2002; Brooks et al., 2003; Tokarz et al., 2004; Caimano et al., 2007; Livengood et al., 2008; Iyer et al., 2015; Caimano et al., 2019). Three microarray analyses have directly compared the transcriptomes of host-adapted *B. burgdorferi* within DMCs compared to *in vitro* cultured spirochetes at 37°C. These studies have demonstrated that gene expression varies substantially between the two growth environments (Revel et al., 2002; Brooks et al., 2003; Iyer et al., 2015). Livengood et al. (2008) identified genes related to spirochete-host interactions by comparing the transcriptomes of *B. burgdorferi* co-cultivated with human neuroglial cells to those cultured in cell-free medium.

Regulators of gene expression

The RpoN/RpoS alternative σ factor cascade (see section above) is activated within ticks during the nymphal blood meal resulting in a sea change of gene expression to allow for transmission and vertebrate infection. RpoS, an alternate σ factor, induces expression of genes required for transmission and maintenance of *B. burgdorferi* in the vertebrate host and represses many tick phase-specific genes. Transcription of *rpoS* is induced during the nymphal blood meal by RpoN, as part of a complex that also includes Rrp2 and the Fur family regulator BosR (see sections above on the RpoN/RpoS alternative σ factor cascade and the Transcription factors BosR and BadR).

The BosR regulon has been examined by microarray by two groups (Hyde et al., 2006; Ouyang et al., 2009b). Hyde et al. (2006) compared the transcriptomes of a *bosR* point mutant sensitive to oxidative stress and corresponding complemented strain. Ouyang et al. (2009b) analyzed the BosR-dependent transcriptome by comparing the transcriptomes of a wild-type strain and *bosR* null mutant.

Fisher et al. (2005) was first to define the RpoN- and RpoS-dependent transcriptome. Their comparative transcriptomics revealed a set of genes regulated through the RpoN/RpoS alternative σ factor cascade as well as subsets of genes that are uniquely regulated by either RpoN or RpoS alone. Ouyang et al. (2008) examined the Rrp2-, RpoN- and RpoS-dependent transcriptomes utilizing microarray analyses. They also identified a large number of genes coordinately regulated by all three factors suggesting a hierarchical coordination of Rrp2, RpoN and RpoS. In contrast to the Fisher et al. (2005) study, however, Ouyang et al. (2008) found only a limited number of genes regulated by RpoN or RpoS alone. In the latter study, the Rrp2 regulon contained >100 genes that were differentially expressed outside of the RpoN-RpoS pathway. Another Rrp2-dependent regulon was identified by Boardman et al. (2008).

Caimano et al. (2007) initially described the RpoS regulon from *B. burgdorferi* strain 297 grown *in vitro* at 37°C and in DMCs, by comparing the transcriptomes of wild type and a

rpoS mutant via microarray analysis. The *in vitro* and DMC RpoS regulons overlapped significantly with many genes being induced by RpoS under both growth conditions. However, they also identified a large cohort of genes that were repressed by RpoS only in DMC, demonstrating that mammalian host-specific signals are necessary for RpoS repression. More recently, Caimano et al. (2019) compared the RpoS regulons of two phylogenetically distinct *B. burgdorferi* strains, 297 and B31, with different origins (human and tick, respectively): both *in vitro* temperature-shifted, and DMC-cultivated spirochetes were used for transcriptome analyses. These studies identified a core set of genes that were differentially regulated in both strains *in vitro* and/or in DMCs as well as RpoS-regulated genes that were unique to each strain. Notably, the core set of genes was primarily encoded on the chromosome, while the strain-specific genes were predominately plasmid-borne. Importantly, RpoS-mediated repression of tick phase-specific genes in both strains occurred only in response to mammalian host-specific signals.

Arnold et al. (2018) employed RNA-seq to examine the RpoN and RpoS regulons and, unexpectedly, detected only one differentially expressed transcript in the *rpoS* mutant, and two in the *rpoN* mutant compared to the wild type. The spirochetes were cultured under conditions that do not induce synthesis of RpoS and therefore levels of the alternate σ factor were assumed to be low in the wild-type strain. This study underscores the impact of growth phase and culture conditions on comparative transcriptome analyses.

The two-component system Hk1/Rrp1 appears to function primarily in ticks during transmission and acquisition (see section above on the Hk1/Rrp1 two-component system and c-di-GMP). In fact, *B. burgdorferi* lacking either Hk1 or Rrp1 do not survive within tick midguts during the blood meal (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011; Caimano et al., 2015). The response regulator Rrp1 is a diguanylate cyclase that synthesizes c-di-GMP, a small signaling molecule that regulates many cellular processes in bacteria (Cotter and Stibitz, 2007; Wolfe and Visick, 2008; Hengge, 2009; Römling et al., 2013; Valentini and Filloux, 2019). Three groups have examined the Rrp1 regulon using both microarray and RNA-seq (Rogers et al., 2009; He et al., 2011; Caimano et al., 2015). Rogers et al. (2009) first identified the Rrp1 regulon using microarray technology and a deletion mutant of *rrp1* in a non-infectious *B. burgdorferi* strain (B31 5A13). He et al. (2011) identified Rrp1-dependent differentially expressed transcripts also using microarray technology but in an infectious wild-type strain. The Rrp1 regulon was also examined by RNA-seq (Caimano et al., 2015).

B. burgdorferi also utilizes the stringent response mechanism to sense and adapt to nutrient limitation by adjusting gene expression that modifies their growth and physiology (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Gaca et al., 2015; Hauryliuk et al., 2015; Liu et al., 2015; Steinchen and Bange, 2016; Gourse et al., 2018) (see section above on the Stringent response and (p)ppGpp). The stringent response is mediated by levels of the alarmones, guanosine pentaphosphate and guanosine tetraphosphate, abbreviated (p)ppGpp. In *B. burgdorferi*, (p)ppGpp levels are controlled by Rel_{Bbu}, a bifunctional synthetase/hydrolase containing domains homologous to RelA and SpoT (Concepcion and Nelson, 2003; Bugrysheva et al., 2005; Drecktrah et al., 2015). RelA typically synthesizes (p)ppGpp in response to amino acid starvation, while SpoT induces accumulation of (p)ppGpp in

response to limiting fatty acids, phosphate, carbon, or iron. The Rel_{Bbu} regulon has been characterized by RNA sequencing and microarray analyses (Bugrysheva et al., 2015; Drecktrah et al., 2015). Bugrysheva et al. (2015) used microarray analyses to characterize the Rel_{Bbu} transcriptome from *in vitro* cultivated *B. burgdorferi* at exponential and stationary growth phase. Drecktrah et al. (2015) used RNA sequencing analyses to describe the Rel_{Bbu} regulon at stationary growth phase, starvation conditions and recovery from starvation.

DksA works in conjunction with (p)ppGpp to affect RNA polymerase activity in *E. coli* (Gourse et al., 2018) (see section above on the Stringent response and (p)ppGpp). Boyle et al. (2019) identified the DksA regulon at mid-logarithmic growth phase and under starvation conditions using microarray analyses in *B. burgdorferi*. The *dksA* mutant has a significant increase in (p)ppGpp at both mid-logarithmic and starvation cultivation conditions, which thwarted an attempt to delineate the role of *dksA* changes in gene expression, independent of (p)ppGpp concentration.

Borrelia host adapted regulator (BadR) is a DNA-binding protein that is necessary for *B. burgdorferi* to colonize mice (Miller et al., 2013; Ouyang and Zhou, 2015) (see section above on the Transcription factors BosR and BadR). BadR binds the DNA upstream of both the *rpoS* and *bosR* genes (Miller et al., 2013; Ouyang and Zhou, 2015). Two transcriptomic studies have examined the BadR regulon. Miller et al. (2013) utilized microarray analyses, while Arnold et al. (2018) performed RNA-seq to compare wild-type and mutant *badR* strains.

The function of the carbon storage regulator A (*csrA*) homolog in *B. burgdorferi* remains unknown and there are contradictory reports as to its influence on the RpoS-RpoN σ factor cascade (Sanjuan et al., 2009; Karna et al., 2011; Sze and Li, 2011; Sze et al., 2011; Karna et al., 2013; Ouyang et al., 2014b; Lin et al., 2018) (see section above on Small regulatory RNAs and RNA chaperones). In other organisms, CsrA is an RNA-binding protein that regulates carbon storage, virulence factors, and other cellular processes (Romeo, 1998; Majdalani et al., 2005; Lucchetti-Miganeh et al., 2008; Vakulskas et al., 2015; Hör et al., 2018; Romeo and Babitzke, 2018). Arnold et al. (2018) utilized RNA-seq to assay the CsrA regulon and compared it to the BadR regulon revealing a cohort of genes regulated by both factors.

Differential gene expression on plasmids

Several plasmids appear to be hotbeds of differential gene expression. Genes on lp54 have been implicated in transmission and borrelial survival in the vertebrate host. Strikingly, many genes on lp54 are reported as differentially expressed in nearly all transcriptome studies. Many genes on lp28–2 are upregulated by the RpoN/RpoS alternative σ factor cascade and downregulated by $RelA_{Bbu}$ in response to starvation (Caimano et al., 2007; Boardman et al., 2008; Ouyang et al., 2008; Ouyang et al., 2009b; Drecktrah et al., 2015; Caimano et al., 2019). These data indicate that induction of genes on lp28–2 may be important for transmission and vertebrate survival or repression of these genes may be needed for adaptation to the tick vector. However, lp28–2 is not necessary for the murine infection via needle inoculation (Purser and Norris, 2000). The cp32 family of circular plasmids also harbor many genes that are differentially expressed under several

environmental conditions and by several regulators, including the RpoN/RpoS alternative σ factor cascade, $RelA_{Bbu}$ and Rrp1 (Caimano et al., 2005; Caimano et al., 2007; Boardman et al., 2008; Ouyang et al., 2008; Ouyang et al., 2009b; Rogers et al., 2009; He et al., 2011; Drecktrah et al., 2015; Caimano et al., 2019). Genes on lp36 are downregulated by RpoS in the DMC and are primarily upregulated by $RelA_{Bbu}$, suggesting phase-specific expression may be important as spirochetes traverse their enzootic cycle (Caimano et al., 2007; Drecktrah et al., 2015; Caimano et al., 2019). Jewett et al. (2007) reported that the lp36 plasmid is not required for survival in the tick but does play a role in mammalian infectivity. As discussed above, the complex plasmid profiles of *B. burgdorferi* pose a significant challenge to identifying bona fide differentially expressed genes harbored on plasmids. In comparative transcriptomic analyses, if all genes on a plasmid are either upregulated or downregulated, then the cause may be plasmid loss in one of the strains.

Identification of sRNAs

The advent of RNA-seq allowed researchers to globally identify sRNAs in *B. burgdorferi* (Arnold et al., 2016; Popitsch et al., 2017) (see section above on Small regulatory RNAs and RNA chaperones). Popitsch et al. (2017) size-selected for sRNAs 50–500 nucleotides in length, and sequenced the sRNA transcriptome from *B. burgdorferi* cultivated *in vitro* at 23°C and after a temperature shift to 37°C, identifying 1005 sRNAs. Arnold et al. (2016) sequenced total RNA from three phases of growth *in vitro* at 37°C and found putative sRNAs from short predicted transcriptional units (<425 nucleotide in length) in non-coding regions of the genome, or antisense to annotated protein coding genes. The Arnold et al. (2016) data set identified 351 sRNAs, but the type of RNA-seq and subsequent analyses do not allow for the identification of sRNAs that overlap an annotated open reading frame. As such, the data set excludes intragenic sRNAs and 5′ UTR-associated sRNAs. In addition, the size selection was performed *in silico* and included predicted transcriptional units. If the intragenic sRNAs and 5′ UTR-associated sRNAs are excluded, then the Popitsch et al. (2017) data set identifies 550 intergenic sRNAs and antisense sRNAs, similar to the findings of Arnold et al. (2016). Further comparison and overlap of the two data sets is difficult due to differences in the sRNA annotations and reference annotations for the genes. In addition, the spirochetes were cultivated in different media and at different growth phases, as well as the two studies used different cDNA library types, construction, and analyses.

The Rel_{Bbu}-dependent sRNA transcriptome revealed that the stringent response regulates the expression of a large number of sRNAs (Drecktrah et al., 2018). Approximately one third of the annotated sRNAs, sans the intragenic sRNA class, were differentially expressed during starvation of *in vitro* cultivated *B. burgdorferi*. 187 sRNAs are upregulated by Rel_{Bbu}, while 54 are downregulated. The non-coding RNA (ncRNA) regulons of CsrA and BadR were also identified using the predicted ncRNAs annotations from Arnold et al. (2016). Antisense RNAs associated with *glpF* and *rpoN* were differentially regulated by both regulators.

Identification of transcriptional start sites (TSSs) and 5′ ends

5′ end-specific RNA-sequencing (5′ RNA-seq) can globally identify the 5′ ends and transcriptional start sites (TSSs) of transcripts. This technique exploits some of the biochemical properties of transcription (Adams et al., 2017), namely that the first nucleotide

transcribed in an RNA contains a triphosphate at the 5′ end, while processed or degraded 5′ ends of RNAs contain a monophosphate or a hydroxyl group. Adams et al. (2017) used 5′ RNA-seq on *in vitro* cultivated *B. burgdorferi* and identified 6042 TSSs. 63% of the TSSs were termed 'internal', denoting that the promoter and 5′ end is within an annotated gene in the same orientation, while 13% were antisense TSSs with the 5′ ends of RNAs encoded opposite to annotated genes. 22% of the TSSs were within 300 nucleotides of the start codon of an annotated open reading frame. Arnold et al. (2016) predicted 5′ ends of transcripts by analyzing total RNA sequencing data from *B. burgdorferi* cultivated *in vitro* at three different growth phases. A custom script was used to determine putative transcriptional units and their transcript boundaries. 600 to 800 putative 5′ ends were reported in each of the growth phases examined. The predicted 5′ ends identified could be associated with TSSs or processed and/or degraded RNAs.

Future perspectives

Transcriptomic studies have significantly contributed toward understanding global gene regulation in *B. burgdorferi*. The accumulated data from studies performed over the last twenty years are laudable, but many challenges still remain to more accurately understand global gene expression during the enzootic cycle of *B. burgdorferi*. More robust *in vivo* transcriptome analyses with higher genomic coverage in both the tick victor and the vertebrate host would paint a more accurate big picture of the changes in gene expression. Specifically, tick tissue-specific (salivary gland and midguts) and mouse tissue-specific (joint, heart, bladder, and others) transcriptomes will shed light on gene expression patterns necessary for *B. burgdorferi* to transmit and persist in the tick and vertebrate. RNA-sequencing technologies continue to evolve with increased sensitivity and coverage yields from significantly less input RNA. Single-cell RNA-seq is an advancing technology that will uncover cell-to-cell heterogeneity in populations of spirochetes that may determine infection and persistence outcomes. Dual RNA-seq analyzes host or vector transcriptomes simultaneously with the bacterial transcriptome to reveal correlations between bacterial and host-specific responses in gene expression. RNA-seq can be coupled to other biochemical approaches to identify RNA targets of RNA-binding proteins (via CLIP-seq, RIP-seq, RIL-seq, and CLASH and Grad-seq), novel ribo-regulators (via Term-seq) and functional antisense RNAs (via dsRNA-seq) (Lybecker et al., 2014b; Lybecker and Samuels, 2017; Saliba et al., 2017). Using and adapting these '-seq' methodologies to *in vitro* and *in vivo* models of infection should rapidly advance our understanding of global gene expression patterns in the Lyme disease spirochete.

Concluding remarks

Gene expression is regulated in *B. burgdorferi* and related species as the spirochetes traverse their natural enzootic cycles, which requires acquisition by a naïve tick from an infected vertebrate, adaptation to and persistence in the tick, transmission from an infected tick to a naïve host, and adaptation to and survival in the vertebrate. Many of the molecular mechanisms controlling gene expression as the spirochete transitions between and adapts to these phases of the enzootic cycle have been experimentally uncovered and genetically dissected, albeit the majority of investigative approaches are restricted to cultured organisms

or an emulation of the genuine environments *B. burgdorferi* experiences. The spirochete employs three regulatory systems to control gene expression, which include a sequential cascade of a pair of alternative σ factors, a couple of two-component systems, and, at the most recent count, three purine second messengers. A trio of transcription factors and three sRNAs are known to activate or fine-tune gene expression at the transcriptional and post-transcriptional levels. High-throughput transcriptomic approaches have identified the genes and defined the phase-specific regulons that are activated or repressed by environmental signals, although their precise roles have yet to be fully elucidated. Transcriptomic studies have revealed a surprising swath of previously unrecognized small RNAs, the vast majority of which have no known function, but are likely responsible for fine-tuning the expression of specific genes throughout the enzootic cycle of the spirochete. Many of the gene products controlled by the RpoN-RpoS σ factor cascade and the Hk1-Rrp1 two-component system function to move the spirochete between the vector and host or to facilitate survival by altering the metabolism of the spirochete or its surface that interfaces directly with the vector or host; however, many of these regulated genes have no homology to genes outside of the deeply branching spirochete phylum (or even genus), so their physiological functions remain a mystery. *B. burgdorferi* is renowned for doing things its own way, but, curiously, the spirochete wields the canonical bacterial response to nutrient deprivation, the stringent response, to survive in the tick between blood meals, which entails shifting the expression of genes encoding various metabolic functions, carbohydrate utilization and surface lipoproteins. Our hope is that this review has shed light into the current state-of-the-art and built a conceptual framework for continuing studies on gene regulation and transcriptomics in the Lyme disease spirochetes.

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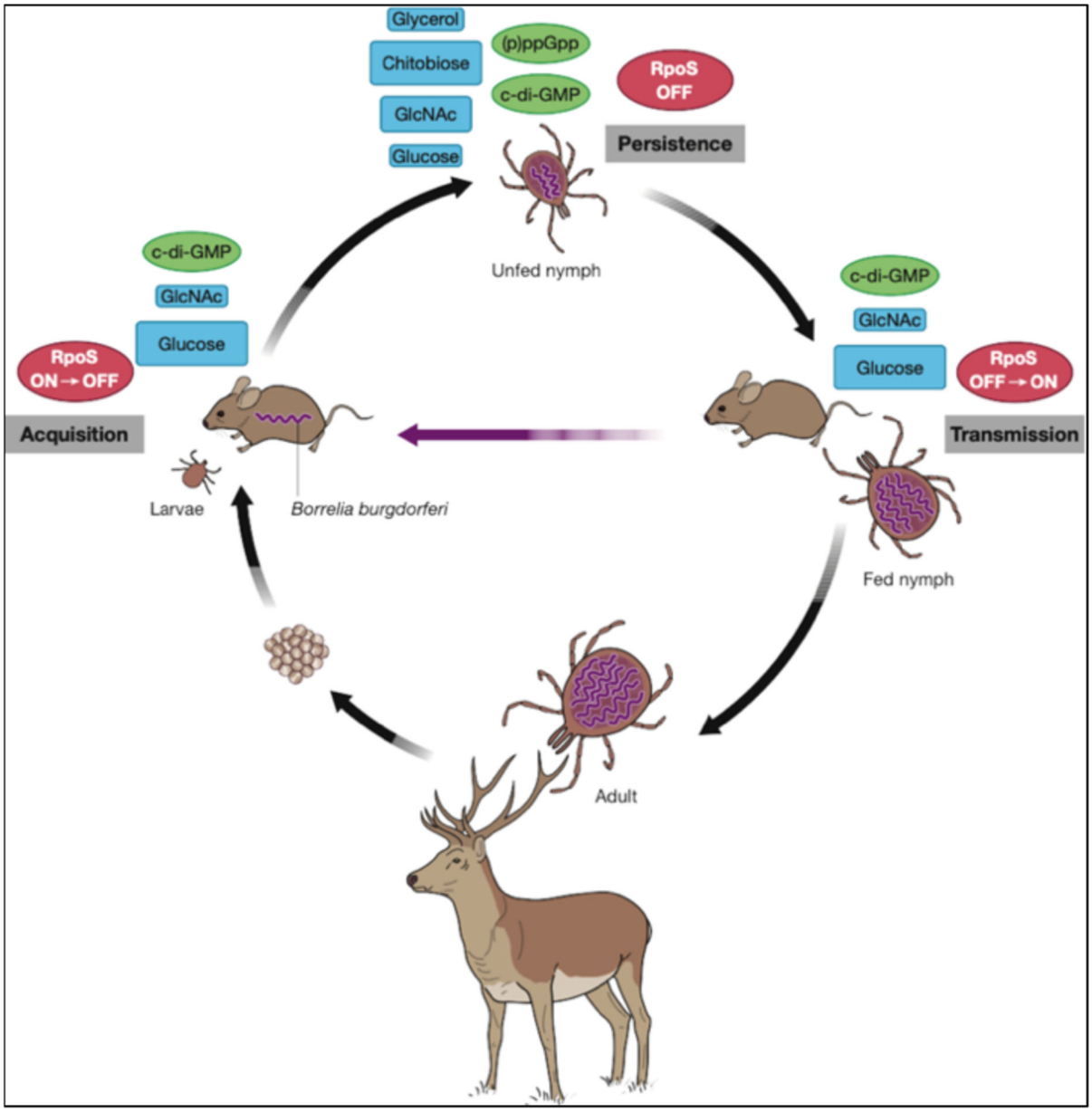

Figure 1.

The enzootic cycle of *B. burgdorferi* showing milestones indicating the action of gene regulatory systems. Acquisition: larval ticks acquire *B. burgdorferi* by feeding on an infected vertebrate. Persistence: intracellular second messengers (p)ppGpp and c-di-GMP regulate persistence in the tick by various mechanisms, including control of genes encoding carbohydrate utilization, while the molecular gatekeeper RpoS is absent in unfed ticks. Transmission: following the molt into nymphs, infected ticks can transmit *B. burgdorferi* to uninfected vertebrates, completing the cycle. Reprinted with permission from Caimano et al. (2016). GlcNAc, *N*-acetyl glucosamine.

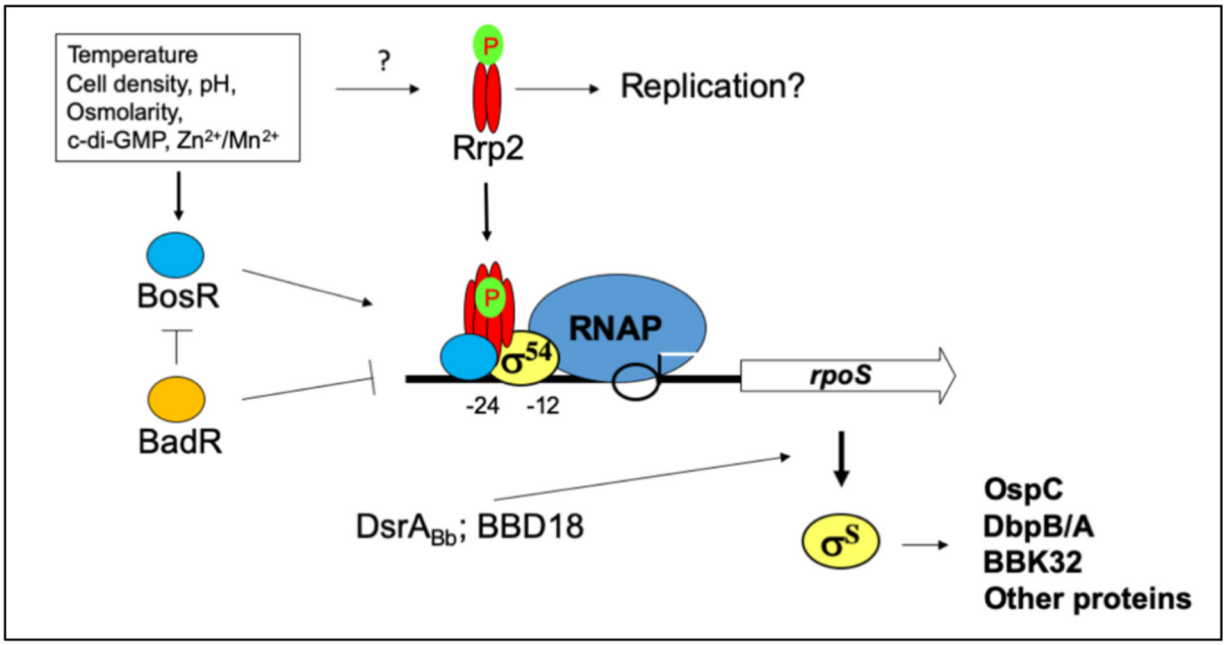

Figure 2.

A model of the RpoN-RpoS σ factor cascade. During tick feeding and mammalian infection, environmental and host signals activate the RpoN-RpoS σ factor cascade. Activation of RpoN requires phosphorylation of Rrp2 and accumulation of BosR. Rrp2 is the sole prokaryotic enhancer-binding protein present in *B. burgdorferi* that is required for RpoN (σ^{54}) activation. Phosphorylation of Rrp2 not only is required for RpoN-RpoS activation, but also is indispensable for cell survival, presumably replication. Levels of BosR respond to environmental signals and accumulation of BosR activates *rpoS* at its RpoN-dependent promoter via an unknown mechanism. BadR represses *rpoS* transcription by directly binding near the RpoN-dependent promoter region. In addition to the major *rpoS* mRNA species transcribed from the RpoN-dependent promoter, a longer *rpoS* transcript is produced at low cell density from an RpoN-independent promoter located within the upstream *flgJ* gene. The sRNA $DsrA_{Bb}$ regulates the efficiency of long *rpoS* mRNA species translation in response to temperature. DDB18 can regulate RpoS (σ^{S}) levels at the post-transcriptional level. Accumulation of *rpoS* transcript leads to the production of OspC, DbpA, DbpB, BBK32, and other mammalian infection-associated proteins.

Figure 3.

Alignment of BosR and Fur proteins using Clustal Omega. Bs, *B. subtilis*; Ec, *E. coli*; Vc, *V. cholera*; Cj, *C. jejuni*; Hp, *H. pylori*. The residues constituting the S1, S2 and S3 metal binding sites are indicated in red, green, and blue, respectively. Residues potentially important for DNA binding are indicated in purple.

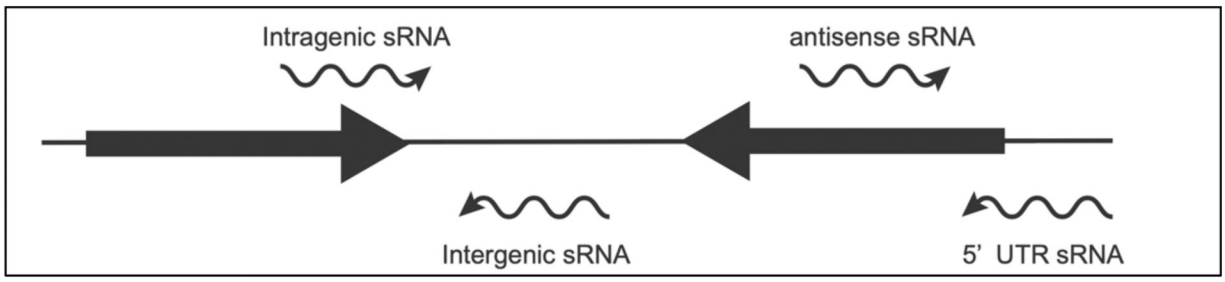

Figure 4.
Classification of sRNAs based on their genomic location. sRNAs (black wavy arrows) were categorized based on their relation to annotated open reading frames (black solid arrows).

Table 1.

Transcriptome studies in *B. burgdorferi*.

Experimental Condition	Strain	Platform	Reference
Increased temperature	B31	Membrane microarray	Ojaimi et al., Infect. Immun., 2003
Comparative analysis of clinical isolates	B31	Membrane microarray	Ojaimi et al., Infect. Immun., 2005
Temperature-shift *in vitro* vs. host adapted DMC	B31	Glass slide microarray	Revel et al., PNAS, 2002
In vitro vs. host adapted DMC	B31	Membrane microarray	Brooks et al. Infect. Immun., 2003
Host mimic: Influence of whole blood *in vitro*	B31	Membrane microarray	Tokarz et al., Infect. Immun., 2004
Monoclonal OspB antibody co-cultivation	B31	Membrane microarray	Anderton et al., Infect. Immun., 2004
Neurological cell co-cultivation	B31	Affymetrix slide microarray	Livengood et al., Infect. Immun., 2008
RpoS regulon (*in vitro* vs. DMC)	297	70 m oligo glass slide microarray	Caimano et al., Mol. Microbiol., 2007
RpoS regulon (*in vitro* vs. DMC)	B31, 297	Illumina RNA sequencing	Caimano et al., Front Microbiol., 2019
RpoN/RpoS regulon (in *vitro*)	B31	70 m oligo glass slide microarray	Fisher et al., PNAS, 2005
BosR regulon (*in vitro*)	B31	Membrane microarray	Hyde et al., Microbiology, 2006
BosR regulon (*in vitro*)	B31	70 m oligo glass slide microarray	Ouyang et al., Mol. Microbiol., 2009
Rrp2 regulon (*in vilro*)	B31	70 m oligo glass slide microarray	Boardman et al., Infect. Immun.,2008
Rrp2/RpoN/RpoS regulon (*in vitro*)	B31	70 m oligo glass slide microarray	Ouyang et al., Microbiology, 2008
CsrA, BadR, RpoN and RpoS	B31	Illumina RNA sequencing	Arnold et al., PLOS One, 2018
Rrp1 regulon (*in vilro*)	B31	70 m oligo glass slide microarray	Rogers et al. Mol. Microbiol., 2009
Rrp1 regulon (*in vilro*)	B31	70 m oligo glass slide microarray	He et al., PLOS Pathog., 2011
Rrp1 regulon (*in vitro*)	B31	Illumina RNA sequencing	Caimano et al., Infect. Immun., 2015
Rel_{Bbu} regulon (*in vitro*)	297	70 m oligo glass slide microarray	Bugrysheva et al., PLOS One, 2015
BadR regulon (*in vitro*)	B31	Nimblegen	Miller et al., Mol. Microbiol., 2013
HrpA regulon (*in vitro*)	B31	Nimblegen	Salman-Dilgimen et al., PLOS Pathog., 2013
Non-human primate tissues	N40, JD1	Glass slide microarray	Narasimhan et al., PNAS, 2003
Fed nymphs	N40	Glass slide microarray	Narasimhan et al., J. Bacteriol., 2002
Infected murine tissues	B31	70 m oligo glass slide microarray	Pal et al., J. Infect. Dis., 2008
Fed larvae and nymphs vs DMCs and *in vitro*	B31	70 m oligo glass slide microarray	Iyer et al., Mol. Microbiol., 2015
GlcNAc starvation	B31	Illumina RNA sequencing	Schneider et al., FEMS Microbiol Lett., 2018
Global 5' end identification	B31	Illumina RNA sequencing	Adams et al., Nucleic Acid Res, 2016
In vitro growth phases	B31	Illumina RNA sequencing	Arnold et al., PLOS One, 2016
Temperature response small RNA regulon	B31	Illumina RNA sequencing	Popitsch et al., BMC genomics, 2016
DNA methylation	B31	Illumina RNA sequencing	Casselli et al., J. Bacteriol., 2018
Rel_{Bbu} regulon and starvation	B31	Illumina RNA sequencing	Drecktrah et al., PLOS Pathogens, 2015
Rel_{Bbu} small RNA regulon	B31	Illumina RNA sequencing	Drecktrah et al., Front. Cell. Infect. Microbiol., 2018
Doxycycline treatment	B31	Ion Torrent RNA sequencing	Caskey et al., Front Microbiol., 2019
EbfC regulon	B31	Illumina RNA sequencing	Jutras et al., J. Bacteriol., 2012
Outer membrane vesicle transcriptome	B31	Illumina RNA sequencing	Malge et al., FEMS Microbiol. Lett., 2018
ittA regulon	B31	Illumina RNA sequencing	Medina et al., PLOS Pathogens, 2020
DksA and starvation regulon	B31	Affymetrix slide microarray	Boyle et al., J. Bacteriol., 2019