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ppGpp Conjures Bacterial Virulence

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INTRODUCTION

To monitor and adapt to their environment, bacteria rely on sensory systems to regulate complex physiological processes. For example, bacteria readily modify their stress tolerance and nutrient utilization pathways in response to local cues. Like all microbes, the goal of every pathogen is to survive and replicate. However, to overcome the formidable defenses of their hosts, pathogens are also endowed with traits commonly associated with virulence, such as surface attachment, cell or tissue invasion, and transmission. A wide variety of pathogens couple their specific virulence pathways with more general adaptations, like stress resistance, by integrating dedicated regulators with global signaling networks, including those critical for carbon and nitrogen metabolism (75, 158). Many of nature's most dreaded bacteria rely on nucleotide alarmones to cue metabolic disturbances and coordinate survival and virulence programs.

Over 40 years ago, Cashel and Gallant first visualized guanosine 5′-diphosphate-3′-diphosphate (ppGpp) and guanosine 5′-triphosphate-3′-diphosphate (pppGpp; collectively referred to as ppGpp) by performing two-dimensional thin-layer chromatography of radiolabeled nucleotides from amino acid-starved *Escherichia coli* cells. The appearance of these "magic spots," synthesized from GDP, or GTP, by pyrophosphoryl transfer from ATP, correlated with the cessation of rRNA synthesis, a process referred to as the stringent response (160). Subsequent research established that bacterial and plant cells that are experiencing nutritional stress synthesize ppGpp to initiate global physiological changes. Although new roles for ppGpp continue to be discovered, the alarmone generally functions to promote the adaptation and resilience of bacterial cells faced with adversity.

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TABLE 1. Stringent response components that contribute to virulence

Pathogen	Stringent response machinery	Pathogenesis-related phenotype(s) associated with ppGpp	Reference(s)
Gammaproteobacteria			
EHEC	RelA, SpoT,[a] DksA	Adherence	143
UPEC	RelA, SpoT,[a] DksA	Adherence	1–3
P. aeruginosa	RelA, SpoT,[a] DksA	Quorum sensing, biofilms, antibiotic tolerance	18, 24, 27, 63, 202, 205
Y. pestis	RelA, SpoT,[a] DksA[d]	Bubonic infection, lung dissemination	190
V. cholerae	RelA, SpoT,[a] RelV, DksA[d]	Mouse colonization	56, 57, 74, 84, 179
E. carotovora subsp. *atroseptica*[b]	RelA, SpoT,[a,d] DksA[d]	Rot in potato tubers	207
S. flexneri	RelA,[d] SpoT,[d] DksA	Intercellular spread	133, 175
S. enterica serovar Typhimurium	RelA, SpoT,[a] DksA	Invasion (SPI1 dependent), intracellular replication (SPI2 dependent)	159, 182, 197, 200, 210, 223
S. enterica serovar Gallinarum	RelA, SpoT,[a] DksA[d]	Invasion (SPI1 independent), intracellular replication (SPI2 dependent)	97
L. pneumophila	RelA, SpoT,[a] DksA	Macrophage transmission	53, 81
F. tularensis	RelA, SpoT,[a] DksA[d]	Phagosome escape in macrophages	43
Actinobacteria			
M. tuberculosis	Rel_{Mtb},[a] CarD	Persistence in mice	52, 185
Firmicutes			
L. monocytogenes	RelA,[a] RelQ,[d] RelP[d]	Adherence, intracellular survival	19, 118, 149, 196
S. aureus	RelA,[a] RelQ,[d] RelP[d]	Essential for viability *in vitro*	44, 71, 114
B. anthracis	RelA,[a] RelQ,[d] RelP[d]	Sporulation	114, 204
C. difficile	RelA[a]	Antibiotic tolerance	62
E. faecalis	RelA,[a] RelQ	Antibiotic tolerance, virulence in *C. elegans*	4, 218
S. pyogenes	RelA,[a] RelQ,[d] RelP[d]		186, 187
S. pneumoniae	Rel_{Spn},[a] RelQ	Pulmonary infection of mice	17
S. mutans	RelA,[a] RelQ, RelP	Biofilms	114–116, 145
Alphaproteobacteria			
Brucella sp.	Rsh, DksA[d]	Macrophage survival, persistence in mice	59, 104
R. etli[c]	RelA,[a] DksA[d]	Nodule formation and nitrogen fixation	25, 39, 137
S. meliloti[c]	RelA,[a] DksA[d]	Nodule formation	211, 213, 214
A. tumefaciens[b]	RelA,[a] DksA[d]	Ti plasmid transfer	206, 221
Epsilonproteobacteria			
C. jejuni	SpoT,[a] DksA	Adherence, invasion, intracellular survival	70, 188, 220
H. pylori	SpoT[a]	Macrophage survival	138, 212, 224
Spirochetes			
B. burgdorferi	SpoT or Rel_{Bbu}[a]	Virulence in mice	34–36

[a] Bifunctional synthetase/hydrolase.
[b] Plant pathogen.
[c] Plant symbiont.
[d] Protein encoded by the species but yet to be studied.

In the heterogeneous environments within mammalian and plant hosts, pathogenic bacteria alter their metabolism and protein repertoire in response to local conditions. Changes in the nutrient supply, alterations in immune responses, or contact with new surfaces can trigger bacterial adaptation. To gain an advantage, pathogenic bacteria may activate specialized secretion systems, motility organelles, or adhesins. Such virulence factors promote survival by equipping microbes to access nutrients, modulate the host cell biology or immune system, or migrate to more favorable locales. In response to local conditions, pathogens utilize dedicated regulators to change their tactics. The expression and activity of many virulence regulators are integrated into a global response mediated by ppGpp, thereby coupling pathogenesis to metabolic status (Table 1). As such, control over cellular ppGpp pools is critical for pathogen survival, replication, and transmission.

METABOLISM OF ppGpp

Levels of ppGpp are modulated by two classes of enzymes: monofunctional synthetase-only enzymes and bifunctional synthetase/hydrolase enzymes (Fig. 1). As the nomenclature of the bifunctional enzymes is not standardized, they can be referred to by their *E. coli* namesakes, RelA (monofunctional) or SpoT (bifunctional), or as RSH (RelA/SpoT homologue) proteins (26, 124, 160, 184). Both the monofunctional RelA- and bifunctional SpoT-like enzymes synthesize ppGpp from either GDP (in the case of guanosine tetraphosphate) or GTP (in the case of guanosine pentaphosphate) and ATP, whereas only the bifunctional enzymes also hydrolyze ppGpp to GDP and pyrophosphate (PP_i) and pppGpp to GTP and PP_i. Most Gram-negative gammaproteobacteria, like *E. coli*, *Salmonella*, *Pseudomonas*, and *Legionella*, encode both RelA and SpoT. Without SpoT, bacteria cannot degrade RelA-derived

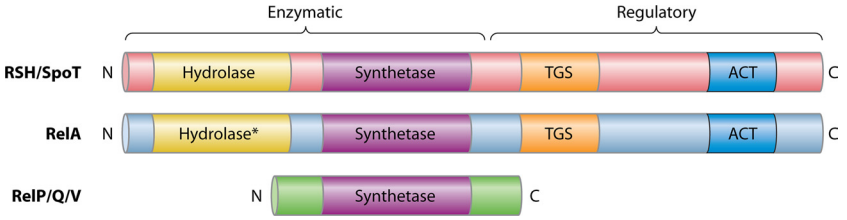

FIG. 1. Domain structure of the enzymes that modulate bacterial pools of ppGpp. Four functional regions have been identified: the ppGpp synthetase domain, the ppGpp hydrolase domain, and the TGS and ACT regulatory domains. The bifunctional SpoT and RSH (RelA/SpoT homologue) proteins contain both synthetase and hydrolase activities in an N-terminal enzymatic domain. RelA proteins behave as monofunctional synthetases. The RelA and SpoT proteins of *E. coli* share 31% amino acid identity, and amino acid divergence renders the hydrolase domain inactive (*). The activity of the SpoT/RSH and RelA proteins is controlled through their TGS and ACT domains. RelP, RelQ, and RelV, referred to as small alarmone synthases (SASs), are monofunctional enzymes with little similarity to RelA or each other at the amino acid sequence level. For example, the *S. mutans* RelP and RelQ proteins share 8% identity with the synthetase domain of *E. coli* RelA and 29% identity with each other (109).

ppGpp, and the unabated accumulation of the nucleotide disrupts cell cycle control. In these species, SpoT function can be studied in the context of *relA spoT* double mutants (often annotated as $ppGpp^0$ cells), which lack all synthetase activity. Many other pathogenic species encode ppGpp synthetase pathways distinct from the two-enzyme RelA/SpoT paradigm. For example, mycobacteria, alphaproteobacterial *Brucella* spp., and epsilonproteobacteria each encode a single bifunctional RSH protein (annotated Rel, RSH, and SpoT, respectively), whereas several Gram-positive *Firmicutes*, such as *Bacillus*, *Listeria*, *Streptococcus*, and *Enterococcus*, encode not only a single bifunctional RSH protein (alternately termed Rel or RelA) but also other small RelA-like synthetase fragments (termed RelP and RelQ) (Fig. 1). Similarly, the gammaproteobacterium *Vibrio cholerae* encodes RelV, another truncated synthetase enzyme (57). The existence of multiple enzymes devoted to alarmone synthesis and hydrolysis illustrates that bacteria have evolved versatile mechanisms to control ppGpp levels.

By regulating the enzyme activities that control the synthesis and degradation of ppGpp, bacteria can coordinate global physiological transformations tailored to distinct metabolic stimuli. It has long been established that the synthetase activity of *E. coli* RelA is elicited at the ribosome by uncharged tRNAs that accumulate during amino acid starvation (160). Likewise, the bifunctional RSH proteins of bacteria harboring only one ppGpp synthetase are activated by amino acid starvation.

In contrast, for bacteria that also encode RelA, their bifunctional SpoT enzymes respond to a variety of stimuli, including phosphate, carbon, and iron starvation, as well as perturbations in fatty acid metabolism (61, 160). The ability of SpoT to respond to fatty acid biosynthesis inhibition is mediated by acyl carrier protein (ACP) (Fig. 2). Although the mechanistic details remain to be discovered, this physical interaction is influenced by the ratio of unacylated to acylated ACP, enabling bacteria to sense the fatty acid biosynthetic capacity of the cell (160). Direct physical interactions between SpoT and ACP have been demonstrated for the *E. coli* and *Pseudomonas aeruginosa* proteins, and genetic evidence suggests that a similar interaction occurs in *Legionella pneumophila* (17, 53). SpoT-dependent responses can reflect changes in either synthetase or hydrolase activity; thus, the catalytic balance of the bifunctional enzymes may be a critical point of control. Fatty acid and carbon starvation each exert allosteric effects on the monofunctional RelV of *V. cholerae*, raising the interesting possibility that other monofunctional stringent response enzymes also sense stresses other than amino acid starvation (57). In addition to nutritional cues, enzymes that govern ppGpp metabolism can be regulated both transcriptionally and post-translationally when pathogens encounter stress during transmission or infection, such as high osmolarity, extreme pH, or chemical onslaughts (4, 149, 212).

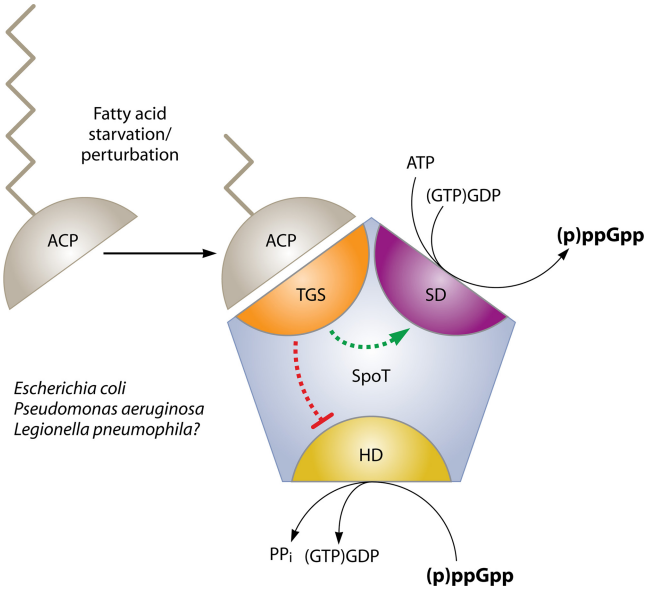

FIG. 2. SpoT activity is regulated through ACP interactions. Gammaproteobacteria that encode both RelA and SpoT have evolved a SpoT-dependent stringent response to fatty acid starvation that is mediated by an interaction between SpoT and ACP. ACP transfers fatty acyl chains to enzymes devoted to phospholipid and secondary metabolite biosynthesis. SpoT interacts with functional acyl-bound ACP at a nonenzymatic region known as the TGS domain. During fatty acid starvation, metabolic signals are transduced through an ACP-SpoT interaction, resulting in an increase in cellular ppGpp pools. It remains to be determined whether, in response to fatty acid stress, the ACP-SpoT interaction specifically modulates the synthetase (SD) or hydrolase (HD) activity of SpoT.

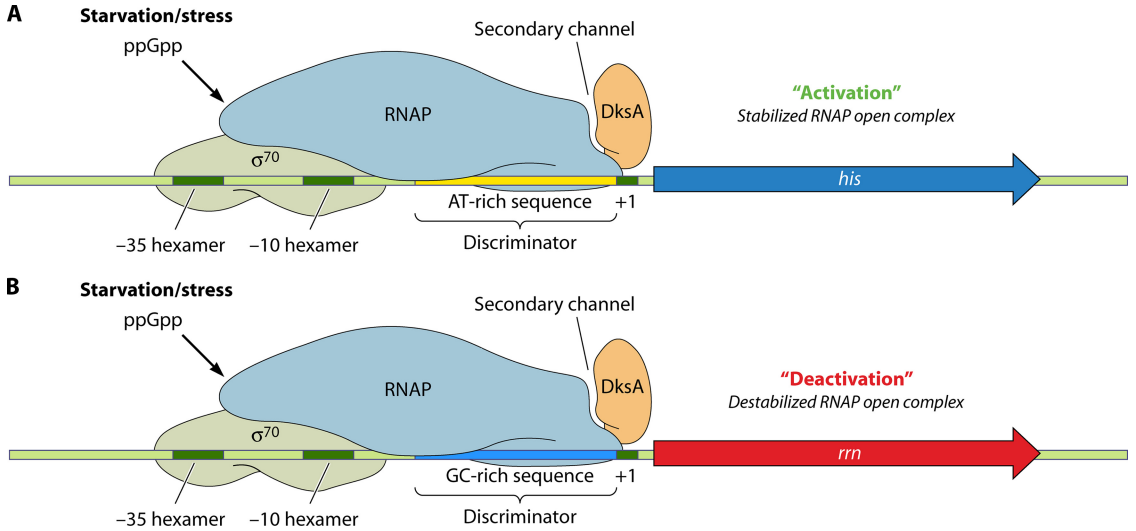

FIG. 3. ppGpp and DksA control transcription directly. In response to stress, gammaproteobacteria use ppGpp and DksA to control RNAP activity at particular promoters. Although DksA is known to bind at the secondary channel of RNAP, a binding site for ppGpp has not been confirmed. In the presence of DksA and elevated ppGpp levels, transcription can be either activated or deactivated. Whether transcription is stimulated or repressed depends upon intrinsic properties of the promoter. Activated targets such as the *E. coli* promoter for the histidine biosynthetic (*his*) operon typically have an AT-rich DNA sequence between the −10 hexamer and the +1 transcriptional start site, known as the discriminator region. Conversely, repressed targets such as the P1 promoter of rRNA (*rrn*) operons typically have a GC-rich discriminator sequence. Promoters controlled directly by ppGpp and DksA generally depend on the housekeeping/vegetative sigma factor σ^{70}.

REGULATORY TARGETS OF ppGpp

The ppGpp alarmone mediates many of its physiological effects by transcriptional control by either direct or indirect mechanisms. In *E. coli*, the direct repression of rRNA operons and the direct activation of amino acid biosynthetic operons by ppGpp occur via an interaction between the nucleotide and RNA polymerase (RNAP) that is not fully understood (160). DksA, a small protein that binds in the RNAP secondary channel, potentiates the effects of ppGpp on transcription (85). Whether a given promoter is directly activated or repressed by ppGpp and DksA is dictated by DNA sequence motifs (Fig. 3). Repressed targets are typically GC rich between the −10-box hexamer and the +1 nucleotide (transcriptional start site), a site known as the discriminator region, whereas activated promoters are typically AT rich in this position.

Indirect transcriptional control by ppGpp and DksA can occur through a process known as sigma factor competition (Fig. 4). In gammaproteobacteria, nearly all direct targets of ppGpp require the vegetative housekeeping sigma factor σ^{70}. During a stringent response, alarmone inhibition of strong σ^{70}-dependent promoters, such as rRNA promoters, increases the availability of core RNAP for transcription by alternative sigma factors (20, 49, 80, 194). In this manner, ppGpp indirectly promotes alternative sigma factor-dependent gene regulation by repressing the transcription of rRNA operons. Together, direct and indirect ppGpp-dependent mechanisms are integrated to mediate the global physiological adaptations of the bacterial cell that comprise the stringent response.

Recently, the dogma that ppGpp and DksA always collaborate to regulate *E. coli* physiology has been challenged. Data from phenotypic studies indicate that the overproduction of DksA by ppGpp^0 bacteria can compensate for the lack of the alarmone (160). Additionally, ppGpp and DksA have opposite effects on certain *E. coli* traits and promoters (1, 125). Furthermore, *in vitro* assays show opposite and independent regulations of some promoters by these two stringent response components (123, 131). Also, some regulatory effects that are predicted by phenotypic assays, such as the expression of fimbriae and flagella, are not recapitulated when transcriptional regulation by ppGpp and DksA is analyzed *in vitro* at the promoters of critical regulators, like FimB, FlhDC, and FliA

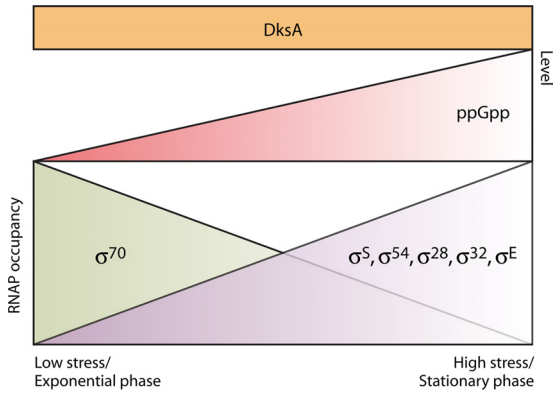

FIG. 4. ppGpp and DksA control transcription indirectly through "sigma factor competition." During exponential growth or favorable conditions, ppGpp levels are low in gammaproteobacteria, and transcription from strong σ^{70}-dependent promoters such as those of rRNA operons is robust. As bacteria exit the exponential phase, or during high stress, ppGpp levels accumulate. DksA, whose levels remain constant during growth, cooperates with ppGpp to repress the transcription of rRNA operons, liberating RNAP to bind alternative sigma factors (σ^S, σ^{54}, σ^{28}, σ^{32}, and σ^E). As a result, transcription from promoters targeted by these sigma factors increases, inducing specialized stress responses. In this manner, ppGpp and DksA contribute indirectly to bacterial adaptation.

(3, 113). Accordingly, other factors likely influence regulation by ppGpp^0 and *dksA* mutant cells. For example, TraR upregulates transcription from amino acid promoters and downregulates transcription from ribosomal promoters in the absence of ppGpp and DksA (22). Therefore, additional work is required before these complex regulatory interactions are fully understood.

Distinct roles for ppGpp and DksA in the bacterial cell also result from interactions between the alarmone and proteins other than RNAP. For example, in *Bacillus subtilis*, ppGpp prevents DNA replication elongation by inhibiting DNA primase activity (208). In *Salmonella enterica* serovar Typhimurium, ppGpp interacts with SlyA, a transcriptional activator of this pathogen's intracellular virulence program, facilitating its dimerization and binding to target promoters (223). In both *E. coli* and *B. subtilis*, ppGpp interacts with Obg, a nucleotide binding protein implicated in a number of physiological processes (33, 156). In *V. cholerae*, the ribosome-associated Obg homologue ("CgtA") appears to regulate ppGpp synthesis. In particular, CgtA is thought to repress the stringent response under nutrient-replete conditions by interacting with and modulating the activity of SpoT, keeping ppGpp levels low (164). A similar interaction was described for *E. coli* (215). Therefore, Obg likely contributes to yet another mechanism to regulate ppGpp levels, at least among some pathogenic members of the gammaproteobacteria. Alarmone production also affects the physiology of some Gram-positive bacteria by an indirect mechanism. In *B. subtilis*, the production of ppGpp is accompanied by a decrease in GTP pools, which affects the activity of GTP binding proteins, including CodY (93). Some cellular GTP is consumed during ppGpp synthesis, but the alarmone itself also inhibits IMP dehydrogenase, an enzyme involved in GTP biosynthesis (68, 119). Since *B. subtilis* utilizes GTP to initiate transcription from rRNA promoters, by reducing GTP pools, ppGpp indirectly represses rRNA production (109, 110). Thus, ppGpp can operate independently of both DksA and RNAP activity, illustrating the alarmone's far-reaching effects. Although the stringent response pathways of the pathogens that we discuss below (Table 1) have yet to be studied in great detail, several concepts learned from the model microbes *E. coli* and *B. subtilis* do apply.

GAMMAPROTEOBACTERIA

Enterohemorrhagic *E. coli*

In the nutrient-rich upper intestine, enterohemorrhagic *E. coli* (EHEC) replicates profusely. Descent into the nutrient-limited lower intestine triggers an increase in surface colonization and slower replication by the bacteria. In particular, the harsh environment of the lower intestine cues the timely expression of the EHEC locus of enterocyte effacement (LEE), a 35-kbp pathogenicity island encoding virulence factors necessary for attachment and colonization (98). The hallmark virulence trait of EHEC is the formation of "attaching and effacing" lesions on intestinal epithelial cells (146). This pathology is initiated when adherent EHEC cells secrete effector proteins across the host cell plasma membrane by using a type III secretion system (T3SS) encoded within the LEE region. By injecting factors that co-opt and interfere with cell processes, the bacteria orchestrate the construction, from host materials, of pedestal-like structures, sites of intimate contact that perturb the architecture of the brush border microvilli (201). From this vantage point, EHEC modulates both epithelial cell biology and the local immune response, promoting its survival within the host intestine and inducing symptoms of disease.

EHEC integrates the core ppGpp signaling system with transcriptional regulators of its more recently acquired virulence machinery, presumably to maximize its fitness in the lower intestine. Aside from horizontally acquired elements like the LEE and genes carried by cryptic prophages, EHEC is nearly identical to *E. coli* K-12 (130). Like this nonpathogenic strain, EHEC encodes a monofunctional RelA enzyme and a bifunctional SpoT enzyme. The accumulation of ppGpp in response to starvation activates LEE gene expression and increases bacterial adherence (143). Along with ppGpp, EHEC employs the transcription factor DksA to regulate LEE expression. Transcriptional targets of ppGpp and DksA include the LEE-encoded regulator (Ler) and the prophage-encoded activators PchA and PchB (143), which amplify *ler* expression (95). The Ler protein, encoded by the first gene in the LEE operon 1 (*LEE1*), coordinates the timely activation of the four remaining LEE operons as well as other T3SS effectors encoded elsewhere in the genome (130).

LEE regulation by the stringent response pathway has been analyzed biochemically and genetically. In *in vitro* transcription assays, ppGpp and DksA are required to activate the *pchA*, *pchB*, and *LEE1* promoters. Importantly, ppGpp and DksA can mediate promoter activation of *LEE1* in the absence of either PchA or PchB. Therefore, EHEC amplifies the starvation stimulus by activating dual systems, ensuring maximal LEE expression (143). In broth cultures, the ppGpp alarmone and DksA protein are required for LEE expression at the onset of stationary phase. However, in late-stationary-phase cells, the activation of the *LEE1* promoter still occurs in a *dksA* mutant but not a ppGpp^0 mutant. This delayed activation has been attributed to the DksA-dependent repression of *LEE1* during long-term starvation (143). Alternatively, late activation may reflect a ppGpp-mediated bypass of DksA, perhaps via an unidentified transcription factor. By integrating the expression of horizontally acquired virulence factors into its native ppGpp regulon, EHEC amplifies nutritional signals received in the lower intestine to enhance surface colonization, withstand nutrient depletion, and subvert host defenses.

Uropathogenic *Escherichia coli*

Acute urinary tract infection caused by uropathogenic *E. coli* (UPEC) can lead to recurrent infections by the same bacterial strain despite antibiotic treatment (168). A mouse model of cystitis suggests that recurrence and resistance may be attributed to the formation of intracellular bacterial communities. The establishment of these biofilm-like communities follows a specific maturation process where loosely associated, rapidly dividing, rod-shaped bacteria mature into slow-growing coccoid bacteria (102). As its microbial intruders replicate, the cytoplasm of the specialized superficial umbrella cell expands, forming protrusions on the bladder epithelium. Here, bacteria begin to express factors critical for motility and flux from the

host cell, processes that promote dispersal throughout the luminal space of the urinary tract (102).

Type I fimbriae are adhesive organelles that promote not only bacterial cell-cell contact during biofilm formation but also the colonization and invasion of bladder epithelial cells by binding to particular host receptors (102). Following invasion, type I fimbriae contribute to the maturation of intracellular communities, likely by facilitating physical interactions between resident bacteria (217). To establish an intracellular niche, UPEC requires the timely activation and repression of adhesive fimbriae in response to specific microenvironments encountered at the surface of the bladder epithelium and within host cells.

To ensure the opportune induction of fimbrial gene expression, UPEC relies on ppGpp and DksA to couple the expression of the *fim* operon with its metabolic status. The control of fimbria expression by ppGpp occurs through the promoter activation of the site-specific recombinase FimB, an enzyme that inverts the 314-bp promoter of the *fimAICDFGH* operon. Following transcriptional activation, FimB flips the *fim* promoter from its "off" orientation to its productive "on" position, thereby activating the transcription of genes encoding the structural components of type I fimbriae (2). Entry into the stationary phase results in the ppGpp-mediated activation of both the *fimB* and *fimA* promoters, increasing the frequency of adherent cells. An identical response occurs when UPEC starvation is triggered by treatment with serine hydroxamate or when ppGpp synthesis is artificially induced by using a truncated derivative of RelA that is constitutively active (2). Suppressor mutations that restore *fimB* expression map to the RNAP subunit *rpoB* and are similar to those that restore the amino acid prototrophy of $ppGpp^0$ mutant bacteria by inducing the expression of the amino acid biosynthetic machinery. Thus, UPEC exploits the classical stringent response alarmone ppGpp to control *fimB* transcription (2).

As with several other promoters that require ppGpp for direct regulation, *fimB* promoter activity is also impacted by DksA. However, recent studies of UPEC and *E. coli* K-12 have challenged the previously ascribed role of DksA during ppGpp-dependent transcriptional regulation (1, 3, 125). DksA not only regulates motility and cell-to-cell adhesion independently of ppGpp but also controls bacterial adherence oppositely of ppGpp (125). Furthermore, whereas $ppGpp^0$ UPEC cells do not express type I fimbriae, *dksA* mutants are hyperfimbriated (3). These distinct phenotypic patterns for *dksA* and $ppGpp^0$ cells are also observed at the level of transcription (3).

Differential regulation by ppGpp and DksA at the *fimB* promoter has been analyzed in detail. The hyperfimbriation of UPEC *dksA* mutants in broth reflects increased levels of *fimB* promoter activity. However, when the components are present together in *in vitro* reactions, ppGpp and DksA stimulate the transcription of *fimB* independently, exerting co-positive regulation. DksA can also enhance RNAP binding to the *fimB* promoter independently of ppGpp (3). Discrepancies between the broth phenotypes of *dksA* mutant UPEC and the DksA activities observed *in vitro* may be attributed in part to an increased occupancy *in vivo* of the RNAP secondary channel by structural homologues of DksA, such as the antipausing factors GreA and GreB, which may be missing from *in vitro* reactions (3). Indeed, increased channel occupancy by GreA and GreB contributes to an elevated level of expression of flagellum-related genes in *dksA* mutant *E. coli* K-12 cells (1). The mechanism by which ppGpp and other secondary channel-interacting proteins control the activation of the *fimB* promoter is highly complex and warrants further study.

Genes other than *fimB* also illustrate nonoverlapping regulation by ppGpp and DksA. For example, numerous genes required for flagellar biosynthesis and directional motility exhibited a greater-than-5-fold difference in relative transcript levels when ppGpp and *dksA* mutants were compared (3). It has become clear that direct transcriptional control by ppGpp and DksA in the bacterial cell is more complicated than previously thought.

Recently, TraR, a DksA homologue (30% identity) encoded by the conjugative F plasmid of *E. coli* K-12, was found to repress rRNA transcription and activate amino acid biosynthetic operons similarly to DksA. However, TraR functions in the absence of ppGpp (22). An understanding of how TraR acts independently of ppGpp will likely provide clues to the mechanisms of ppGpp- and DksA-dependent transcriptional control. Its self-sufficiency and presence on a conjugative plasmid, together with the general propensity of bacteria to integrate ppGpp into the regulation of pathogenicity island genes, make TraR, like other DksA homologues, a candidate activator of specialized bacterial virulence systems (22).

Shigella flexneri

The Gram-negative bacterium *Shigella flexneri* causes shigellosis in humans. The bacterial invasion of colonic mucosa elicits a robust inflammatory response and results in the destruction of the host epithelium. *S. flexneri* crosses the epithelial layer through epithelial M cells and accesses the basolateral surface (153), where bacteria induce uptake through membrane ruffling. Following ingestion by host cells, *S. flexneri* promptly escapes the endocytic vacuole and enters the cytoplasm. Cytosolic bacteria divide rapidly, with generation times of 40 min. To spread to neighboring cells, the pathogen induces a rapid, polar assembly of host actin, which propels *Shigella* through the cytosol and into double-membrane protrusions formed at the lateral surface of epithelial cells, enabling dissemination to adjacent cells.

Although highly similar to *E. coli* K-12, *S. flexneri* has not only lost and gained chromosomally encoded functions but also acquired extrachromosomal elements such as a virulence plasmid, prophage elements, and other insertion sequences that contribute to its fitness *in vivo*. To control the expression of many of these horizontally acquired virulence determinants, *S. flexneri* employs DksA.

S. flexneri DksA is 98% identical at the amino acid sequence level to those of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium. In a tissue culture model of infection, *S. flexneri* specifically requires DksA for intercellular spread but not for invasion or intracellular multiplication (133). DksA apparently controls *S. flexneri* dissemination via an RpoS-independent mechanism, as *rpoS* is dispensable for spread. The cell-to-cell transmission of *Shigella* requires the polar localization of IcsA, a protein that induces the polymerization of the epithelial cell's actin to propel the microbe unidirectionally through the cytosol. The ability of *S. flexneri* to localize IcsA to a single pole

requires DksA, as *dksA* mutants exhibit an increased propensity to distribute the protein evenly onto its surface, likely contributing to aberrant intercellular spread during infection (175).

Global gene expression analysis during exponential growth in broth demonstrates that DksA exerts positive control over a number of plasmid-carried and chromosomally carried *S. flexneri* virulence genes. The levels of mRNAs of *icsA*, *virF* (encoding a transcriptional regulator of virulence), and certain T3SS genes are each decreased in *dksA* mutant bacteria relative to wild-type (WT) bacteria. Additionally, DksA positively controls the expression of the chromosomally encoded regulators RpoS, Fur, Hns, and Hfq (175).

To spread from cell to cell, *S. flexneri* requires DksA for the direct transcriptional activation of Hfq (175), a regulatory protein that promotes the hybridization of small RNAs to their target mRNAs. The exponential-phase activation of *hfq* transcription by DksA is direct, since the addition of DksA to *in vitro* reaction mixtures increases *hfq* transcripts 4-fold (175). DksA-dependent activation is likely enhanced during starvation, because the addition of ppGpp to reaction mixtures results in additional increases in *hfq* transcript levels. Control over *hfq* activation is important during the dissemination of *S. flexneri*, based on the observation that the experimental induction of *hfq* to physiological protein levels is sufficient to bypass the requirement for *dksA* during intercellular spread. Thus, DksA activates the transcription of *hfq* during infection to promote cell-to-cell transmission.

Surprisingly, a role for ppGpp during cell-to-cell spread has not been tested. It will be interesting to determine if $ppGpp^0$ mutant *S. flexneri* exhibits dissemination defects similar to those of *dksA* mutant bacteria during infection. If so, starvation may trigger *Shigella* escape from nutrient-depleted epithelial cells, a strategy employed by *L. pneumophila* (51).

Pseudomonas aeruginosa

In addition to surviving in a variety of environmental niches, including soil and water, the ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* infects the lungs of individuals with cystic fibrosis (CF). To do so, *P. aeruginosa* is thought to persist within biofilms that are recalcitrant to antibiotic treatment (152). As such, the establishment of chronic infection often coincides with the emergence of strains that display a heritable mucoid phenotype characterized by the overproduction of the extracellular biofilm-associated polymer alginate. Also contributing to virulence is an extensive repertoire of exoproduct virulence factors, including toxins, secreted proteases such as elastase, toxic secondary metabolites including pyocyanin, and biofilm-related factors such as rhamnolipid (203).

Like *E. coli*, *P. aeruginosa* harbors both a bifunctional SpoT and a RelA, which is required for ppGpp accumulation during amino acid starvation. *P. aeruginosa relA* mutant strains showed reduced virulence in a *Drosophila melanogaster* model of infection (63), which may in part reflect a perturbation of quorum sensing (QS). QS equips *P. aeruginosa* to coordinate the expression of secreted virulence factors with antibiotic and stress tolerance phenotypes. In particular, a hierarchical acyl-homoserine lactone (AHL) QS cascade comprised of the Las and Rhl systems contributes to pathogenesis. The Las pathway positively regulates the Rhl system, and they both affect the expression of genes important for biofilm development, motility, and virulence-associated exoprotein expression. *P. aeruginosa* also harbors a third QS system, mediated by the *Pseudomonas* quinolone signal (PQS), which intersects with the AHL systems and also controls the stationary-phase production of exoprotein virulence factors. In addition to *relA*, functional QS systems are required for the full virulence of *P. aeruginosa* (108, 154).

The stringent response influences QS signaling mechanisms in *P. aeruginosa* that are necessary for infection. When cultured in minimal medium, differences in levels of AHL production between WT and *relA P. aeruginosa* strains are negligible. However, under conditions of Mg^{2+} limitation in minimal medium, conditions that induce *relA* expression (79) and that may be encountered in the CF lung, *P. aeruginosa* produced high levels of AHL by a *relA*-dependent pathway, even at low cell densities (63). In contrast, when amino acid starvation was initiated with serine hydroxamate, PQS levels decreased by a *relA*-dependent mechanism (63).

Consistent with altered QS signal production, a *P. aeruginosa relA* mutant dysregulated the expression of QS-controlled virulence factors such as pyocyanin and elastase (60). Specifically, the expression of elastase is controlled by the Las QS system, and a *lasR* mutant displays an elastase-negative phenotype. Elastase production by *lasR* mutants is restored by a second-site suppressor mutation that increases the level of expression of the autoinducer synthase gene *rhlI*. Interestingly, DksA appears to inhibit *rhlI* expression by this suppressor strain, since multiple copies of *dksA* abolish its elastase production and reduce *rhlI* transcript levels (27). The levels of production of rhamnolipid and LasB elastase are also reduced in a *dksA* mutant; however, this regulation was proposed to occur by a posttranscriptional mechanism (101). Taken together, these observations indicate that ppGpp regulates QS-mediated virulence factor expression in *P. aeruginosa*, likely by controlling the expression of QS enzymes such as autoinducer synthases.

In *P. aeruginosa*, the control of biofilm formation appears to be complex, with many regulatory elements interconnected with QS in a manner that depends on environmental cues (105). Some observations suggest that the stringent response pathway also affects *P. aeruginosa* biofilm development. For example, the contribution of QS to biofilm formation depends on nutrient conditions (178). When nutrients are limiting, bacteria induce the expression of the AHL autoinducer synthases in the exponential phase rather than the early stationary phase (60). Furthermore, ppGpp can induce the expression of QS-regulated virulence factors independent of cell density, causing the premature production of HSL autoinducers (202). Although Erickson et al. did not observe a difference in biofilm formation by *relA* mutants compared to the WT (63), the strong influence of environmental cues indicates that *P. aeruginosa* biofilm formation is governed by ppGpp under particular growth conditions that remain to be identified. Alternatively, the basal ppGpp pool resulting from the synthetase or hydrolase activity of SpoT may be more critical than RelA-controlled ppGpp levels for *P. aeruginosa* biofilm formation. The stringent

response pathway may also influence the starvation-induced dispersal of *P. aeruginosa* biofilms (92, 172).

As expected, ppGpp also affects stress tolerance phenotypes in *P. aeruginosa*. In particular, SpoT positively regulates the expression of *usp* genes, encoding universal stress proteins essential for survival under conditions such as the anaerobic stationary phase (24). The response to membrane perturbation also appears to be coordinated by ppGpp, as mutations that affect the membrane composition of *P. aeruginosa* lead to increased levels of transcription of *relA* as well as the *relA*-dependent activation of the AHL QS systems (18).

The stringent response also influences the expression of other regulators of *P. aeruginosa* virulence. For example, ppGpp may influence stationary-phase survival by controlling the expression of RpoS, since *relA* mutants contain less RpoS than do WT bacteria (63). *P. aeruginosa* also requires ppGpp and DksA for expression from RpoN-dependent promoters, possibly due to a stringent repression of σ^{70} promoters and the subsequent release of RNA polymerase to bind alternative sigma factors (Fig. 4) (194). RpoN-dependent virulence genes include those required for type IV pilus biogenesis, motility, and alginate synthesis (161).

An extensive repertoire of regulatory factors equips *P. aeruginosa* to detect and respond to numerous environmental conditions. Among these are components of the stringent response, which affect antibiotic and stress tolerance, biofilm formation, and the production of exoproducts associated with virulence. By coupling the stringent response pathway to other regulatory mechanisms, such as QS and alternative sigma factors, this versatile pathogen integrates nutrient cues with virulence factor expression.

Yersinia pestis

Prior to the advent of antimicrobials, plague was a devastating infectious disease affecting mankind. Although the number of cases reported annually has decreased dramatically, *Yersinia pestis*, the etiological agent of the disease, still threatens humans as a potential agent of bioterrorism. Natural reservoirs for *Y. pestis* include rodents and fleas, which disseminate bacteria to humans (155). Plague is manifested in bubonic and pneumonic forms. Bubonic plague is caused by the transmission of bacteria into the human lymphatic system by fleas that have fed on infected rodents. Mammals can also transmit bacteria via aerosols, the inhalation of which results in pneumonic infection and rapid mortality (155).

Y. pestis carries three plasmids that contribute to pathogenesis. One virulence plasmid encodes a T3SS required to evade phagocytosis and limit host inflammatory responses (155). Another plasmid is thought to promote survival in fleas (89). The third plasmid encodes factors that facilitate the invasion of host cells and dissemination into the circulatory system of mammals (31, 50). To express a number of plasmid-borne virulence factors, *Y. pestis* employs ppGpp.

Y. pestis utilizes two ppGpp synthetases to respond to distinct starvation stimuli during growth at flea and mammalian body temperatures. *Y. pestis* encodes a monofunctional RelA (~84% identical to *E. coli* K-12 and *S. enterica* serovar Typhimurium) and a bifunctional SpoT (~91% identical to *E. coli* K-12 and *S. enterica* serovar Typhimurium). As in *E. coli*, *Y. pestis* requires RelA to respond to amino acid starvation and SpoT to respond to carbon starvation (190). A source of ppGpp is critical for optimal *Y. pestis* growth in heart infusion broth, as *relA spoT* double mutants, but not *relA* single mutants, exhibit an increased lag phase and do not achieve WT cell densities at either 26°C (typical of fleas) or 37°C. Double-mutant bacteria also demonstrate increased autoaggregation at 26°C but not at 37°C. Excess autoaggregation is due to the absence of *spoT*, as the expression of *spoT in trans* is sufficient to reduce the enhanced autoaggregation phenotype in a dose-dependent manner (190).

Y. pestis also requires a source of ppGpp in a mouse model of bubonic infection. After subcutaneous infection with either WT or *relA* mutant bacteria, mice die synchronously, with 100% mortality by 8 days postinfection (190). SpoT-dependent ppGpp levels are critical for lethality, as mice infected with *relA spoT* mutant *Y. pestis* cells exhibited a decreased mortality rate, with 80% of mice surviving at 6 days. Additionally, the lethal dose of *relA spoT* mutant bacteria required to kill 50% of mice (LD_{50}) is ~100,000-fold greater than the LD_{50} of WT and *relA* mutant *Y. pestis* bacteria. Moreover, the experimental induction of *spoT* fully complements the virulence defect of $ppGpp^0$ bacteria (190). *relA spoT* mutants exhibit WT colonization patterns in the blood, spleen, and liver; in contrast, the stringent response pathway may play a modest role in dissemination into the lungs, as judged by enumerating CFU in mouse tissues 3 days after infection. Although ppGpp is dispensable for initial colonization, a CFU decrease 7 days postinfection suggests that mice clear *relA spoT* mutant *Y. pestis* bacteria.

The defects of *Y. pestis relA spoT* mutants during mouse infections may reflect decreased quantities of plasmid-encoded virulence proteins. While expression studies by reverse transcription (RT)-PCR analysis of Yops (*Yersinia* outer proteins) and other T3SS effectors reveal WT patterns of transcription in *relA* mutant and *relA spoT* double-mutant bacteria, secreted protein levels are reduced in *relA spoT* mutants but not *relA* bacteria (190). Therefore, SpoT is sufficient to elicit a post-transcriptional mechanism that induces the expression of *Y. pestis* virulence factors directly or indirectly. Specifically, ppGpp induces the expression of three T3SS effectors: YopE and YopH, effectors that disrupt the host cell cytoskeleton and facilitate the resistance of phagocytosis (157, 183, 190), and LcrV, a factor that triggers interleukin-10 (IL-10) release and suppresses the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (142, 150). As predicted, IL-10 levels in sera of mice inoculated with *relA spoT* mutants were diminished compared to those in the sera of mice inoculated with the WT. However, *relA spoT* bacteria may harbor a sufficient amount of LcrV to suppress TNF-α and IFN-γ production, as these cytokines are not detected in mice infected with either WT or alarmone-deficient *Yersinia* strains. In addition to altered T3SS effector levels, stringent-response-defective *Y. pestis* exhibits reduced levels of Pla (190), a protease essential during bubonic infection that cleaves host plasminogen (191) and promotes the invasion of epithelial cells (112, 174). Thus, the ability of *Y. pestis* to disseminate into the lungs and kill its mammalian host during bubonic infection requires the ppGpp-dependent post-transcriptional control of virulence factor production (190).

The phenotypic patterns of *Y. pestis relA* single-mutant and

relA spoT double-mutant bacteria *in vivo* are similar to those observed for other Gram-negative bacteria that possess both monofunctional and bifunctional stringent response enzymes. As detailed below, *relA* is dispensable and *spoT* is sufficient during both *Salmonella enterica* serovar Typhimurium infection of mice and *Legionella pneumophila* infection of macrophages. Together, these patterns raise the possibility that a classical, RelA-dependent stringent response to amino acid starvation is not demanded within mammalian hosts. Perhaps, during each of these bacterial infections, stimuli that trigger ppGpp accumulation *in vivo* are sensed by SpoT specifically. It is also possible that a modest amount of alarmone is sufficient to induce this class of virulence factors, since SpoT is a weaker synthetase than RelA in some bacteria (160). Alternatively, for bacteria to survive in hostile host environments, the ability of SpoT to balance alarmone levels through hydrolysis may outweigh the need for robust ppGpp synthesis.

Vibrio cholerae

As it transits between aquatic reservoirs and the human gastrointestinal tract, enterotoxigenic *Vibrio cholerae* adapts to each environment by expressing its virulence factors at the appropriate time and place. *V. cholerae* is thought to persist in aquatic environments by differentiating into stress-tolerant forms, such as biofilms or a viable but nonculturable (VBNC) state (6). Following ingestion, aquatic-adapted *V. cholerae* induces the expression of a virulence program consisting of toxins such as RTX (repeats in toxin), cholera toxin (CT), hemagglutinin (Hap), hemolysin (HlyA), and the toxin-coregulated pilus (TCP) as well as factors that allow transit through the gastrointestinal tract (128).

While there have been reports of the cell density-dependent expression of virulence factors by QS systems, there is also evidence that *V. cholerae* integrates particular virulence traits with growth phase regulation programs governed by ppGpp. For example, the RTX toxin is not secreted during the stationary phase, and the promoters of the genes encoding both the toxin and cognate secretion system contain a GC-rich discriminator region, which is a hallmark of negatively controlled stringent promoters (Fig. 3) (23). Furthermore, *relA* transcripts are upregulated ~3-fold when cells are in the resilient VBNC state (74). However, while the current body of evidence points to the involvement of ppGpp in *V. cholerae* pathogenesis, there have been conflicting observations on the extent of the impact of ppGpp in this process. The recent identification a third ppGpp synthetase homologue in *V. cholerae*, named RelV (57), suggests that this pathogen controls ppGpp levels by using mechanisms not typical of Gram-negative pathogens that harbor only one or two RSH homologues. Although these complex mechanisms remain to be understood, the identification of RelV does provide a potential explanation for the conflicting reports in the literature.

RelA was the first *V. cholerae* ppGpp synthetase shown to be critical for virulence (84). A *relA* mutant defective for ppGpp accumulation during amino acid starvation expressed significantly lower levels of two major virulence regulator proteins, ToxR and ToxT. As predicted, the levels of expression of both CT and the TCP were reduced in *relA* mutants compared to the WT strain under *in vitro* toxin-inducing conditions; the ratio of the ToxT-regulated porins OmpU and OmpT was also altered. Motility, another virulence trait of *V. cholerae*, is affected in the *relA* mutant. Finally, consistent with its lower levels of expression of factors known to be required *in vivo*, such as TCP, the *relA* mutant is significantly attenuated for the colonization of suckling mice (79). In contrast, the mutant grows as well as the WT in rich broth. Thus, RelA-dependent ppGpp levels regulate several virulence phenotypes.

RelA also equips *V. cholerae* to adapt to nutrient limitation, since a *relA* mutant is also defective for growth in minimal medium (179). However, in contrast to previous work, that same study found that the *relA* mutant was motile and able to colonize the suckling mouse intestine as well as the WT strain. The mutant also produced normal biofilms and expressed hemagglutinin/protease at WT levels (179, 180); however, the overexpression of RelA did diminish the level of production of this enzyme. Perhaps, differences in SpoT synthetase or hydrolase activities between the two El Tor strains used for these studies are responsible for the conflicting results (179). It is also possible that basal levels of ppGpp, controlled by SpoT (or the newly identified RelV protein [described below]), are sensitive to conditions not yet identified that differ in these studies. As such, the maintenance of basal levels of ppGpp, rather than the RelA-dependent stringent response, may have a greater influence on the expression of virulence factors by *V. cholerae* (179).

V. cholerae also encodes a third enzyme that generates ppGpp. As predicted by data for *E. coli* reported in the literature, *relA* mutant cells do not accumulate ppGpp under conditions of amino acid starvation, but the *V. cholerae* mutants still respond to carbon source starvation. However, a *relA spoT* double mutant still accumulates ppGpp during glucose starvation (56). Furthermore, the *relA spoT* double mutant grows in M9 minimal medium; is resistant to 3-amino-1,2,4-triazole, a reagent that induces histidine limitation; and exerts a stringent control of stable RNA synthesis when glucose is limiting. Since the double mutant lacks several phenotypes expected for a $ppGpp^0$ strain, investigators postulated that *V. cholerae* encodes another ppGpp synthetase that has yet to be identified. Repeated subculturing of *relA spoT* double mutants identified a suppressor strain that no longer accumulated ppGpp in response to carbon source starvation (57). The genetic lesion mapped to a locus, now named *relV*, that encodes a protein homologous to the catalytic domain of *E. coli relA* and appears to be restricted to the vibrios. Analysis of a *relA spoT relV* triple mutant confirmed that this novel ppGpp synthetase was the cryptic source of ppGpp in the *relA spoT* double mutant. Further work is needed to determine whether RelV enables *V. cholerae* to adapt to environments critical for pathogenesis and whether this third enzyme has contributed to the variations in *relA* mutant phenotypes reported by different groups. Although both the metabolism of ppGpp and its effect on pathogenesis have proven to be complex, work to date indicates that the alarmone affects the ability of *V. cholerae* to thrive in animal hosts and in aquatic environments between infections.

Salmonella enterica

Salmonella enterica serovars are responsible for a wide spectrum of human infections, ranging from systemic disease such as typhoid fever to a gastroenteritis known as salmonellosis.

Accordingly, the bacterium confronts a variety of environments to which it must adapt. Following oral ingestion within contaminated food or water, *Salmonella enterica* serovar Typhimurium passes through the stomach and enters the small intestine. Here, environmental cues prompt the activation of a T3SS encoded within the chromosomal *Salmonella* pathogenicity island 1 (SPI1). When the pathogen encounters specialized epithelial cells known as M cells, the SPI1 T3SS secretes effectors that induce membrane ruffling and the uptake of the bacterium (83). After breaching the epithelium by transcytosis across M cells, salmonellae are ingested by macrophages and trafficked to a hostile, acidic phagosome known as the *Salmonella*-containing vacuole (83). Conditions within this compartment trigger the activation of a second, chromosomally encoded T3SS within *Salmonella* pathogenicity island 2 (SPI2), whose effectors convert the vacuole to an environment suitable for replication. As protected macrophage residents, salmonellae then enter the lymphatic system and disseminate to the liver and spleen, where they may cause enteric (typhoid) fever (83).

Its life cycle within the host requires that *S. enterica* respond to environmental conditions to cue the expression of invasive and intracellular virulence programs: ppGpp signaling coordinates these systems. *S. enterica* encodes both a monofunctional RelA, responsible for a classic stringent response to amino acid starvation, and a bifunctional SpoT, which responds to carbon starvation (182). In an animal model of systemic disease following oral infection of BALB/c mice, WT and *relA* mutant *S. enterica* serovar Typhimurium strains caused morbidity within 7 to 10 days, whereas *relA spoT* double-mutant bacteria did not trigger signs of illness until 30 days. Furthermore, bacterial colonization of the liver and spleen was completely abolished by the *relA spoT* mutation (159). In tissue culture models, these ppGpp^0 mutants were defective for invasion and replication in intestinal epithelial cells as well as for replication and survival in murine macrophage cell lines (159, 197). Thus, the attenuation of ppGpp^0 mutants *in vivo* may be attributed to defects in virulence programs that are critical for invasion, survival, and replication in host cells. The bifunctional SpoT enzyme is sufficient during infection, suggesting that *S. enterica* serovar Typhimurium may employ SpoT to both synthesize and balance ppGpp pools through the hydrolysis of the alarmone, a control mechanism to ensure that virulence traits are expressed at appropriate sites within the host.

To coordinate entry into epithelial cells, *S. enterica* responds to external stimuli within the intestinal lumen by activating the expression of SPI1. In broth, *S. enterica* serovar Typhimurium induces SPI1 genes in response to ppGpp levels generated by SpoT activity during carbon starvation or exposure to excess short-chain fatty acids (159, 182). Both carbon starvation and oxygen limitation cause the ppGpp-dependent activation of *hilA* (182, 197), which encodes a transcription regulator of the OmpR/TotT family. In turn, HilA controls the expression of the InvF transcriptional regulator and other factors that together coordinate the expression of SPI1 (55, 121).

Whether the alarmone induces particular SPI1 genes depends on the context or the amount of ppGpp. For example, RelA-dependent ppGpp is not sufficient to activate the expression of the *hilA* SPI1 activator but is sufficient to activate *hisG* of the histidine biosynthetic operon (182). The full activation of *hilA* requires ppGpp, but another mechanism must also contribute, since the overexpression of the SPI1-encoded two-component system HilD/HilC can bypass the ppGpp requirement (159). The only sigma factor known to contribute to the expression of SPI1 genes is the housekeeping sigma factor σ^{70}. On the other hand, both SPI1 and SPI2 regions are AT rich, raising the possibility of direct positive regulation by ppGpp (Fig. 3) rather than passive control through sigma factor competition (Fig. 4) (159).

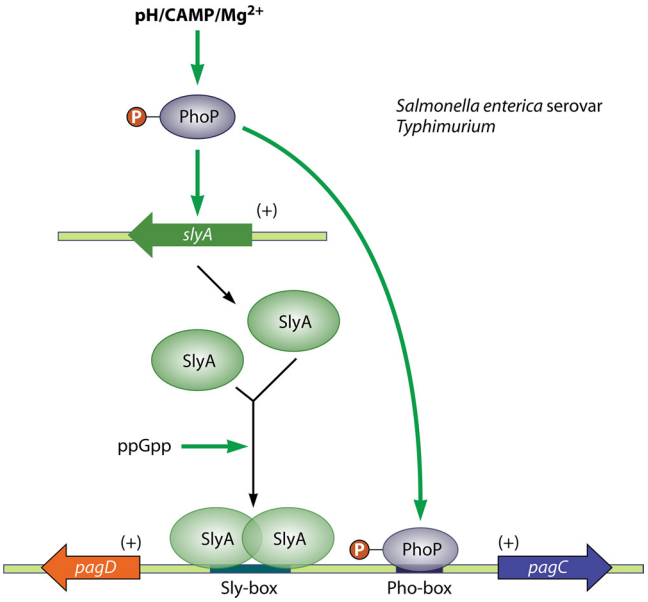

FIG. 5. ppGpp controls dimerization and DNA binding of SlyA. In addition to controlling RNAP activity, ppGpp can directly control transcriptional activators. After phagocytosis, *S. enterica* serovar Typhimurium encounters an acidic, magnesium-limited environment with high levels of cationic antimicrobial peptides (CAMP), activating the PhoP/PhoQ two-component system. The PhoP response regulator acts with the transcriptional activator SlyA in a feed-forward loop. PhoP itself activates the transcription of the *slyA* gene; together, the PhoP and SlyA proteins control the expression of SPI2 genes, which are critical for intracellular survival and replication. SPI2 gene expression is intimately linked to ppGpp signaling, as the alarmone facilitates dimerization and DNA binding of SlyA, facilitating the SlyA- and PhoP-dependent activation of several divergent operons that promote the pathogen's intracellular virulence program, including the SPI2 genes *pagC* and *pagD*.

Within its vacuole, *S. enterica* serovar Typhimurium encounters an acidic, magnesium-limited environment with high levels of cationic antimicrobial peptides. These conditions activate the PhoP/PhoQ two-component system, which represses SPI1 and activates SPI2, resulting in the repression of virulence factors involved in bacterial uptake, increased antimicrobial resistance, and the remodeling of the vacuole to a replicative compartment (83). Although ppGpp does not seem to play a direct role in the repression of SPI1, the bacteria do require the alarmone for the rapid PhoP-dependent activation of SPI2. The PhoP response regulator acts in a feed-forward loop with SlyA, another transcriptional regulator of SPI2 (Fig. 5). PhoP itself activates the transcription of *slyA*. Together, the PhoP and SlyA proteins then activate the transcription of SPI2 in response to distinct metabolic signals. In particular, low pH, Mg^{2+}, and cationic antimicrobial peptides activate the PhoQ sensor kinase, whereas ppGpp induces SlyA activity (176, 223).

For example, in the presence of low Mg^{2+} and ppGpp, PhoP and SlyA control the transcription of a divergent operon carrying two SPI2 genes, *pagC* and *pagD* (223). The alarmone does not impact the level of the PhoP or SlyA protein. Instead, *in vitro* assays demonstrated that ppGpp interacts directly with SlyA to facilitate its dimerization and binding to target promoters (Fig. 5). Regulation by ppGpp appears to be specific to SlyA, since the alarmone does not affect PhoP-dependent, SlyA-independent gene transcription (223). Ten other loci, including seven divergent operons and three unpaired genes, exhibit both SlyA and PhoP binding motifs as well as ppGpp dependence, demonstrating the breadth of this mechanism (223). Similar physical interactions between ppGpp and DNA binding transcription factors may govern the expression of SPI1 in *Salmonella* as well as the virulence programs of other pathogens.

S. enterica serovar Gallinarum causes typhoid fever in chickens and seriously threatens the poultry industry in South America and Asia (177). *S. enterica* serovar Gallinarum encodes SPI1 and SPI2, which share significant homology with other serovars. As in *S. enterica* serovar Typhimurium, ppGpp controls both invasion and replication in nonphagocytic and phagocytic cells, likely through the regulation of expression of and secretion by both SPI1 and SPI2 T3SSs. In contrast to *S. enterica* serovar Typhimurium, *S. enterica* serovar Gallinarum exhibits maximal invasion when cultured under nonstarvation conditions in the presence of oxygen (97). Under these conditions, the expression levels of both *hilA* and its target SPI1 are low, suggesting that ppGpp controls the invasion of *S. enterica* serovar Gallinarum by a mechanism that does not require SPI1 (97).

The stringent response transcription factor DksA also contributes to the virulence of *Salmonella enterica* serovar Typhimurium during infection of both chickens and mice. For example, mutants with transposon insertions in *dksA* are less often lethal in 1-day-old chicks and poorly colonize the intestines of 3-week-old animals (200). Bacteria lacking DksA also exhibit a 5-log increase in the LD_{50} in a mouse model of infection (210).

Similar to *E. coli*, in *S. enterica* DksA is a component of the general stress response that promotes adaptation to environmental hazards. The DksA protein of *S. enterica* serovar Typhimurium is 97% identical to that of *E. coli* K-12. As in *E. coli*, DksA regulates amino acid biosynthesis and mediates the repression of rRNA expression (200). In addition, DksA promotes the accumulation of the alternative sigma factor RpoS (σ^S) in the stationary phase and in response to acid stress (210). The σ^S protein controls the expression of many factors crucial for acid tolerance and is also required during infection (65). Specifically, DksA facilitates the translation of the *rpoS* transcript, but not mRNA production, to generate sufficient protein for the efficient transcriptional control of the RpoS regulon (210). DksA also increases the levels of expression of several other *Salmonella enterica* serovar Typhimurium proteins, which accumulate independently of *rpoS*. Therefore, DksA acts both cooperatively with and independently of RpoS (210). Although a function for DksA in RpoS-mediated regulation in *Salmonella enterica* serovar Typhimurium has been demonstrated, a direct link between ppGpp and DksA in the control of key virulence factors, like those encoded within SPI1 and SPI2, has not.

The ppGpp alarmone is the first regulatory molecule shown to play a comprehensive role in *S. enterica* serovar Typhimurium virulence, controlling both SPI1 and SPI2. *S. enterica* serovar Typhimurium employs ppGpp not only to redirect RNAP during starvation but also to control transcription directly and independently of RNAP by modulating activator binding (223). It is exciting to think that other pathogens may also utilize direct physical interactions of ppGpp with transcriptional regulators of their specialized secretion systems, thereby coordinating the assembly of these virulence machineries with the production of the secreted effectors.

Legionella pneumophila

The inhalation of aerosols from manufactured water systems containing *Legionella pneumophila* puts immunocompromised individuals at risk of contracting Legionnaires' disease. For millions of years, environmental legionellae have coevolved with freshwater protozoa that graze on biofilms. Selective pressure on phagocytosed bacteria to avoid digestion has driven the evolution of survival strategies that enable *L. pneumophila* to opportunistically infect human alveolar macrophages. A hallmark virulence trait of *L. pneumophila* is the ability to differentiate between morphologically and phenotypically distinct states within host cells, including replicative and transmissive cell types (32, 134). In some protozoan hosts, transmissive *L. pneumophila* differentiates further into "mature intracellular forms" suited for environmental persistence (66).

The regulation of the *L. pneumophila* life cycle in both protist and mammalian host cells requires a strict control of ppGpp metabolism. Increased levels of ppGpp cue the differentiation of *L. pneumophila* to a motile, coccoid, transmissive form that exhibits increased resistance to stress and the ability to evade lysosomal degradation (134). In the transmissive state, *L. pneumophila* effector proteins are increasingly transcribed and translocated into the host cell by the Dot/Icm type IV secretion system (T4SS) (32, 141, 163, 199). In particular, *L. pneumophila* relies on the Dot/Icm system to avoid fusion with the endosomal pathway and to establish a replication niche in a compartment derived from the endoplasmic reticulum (ER) (94). When nutrients are abundant, transmissive bacteria hydrolyze ppGpp, resulting in the initiation of cell division and repression of transmission factors (53, 135). As a consequence, in mouse macrophages, the block to phagosome-lysosome fusion is relieved, and the replication vacuole matures into an acidic lysosomal vacuole (189). As the replicating bacteria consume nutrients, vacuolar conditions presumably deteriorate and stimulate ppGpp production, prompting the progeny to reenter the transmissive state (53). For example, elevated levels of ppGpp trigger the expression of factors leading to bacterial cytotoxicity to macrophages, motility, stress resistance, and the ability to evade lysosomes, traits which promote the transmission of the pathogens from the exhausted host cell and the infection of naïve ones. Thus, ppGpp potentiates *L. pneumophila* cell-to-cell transmission.

L. pneumophila is equipped with two ppGpp synthetases that coordinate differentiation to the transmissive state in response to distinct metabolic cues (53, 61). Its monofunctional RelA enzyme is 44% identical and 63% similar to its *E. coli* K-12 counterpart, and its bifunctional SpoT is 53% identical and

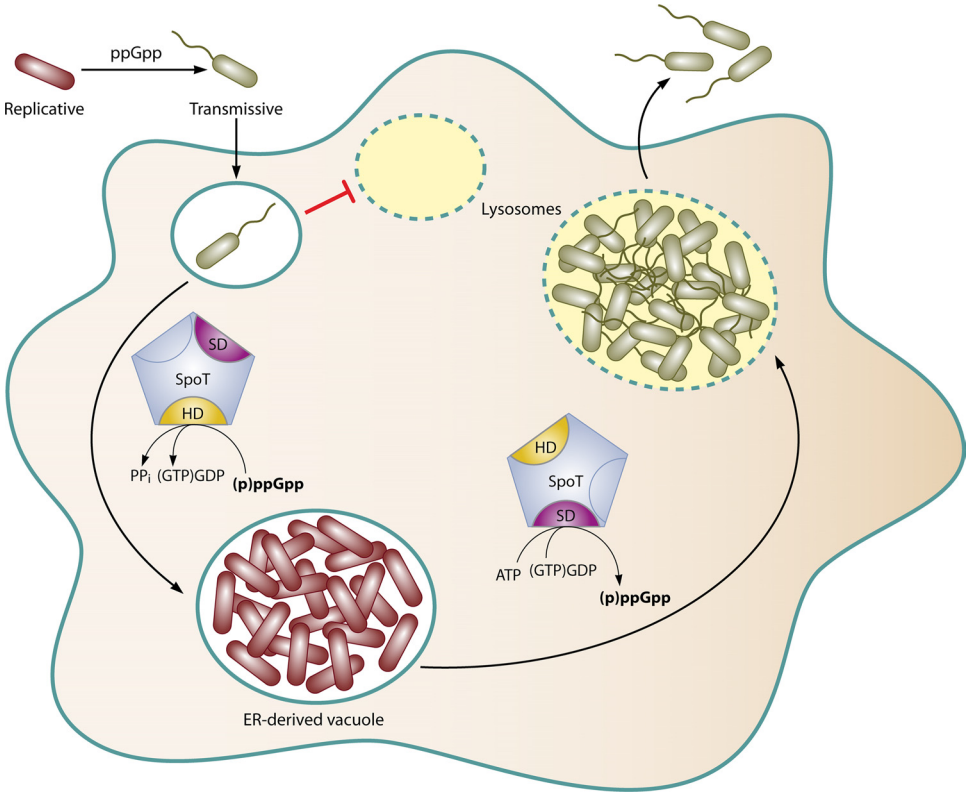

FIG. 6. SpoT governs the *Legionella* life cycle in macrophages. During its life cycle, the intracellular pathogen *L. pneumophila* differentiates between two forms, replicative and transmissive. When nutrients become scarce, ppGpp levels increase, coordinating the differentiation of replicative bacteria to the highly resilient, motile, transmissive form. After phagocytosis, transmissive bacteria inhibit fusion with degradative lysosomes (small, dashed, empty vacuole). To convert to the replicative form, *L. pneumophila* must sense favorable vacuolar conditions that stimulate the bifunctional SpoT enzyme to reduce alarmone pools via ppGpp hydrolysis (HD). In a vacuole derived from the ER, replicative *L. pneumophila* cells divide exponentially. Gradually, the replication vacuole acidifies and acquires lysosomal markers. Deteriorating vacuolar conditions elicit SpoT synthetase (SD) activity, cueing replicative bacteria to differentiate back to the transmissive form. Transmissive *L. pneumophila* cells resist lysosomal degradation and migrate to a naïve host cell, primed to establish a new infection.

71% similar to the *E. coli* enzyme. In broth culture, RelA synthesizes ppGpp in response to amino acid starvation. SpoT-dependent ppGpp accumulates following perturbations in fatty acid metabolism. In fact, either the inhibition of fatty acid biosynthesis or the addition of excess short-chain fatty acids triggers SpoT activity in *L. pneumophila* (61). Alarmone synthesized from either enzyme can be detected at the transition from the exponential to the postexponential phase in rich broth, and the artificial induction of ppGpp synthesis by cells replicating in rich medium is sufficient to trigger rapid differentiation to the transmissive form (53, 81). In macrophages, the alarmone is dispensable for replication (5, 51), but it is essential for transmission to a new host cell, since *relA spoT* double-mutant *L. pneumophila* cells replicate intracellularly but are subsequently degraded during the period when WT bacteria undergo transmission.

By virtue of its dual enzymatic activities, SpoT regulates both replicative and transmissive functions in macrophages and in microbiological medium (53). Following uptake by macrophages, a hydrolase-competent SpoT enzyme is essential for transmissive *L. pneumophila* to initiate replication (Fig. 6). Likewise, when plated onto rich medium, transmissive *L. pneumophila* bacteria require SpoT to form colonies. Furthermore, the induction of plasmid-borne SpoT, but not RelA, promotes the transmission of *relA spoT* mutant bacteria between macrophages. In contrast, *relA* single mutants still transform to motile, lysosome-resistant, transmissive forms in broth, and they exhibit no intracellular growth defects in macrophages (51, 225). Therefore, *L. pneumophila* bacteria use SpoT first to initiate replication when the vacuole is suitable for propagation and later to engage transmission when vacuolar conditions deteriorate (Fig. 6). Thus, control over ppGpp accumulation and degradation by SpoT is critical for the *L. pneumophila* life cycle.

L. pneumophila also encodes a DksA homologue that is 72% identical to the DksA proteins of *E. coli* K-12 and *S. enterica* serovar Typhimurium that contributes to differentiation in broth and growth in amoebae (54). Genetic analysis revealed that DksA is critical for *L. pneumophila* differentiation to the transmissive form, including flagellar gene activation, evasion of lysosomes, and cytotoxicity toward macrophages. The roles of DksA and ppGpp depend on the context. For transmission between macrophages, ppGpp is essential, whereas DksA is dispensable; therefore, ppGpp is sufficient to coordinate transmission in macrophage cultures. In broth, DksA promotes differentiation when ppGpp levels increase or in response to fatty acid stress, suggesting that ppGpp-independent signals cue DksA activity. In the flagellar cascade, ppGpp and DksA

act both cooperatively and independently. For the basal expression of the alternative sigma factor *fliA* (σ^{28}) in the exponential phase, DksA functions independently of ppGpp. Furthermore, the experimental induction of *dksA* expression is sufficient to restore flagellar synthesis and macrophage cytotoxicity to ppGpp^0 mutant *L. pneumophila* cells. When alarmone levels increase, DksA cooperates with ppGpp to control the activation of at least three flagellar gene classes, generating a pulse of early rod transcripts and the prolonged activation of late sigma factor (σ^{28}) and flagellin RNAs. Thus, DksA responds to the level of ppGpp and other stress signals to coordinate the differentiation of replicating *L. pneumophila* to the transmissive form.

Like other gammaproteobacteria, *L. pneumophila* may have evolved a SpoT-dependent mechanism to monitor fatty acid metabolism that is mediated by a physical interaction between SpoT and ACP (Fig. 2). ACPs transfer acyl groups to enzymes involved in either phospholipid or secondary-metabolite biosynthesis. The ACP-SpoT interaction is conserved in bacteria possessing two ppGpp synthetase enzymes (i.e., RelA and SpoT) and appears to be highly specific (17). In particular, SpoT interactions are restricted to ACPs that are encoded within fatty acid biosynthesis operons. SpoT binds specifically to functional ACPs, namely, those that have been posttranslationally modified to carry a fatty acid intermediate (16). Furthermore, ACP interacts with SpoT but not RelA. In *E. coli*, the interaction of ACP with SpoT during growth in nutritionally replete medium appears to inhibit the ppGpp synthetase domain of SpoT, skewing the balance of ppGpp metabolism by this enzyme toward hydrolysis. Upon fatty acid starvation, in some manner, ACP interactions with the TGS domain (a domain found in *t*hreonyl-tRNA synthetase, *G*TPase, and *S*poT proteins) of SpoT promote the accumulation of ppGpp in the cell (Fig. 1 and 2). In *E. coli*, the mutation of specific amino acids within the regulatory domain of SpoT (A404E and S587N) abrogates the ACP interaction and eliminates the cellular response to fatty acid biosynthesis inhibition (16).

A similar SpoT-ACP interaction may govern *L. pneumophila* differentiation. First, the pathogen requires an identical SpoT amino acid residue (alanine 413) to elicit a stringent response to perturbations in fatty acid biosynthesis (53). In addition, genetic studies indicate that an ACP-SpoT interaction may influence ppGpp hydrolysis in *L. pneumophila*. When highly expressed, the SpoT(A413E) mutant protein confers a phenotype similar to that caused by RelA overexpression: in both cases, the cells are locked in the transmissive state and are unable to initiate replication in broth or macrophages (53). Thus, some bacteria have evolved mechanisms to monitor fatty acid metabolism and control ppGpp levels through an ACP-SpoT interaction, a scheme analogous to the tRNA-RelA interaction that activates alarmone production when amino acids become scarce.

From atop a complex regulatory cascade, ppGpp exerts both direct and indirect control over downstream activators and repressors of *L. pneumophila* transmission (134). To respond to elevated ppGpp levels, *L. pneumophila* requires the LetA/LetS two-component system (82). The mechanism by which ppGpp activates the LetA/LetS system remains unknown. The LetA transcriptional activator binds upstream of two genes encoding small regulatory RNAs, RsmY and RsmZ, initiating their transcription (163, 169). Both RsmY and RsmZ interact with CsrA, an RNA binding protein and repressor of *L. pneumophila* transmission, relieving its ability to bind transcripts critical for the transmissive phenotype, such as effectors of the Dot/Icm system. Several observations indicate that the ppGpp alarmone likely contributes to the ability of LetA to positively regulate the transcription of the RsmZ regulatory RNA: the activation of the *rsmZ* promoter requires ppGpp, RsmZ RNA accumulates immediately following ppGpp synthesis, and the overexpression of *rsmZ* bypasses the requirement for ppGpp to activate motility, toxicity toward macrophages, and lysosome evasion (Z. D. Dalebroux, unpublished data). It is conceivable that ppGpp regulates the ability of LetA to control the activation of *rsmZ* transcription.

L. pneumophila may also employ ppGpp to control the expression of alternative sigma factors directly and/or to increase their activity indirectly. The stationary-phase sigma factor RpoS (σ^S), the flagellar sigma factor FliA (σ^{28}), and the alternative sigma factor RpoN (σ^{54}) are each known activators of transmission traits (11, 12, 88, 91, 96). In the transmissive phase, ppGpp is predicted to influence the activity of alternative sigma factors passively. According to studies of *E. coli*, by deactivating transcription at σ^{70}-dependent rRNA promoters, ppGpp and DksA increase the amount of RNAP available to alternative sigma factors (Fig. 4) (160). Indeed, at the onset of starvation, *L. pneumophila* RpoN and FliA contribute to flagellar gene expression and synthesis (88, 96). Unlike *rpoN*, *fliA* is highly induced in the transmissive phase by ppGpp and DksA (54), making it a likely candidate for direct regulation. Another candidate for direct or indirect regulation by ppGpp and DksA is RpoS. RpoS is essential for the intracellular replication of *L. pneumophila* in *Acanthamoeba castellani* and is partially required for growth in macrophages (12, 91). Although RpoS translation efficiency and protein levels in *L. pneumophila* have not been monitored, the *rpoS* transcript is more abundant in the exponential phase than in the stationary phase (13). As with *E. coli*, the transcription of *rpoS* in *L. pneumophila* is sensitive to ppGpp pools, since transcript levels increased following the artificial induction of the alarmone (30). Thus, it appears that ppGpp controls RpoS expression to affect transmission and replication in *L. pneumophila*; however, specifics of the interplay in this complex regulatory mechanism remain to be clarified.

RpoS is also integrated into the *L. pneumophila* CsrA regulatory system. In particular, this sigma factor is required for maximal *rsmY* and *rsmZ* gene expression (91, 163). Therefore, by mechanisms yet to be defined, ppGpp, LetA/LetS, and RpoS cooperate to relieve the CsrA-mediated repression of transmissive transcripts. These and other ppGpp-dependent regulatory mechanisms, such as sigma factor competition, enable *L. pneumophila* to transition rapidly between replicative and transmissive virulence programs in host cells. Its reliance on ppGpp and the bifunctional SpoT enzyme makes *L. pneumophila* an attractive experimental model to understand how metabolic cues are transmitted by second-messenger signaling pathways to govern bacterial virulence.

Francisella tularensis

Francisella tularensis, the etiological agent of tularemia, is a Gram-negative, facultative, intracellular bacterium that infects

mammals, fish, insects, amphibians, and protozoa. Its ability to disseminate readily among a variety of hosts along with its high infectivity and mortality rates make *F. tularensis* a bioterrorism threat (170). *F. tularensis* can infect humans by many routes, including inhalation and skin abrasions. Most commonly, arthropods that have fed on an infected mammal transmit bacteria to humans through bites. To develop treatment and prevention strategies, much effort has focused on understanding the interaction between *F. tularensis* and the mammalian macrophage, which serves as an intracellular replication niche during infection.

Following macrophage uptake by asymmetric, spacious pseudopod loops, *F. tularensis* is contained in a specialized phagosome. At 1.5 h postinfection, the bacteria induce the expression of MglA, a protein sharing 20% identity to the stringent starvation transcriptional regulator SspA of *E. coli* (14). MglA interacts with another SspA homologue (referred to as SspA), and the MglA-SspA complex associates with RNAP to control the transcription of genes within the *Francisella* pathogenicity island (FPI). Factors encoded by the FPI cue the arrest of phagosome maturation and the escape of bacteria into the cytosol, where *F. tularensis* replicates. The FPI carries ~100 genes essential for intracellular survival, including a type VI secretion system (122). To integrate nutritional cues into virulence regulation, *F. tularensis* uses ppGpp to promote physical interactions between the RNAP-associated MglA-SspA complex and the putative DNA binding factor PigR to control the activation of FPI (43). In this manner, ppGpp contributes to phagosome escape and the intracellular survival of *F. tularensis*.

F. tularensis encodes both a monofunctional RelA and a bifunctional SpoT enzyme. In the live vaccine strain of *F. tularensis* subsp. *holarctica*, *mglA* mutant and *relA spoT* double-mutant ($ppGpp^0$) bacteria exhibit overlapping defects in FPI gene activation, growth in macrophages, and virulence in mice, suggesting that ppGpp works with MglA and SspA to control virulence expression (43). A SpoT-dependent ppGpp pool is sufficient for *F. tularensis* virulence, since *relA* mutant bacteria show only modest defects relative to WT bacteria. The ppGpp alarmone does not influence the abundance of either MglA or SspA or affect the MglA-SspA-RNAP interaction. Instead, ppGpp controls MglA-SspA-dependent gene expression through PigR, another regulator of FPI critical for intracellular growth and virulence. PigR regulates the MglA-SspA complex in a manner that requires ppGpp, as the interaction was not observed in bacteria lacking the alarmone (43). Furthermore, the autoactivation of *pigR* requires both ppGpp and the MglA-SspA complex, indicating that the alarmone also amplifies PigR-dependent gene expression. This paradigm can likely be extended to include *F. tularensis* subsp. *novicida*, as the PigR orthologue FevR is also required for virulence in macrophages and mice (29). It remains to be determined if ppGpp allosterically impacts the interaction between PigR and the RNAP-associated MglA-SspA complex through the alarmone's ability to control RNAP itself. Alternatively, ppGpp may interact directly with PigR to facilitate its binding to the MglA-SspA complex. In either case, *F. tularensis* is a striking example of how ppGpp modulates critical regulatory proteins to control virulence expression during infection.

ACTINOBACTERIA

Mycobacterium tuberculosis

A hallmark of the life-style of *Mycobacterium tuberculosis* is its entry into a state of dormancy that withstands an intense immune response until conditions favor reactivation. When humans inhale tubercle bacilli, alveolar dendritic cells ingest invading bacteria and then initiate a potent cell-mediated immune response. To counter host defenses, *M. tuberculosis* blocks phagosome-lysosome fusion to establish an intracellular replication niche. Nevertheless, released bacterial products continue to stimulate a local immune response. After several weeks, chronic cytokine stimulation leads to granuloma formation and bacterial containment. Surrounded by a hostile environment, *M. tuberculosis* transforms to a latent, hypometabolic state (58, 127). During latency, which can extend for decades, bacilli dramatically reduce their replication rate, adopt a granular appearance, and become refractory to acid-fast staining and more resistant to antimicrobial agents (72, 147, 148). If the immune system wanes, the granuloma undergoes caseous necrosis, causing the death of most bacilli and substantial tissue destruction (78, 171). The erosion of air cavities permits access to the respiratory tree, enabling bacteria to spread to other humans through aerosols (72). Worldwide, 2 billion people are infected with latent *M. tuberculosis*. Reactivation typically occurs in ~5% of these individuals, but this rate increases to 50% for those coinfected with HIV (171).

To initiate dormancy and establish latent infection, *M. tuberculosis* employs its stringent response enzyme, RSH. Annotated as *M. tuberculosis* Rel (Rel_{Mtb}), this enzyme increases bacterial resilience to antimicrobial mechanisms, alters central metabolism, and modulates the immune response of the host (52). The 738-amino-acid protein exhibits both synthetase and hydrolase activities and is most similar to Rel proteins of the Gram-positive actinomycetes, i.e., *Corynebacterium glutamicum* (67% identity), *Streptomyces antibioticus* (66% identity), and *Streptococcus equisimilis* (62% identity), and less like those of Gram-negative organisms like *E. coli* (9, 162). After aerosol inoculation of C57BL/6 mice with rel_{Mtb}-null mutants constructed in the virulent H37Rv strain background, bacterial growth in the lung was normal initially. As expected, the enzyme is also dispensable for replication in the THP-1 macrophage cell line (162). However, Rel_{Mtb} is essential for chronic persistence *in vivo* (52). Beginning 5 to 7 weeks after infection, rel_{Mtb} mutant viability declined drastically in the lungs and spleens of infected mice (52). The persistence defect correlates with reduced histopathology in the lungs of animals 15 and 38 weeks after infection with rel_{Mtb} mutant bacteria. Therefore, Rel_{Mtb} function is critical for *M. tuberculosis* latency.

The requirement for Rel_{Mtb} at the onset of latency suggests that the enzyme responds to environmental conditions particular to this stage of infection. The level of ppGpp is known to increase during the stationary phase, carbon source starvation (0.2% glucose), and inhibition of the respiratory chain by sodium azide in broth cultures of *M. tuberculosis* (162). Mutants lacking rel_{Mtb} are also vulnerable *in vitro* to long periods of nutrient deprivation (100 days) and hypoxia (24 h). When the lipid dipalmitoylphosphatidylcholine (DPPC) is provided as a nutrient source in synthetic liquid medium, rel_{Mtb} mutant my-

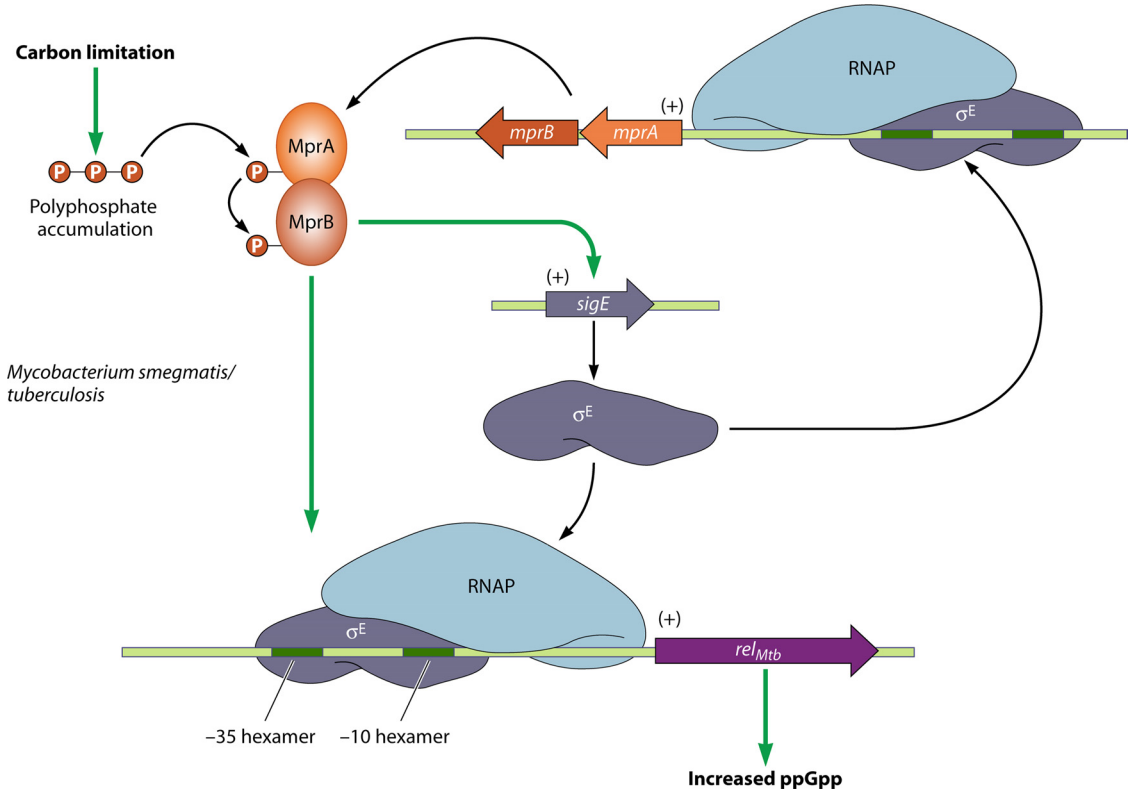

FIG. 7. rel_{Mtb} expression is induced in response to carbon stress. In addition to controlling the activity of ppGpp synthetases, some bacteria regulate the transcription of the gene that encodes this enzyme. In response to carbon limitation, *M. smegmatis* and *M. tuberculosis* accumulate polyphosphate. This high-energy molecule donates phosphate to the MprA/MprB two-component system. MprA/MprB and the alternative sigma factor σ^E comprise a positive-feedback loop: MprA/MprB activates the transcription of the *sigE* gene; in return, the σ^E protein controls the activation of the *mprA mprB* locus. Together, MprA/MprB and σ^E activate the transcription of rel_{Mtb}, thereby increasing levels of ppGpp in the cell. This positive-feedback mechanism amplifies the response to carbon limitation, leading to a robust activation of the stringent response.

cobacteria grow as well as the WT, whereas the mutants are attenuated for growth on either peptone or oleic acid (162). The observation that rel_{Mtb} is dispensable during replication in macrophages is consistent with the idea that *M. tuberculosis* exploits lipids as a carbon source during lung infection. Together, current data suggest that after a period of intracellular replication, Rel_{Mtb} ppGpp synthetase activity is elicited at the onset of hypoxia, perhaps as a result of a major shift in carbon metabolism.

M. tuberculosis also employs an RNAP-interacting protein, CarD, to activate its stringent response by repressing the transcription of components of the translation machinery (185). Transcript levels of *carD* increase in response to genotoxic, oxidative, and nutritional stress, and *M. tuberculosis* requires CarD to survive these hostile conditions. Similar to Rel_{Mtb}, CarD is required for *M. tuberculosis* to persist in mice. However, unlike rel_{Mtb}, the *carD* gene is also essential for *M. tuberculosis* viability *in vitro* and replication in mice, indicating that CarD plays a broader role than Rel_{Mtb} (185). Although CarD can substitute for DksA function to repress *E. coli* rRNA synthesis, the two proteins regulate RNAP by different mechanisms. Specifically, CarD binds the N terminus of the RNAP β subunit and not the secondary channel. The ability of factors like CarD to exert a transcriptional repression of the ribosomal machinery may explain why *dksA* homologues are not obvious in the genomes of *M. tuberculosis* and other nonproteobacteria.

Like the enzyme activity, the transcription of the rel_{Mtb} gene is induced during stress, ensuring a robust activation of the stringent response regulon to counter nutritional adversity. The transcriptional activation of rel_{Mtb} requires a complex signaling pathway involving the MprAB two-component system, σ^E, and polyphosphate (192). Polyphosphate is a ubiquitous, anionic, high-energy molecule that participates in numerous biological processes. In *E. coli*, ppGpp acts upstream of polyphosphate, inhibiting its degradation by PPX, a polyphosphate hydrolase. As polyphosphate accumulates, it increases both RecA and RpoS levels, thereby activating the SOS response and increasing general stress resistance of the bacteria (111).

In mycobacteria, polyphosphate instead acts upstream of ppGpp (Fig. 7). Under carbon-limiting conditions, *Mycobacterium smegmatis rel* promoter activity increases dramatically. Both *in vitro* transcription and transcriptional reporter assays showed that *rel* induction is directly dependent on σ^E. In particular, σ^E and MprAB comprise a positive-feedback loop: the two-component system activates the transcription of *sigE*, and *mprAB* itself is part of the σ^E regulon (192). Polyphosphate can both act as a phosphodonor to MprAB and activate the transcription of *mprAB*, *sigE*, and *rel*. Thus, polyphosphate pro-

motes *rel* transcription, which then increases ppGpp levels (192). A similar signaling pathway was verified for pathogenic *M. tuberculosis* by using antisense technology to reduce the level of expression of the gene encoding polyphosphate kinase, *ppk1* (192). Mycobacterial latency is a striking illustration of the capacity of bacteria to adapt to stress through an interplay between two-component phosphorelay systems, sigma factors, and the ppGpp alarmone.

In addition to factors regulating Rel_{Mtb} expression and activity, many downstream *M. tuberculosis* targets of ppGpp have been identified. In particular, ppGpp alters central metabolism, confers increased resilience to host antimicrobial strategies, and controls the expression of factors that modulate the immune response (52). Consistent with this cellular differentiation, microarray analysis determined that Rel_{Mtb} downregulates the translational apparatus and influences the expression of factors associated with virulence, including macrophage entry proteins, components required to use nitrate as a terminal electron acceptor, and proteins that engage isocitrate lyase (52). Rel_{Mtb} also affects the expression of cell wall modification factors, polyketide synthases, proteins critical for antigenic variation, and several potent mycobacterial antigens that actively shape the immune response during infection. Thus, from the long course of coevolution of *M. tuberculosis* with its human host, Rel_{Mtb} and the ppGpp alarmone have emerged to coordinate global physiological adaptations not only to survive during latency but also to tolerate and modulate the immune response to secure its place among the human population.

FIRMICUTES

In Gram-positive bacteria, levels of ppGpp are fine-tuned by using mechanisms distinct from those typical of the gammaproteobacteria. For instance, most pathogenic firmicutes encode a bifunctional *spoT* homologue, sometimes annotated as *rel* or *relA*, in addition to one or two small alarmone synthases (SASs) (144), often called *relP* or *relQ* (Fig. 1). The SAS proteins account for *relA*-independent ppGpp synthesis in these bacteria (116). Alarmone synthesis can also reduce the GTP pool in both Gram-negative and -positive bacteria either via consumption during ppGpp synthesis or by the ppGpp-dependent inhibition of IMP dehydrogenase, an enzyme involved in guanine nucleotide biosynthesis (68, 120). Whereas the capacity of ppGpp to control promoter selection by RNA polymerase in Gram-negative bacteria has been well documented, the literature on firmicutes more often describes an indirect control of regulatory proteins by ppGpp. Two such regulators are σ^B, the sigma factor that controls the general stress regulon (222), and CodY, a global regulatory protein (181).

The bifunctional ppGpp synthetase/hydrolase SpoT is central to the biology of many Gram-positive pathogens. For example, the *Staphylococcus aureus spoT* homologue (referred to as *relA*) is essential for viability *in vitro* (44, 71). A number of the obligately parasitic and genetically streamlined species of the genus *Mycoplasma* accumulate ppGpp and exhibit stringent control (73). Other members of this phylum rely on the stringent response to regulate global physiological changes implicated in pathogenesis, such as environmental persistence and antibiotic resistance. *Bacillus anthracis*, causing pulmonary anthrax, survives long periods in the environment as stress-tolerant spores, and its differentiation into spores appears to be *relA* dependent (204). *Clostridium difficile*, an increasingly prevalent nosocomial pathogen that is frequently exposed to antimicrobial agents, upregulates a putative ppGpp synthesis/degradation protein in the presence of both amoxicillin and clindamycin (62). Here we discuss more extensive evidence for the direct involvement of ppGpp in the survival and pathogenesis of three firmicutes, the food-borne pathogen *Listeria monocytogenes*, hospital-acquired *Enterococcus faecalis*, and the versatile *Streptococcus* spp.

Listeria monocytogenes

L. monocytogenes is associated with a rare, but potentially fatal, food-borne illness termed listeriosis. Although infection may manifest only as noninvasive gastroenteritis, severe cases can lead to septicemia and meningitis. Such infections likely reflect the potential of this pathogen to invade host cells and promote cell-to-cell spread. *L. monocytogenes* can induce its uptake by macrophages, which is followed by listeriolysin O-mediated escape from its phagosome and intracellular replication in the cytosol (67). Cell-to-cell spread is then achieved by inducing host actin polymerization, which propels the bacterium into adjacent cells. *L. monocytogenes* adapts to nutritional challenges posed by replication within the host cytosol. Furthermore, as a food-borne pathogen, *L. monocytogenes* can also survive in contaminated food products, such as unpasteurized cheeses, where it adapts to high salt and low temperatures. The adherence of *L. monocytogenes* to surfaces during food preparation and storage also promotes survival (196).

Although *relQ* and *relP* homologues have been identified in the genome of *Listeria* spp. (114), the only enzyme characterized to date is the bifunctional *spoT* homologue, interchangeably termed Rel or RelA. When amino acid starvation was induced with serine hydroxamate, these *relA* mutant *L. monocytogenes* strains still produced ppGpp (149), suggesting that its *relQ* or *relP* may contribute. Carbon starvation induced by alpha-methyl glucoside does not affect ppGpp levels in either WT or *relA* mutant *L. monocytogenes* (149). On the other hand, genetic screens seeking factors important for tolerance to low temperatures and high osmolarity have implicated RelA function in stress survival (118, 149). *L. monocytogenes* also tolerates cold temperatures, allowing survival on refrigerated food products. In *E. coli*, ppGpp levels are negatively correlated with the induction of cold shock genes following a shift to low growth temperatures (99). Likewise, *L. monocytogenes* strains that harbor a transposon insertion in *pghH*, encoding a putative metal-dependent phosphohydrolase (118), are cold sensitive and contain increased levels of ppGpp. Furthermore, at 4°C, stationary-phase *L. monocytogenes* cells contain lower levels of *relA* transcript than do their log-phase counterparts rather than the higher levels usually associated with stress tolerance (42). Therefore, *L. monocytogenes* may decrease its ppGpp pool to adapt to the cold temperatures encountered in refrigerated food products. The PghH phosphohydrolase may be a critical component of this mechanism by mediating the degradation of ppGpp in response to low-temperature conditions. Finally, ppGpp has also been implicated in *L. monocytogenes* biofilm growth: a *relA* mutant was defective for growth following ad-

herence to a surface, and *relA* transcription was induced upon surface attachment (196).

Intracellular survival and replication contribute to *L. monocytogenes* virulence, which sometimes causes fatal septicemia and meningitis. However, the contribution of ppGpp to the *in vivo* adaptation of *L. monocytogenes* appears to be complex and involves interactions with the pleiotropic regulator CodY. *L. monocytogenes* mutants lacking *relA* exhibit reduced survival in culture cell lines, including Caco-2 intestinal epithelial cells and J774.A1 macrophages (19). The defect appears to be specific to intracellular survival, as the mutant escapes the phagosome and induces host actin polymerization similarly to WT bacteria. The contribution of the stringent response to *L. monocytogenes* during infection is not yet clear, as two mouse infection studies reported conflicting results (149, 196). However, the *relA* mutant strain that maintained virulence also retained significant ppGpp synthetase activity (149), whereas the attenuated *relA* mutant assessed by Taylor et al. exhibited decreased ppGpp production (196). Studies of bacteria grown in broth and in cultured cells also indicated that ppGpp equips *L. monocytogenes* to adapt to conditions likely encountered during infection of the host (19, 149).

In Gram-positive bacteria, the global regulator CodY negatively regulates genes required for adaptation to low nutrient availability, stationary phase, and sporulation in response to levels of branched-chain amino acids and, in some cases, GTP pools (181). The CodY regulon of *L. monocytogenes* comprises genes involved in amino acid metabolism, nitrogen assimilation, sugar uptake and incorporation, and virulence; members of the regulon are also induced upon entry into stationary phase *in vitro* (19). The attenuation of an *L. monocytogenes relA* mutant may be due to its inability to express the virulence-associated CodY regulon, as this genetic circuit was continually repressed in a *relA* null mutant (19). Thus, an increase in ppGpp levels mediated by RelA may be necessary for the derepression of the CodY regulon under low-nutrient conditions, likely through the depletion of the GTP pool. However, the attenuation of the *relA* mutant cannot be explained solely by an inability to derepress the CodY regulon: the introduction of a *codY* mutation into a *relA* background does not fully restore virulence, and *codY* mutants are as fit as WT cells within cultured host cells. Thus, the effect of the ppGpp alarmone on *L. monocytogenes* pathogenesis appears to be multifactorial.

Enterococcus faecalis

Nosocomial enterococci such as *Enterococcus faecalis* cause diverse opportunistic infections ranging from urinary tract infections to life-threatening conditions such as meningitis and bacteremia. As such, this pathogen must have the capacity to adapt to many niches within a host. In addition, enterococci can tolerate inhospitable conditions common in clinical settings, such as sanitizer exposure and treatment with antibiotics, including vancomycin. Interestingly, *E. faecalis* accumulates ppGpp in response to numerous stresses typically encountered in a hospital, such as heat or alkaline shock, vancomycin exposure, and amino acid starvation (4).

E. faecalis harbors a bifunctional SpoT enzyme, termed RelA, and an SAS RelQ homologue. A *relA* mutant is more susceptible to stress conditions such as heat shock, low pH, high osmolarity, and oxidative stress, indicating that *relA*-dependent ppGpp synthesis contributes to stress tolerance (4). Since the inactivation of *relQ* in the *relA* mutant restores several WT phenotypes, the capacity of this RelA enzyme to hydrolyze ppGpp contributes to adaptation to such stresses. In contrast, vancomycin tolerance is enhanced in the *relA* mutant but reduced in either a *relQ* mutant or a *relA relQ* double mutant (4). Furthermore, the *relA* mutant is more resistant to bile salts, low pH, and ethanol treatment (218). High ppGpp levels may also reduce tolerance to oxidative stress while increasing vancomycin resistance. Thus, ppGpp likely has reciprocal effects on certain stress phenotypes in *E. faecalis*. It is conceivable that certain decontamination procedures inadvertently contribute to antimicrobial resistance by modulating ppGpp levels, a notion that warrants further investigation as the burden of nosocomial infections recalcitrant to treatment rises. Nonetheless, ppGpp appears to contribute to the virulence of *E. faecalis*, as judged by the observation that a $ppGpp^0$ (*relA relQ*) mutant was attenuated in a *Caenorhabditis elegans* infection model (4).

Streptococcus pyogenes

Group A streptococci (GAS) also adapt to a wide range of environments in the human host. Their versatility is exemplified by the variety of GAS-mediated infections, including impetigo (affecting skin), pneumonia, and meningitis. To do so, the expression of dedicated virulence factors, including secreted enzymes and exotoxins, is coordinated with factors involved in nutrient metabolism in a growth-phase-dependent manner, possibly utilizing ppGpp (45). Like other Gram-positive species, *Streptococcus pyogenes* harbors *relQ* and *relP* SAS homologues in addition to a bifunctional *spoT* homologue (114). Accordingly, GAS can mount a *relA*-independent stringent response (186, 187).

Although its *relA* transcript is induced 2-fold upon entry into stationary phase (45), much of the work exploring the *S. pyogenes* stringent response has focused on the *relA*-independent response to amino acid starvation. This pathway affects the expression of genes important for virulence. Examples include the growth-phase-controlled regulator *ropB*, which governs the expression of the secreted protease exotoxin B, encoded by *speB*; the two-component regulatory system *covRS* that controls the expression of streptolysin S, streptokinase (77), and exotoxin B; factors involved in the uptake and processing of oligopeptides, such as the *opp* and *dpp* permease systems; and the *pepB* processing gene (186). Amino acid starvation also induces the *fas* operon, which regulates virulence, and the gene encoding the autoinducer-2 production protein (187). Genes under negative stringent control and independent of *relA* include transporters, metabolic enzymes, and at least two virulence genes (126). Thus, ppGpp levels and their indirect influence on the activity of regulators such as CodY are predicted to modulate a wide array of accessory and dedicated virulence genes. Genetic and biochemical studies of the three stringent response enzymes and their target promoters can now examine the mechanistic details.

Streptococcus pneumoniae

Like enterococci and GAS, alpha-hemolytic streptococci such as *Streptococcus pneumoniae* and *Streptococcus suis* also exhibit broad tissue tropism, causing infections ranging from endocarditis to otitis media and bacteremia. *S. pneumoniae* is a notorious cause of pneumonia, and a signature-tagged mutagenesis screen identified a bifunctional *rel* gene as being essential for full virulence in a murine model of pneumococcal pneumonia (86). *S. pneumoniae* also encodes a RelQ homologue that was demonstrated to synthesize ppGpp in a heterologous system (17) but not in *S. pneumoniae* (103). The *rel*-dependent stringent response in *S. pneumoniae* strain D39 includes a significant induction of *ply* (103). This gene encodes a pneumolysin toxin that contributes to early infection and invasion from pulmonary tissues into the blood by both cytotoxic and proinflammatory mechanisms (46); *ply*-null *S. pneumoniae* cells are attenuated following intranasal challenge of mice (21). A *rel* mutant of D39 was also avirulent in a murine model of pulmonary infection (103), perhaps because of the aberrant expression of multiple genes, including those affecting metabolic enzymes (103). Although *ply* may also be regulated by other pathways, it is one example of the *rel*-dependent regulation of an *S. pneumoniae* virulence factor. The related pathogen *S. suis*, responsible for both infections of pigs and zoonotic disease of humans, upregulates the *relA* transcript upon iron limitation. Thus, ppGpp may also mediate the adaptation of *S. suis* to low-iron conditions encountered within the host (117).

Streptococcus mutans

Streptococcus mutans is a major contributor to dental caries. To adapt to the oral cavity, this pathogen forms biofilms, tolerates low pH and fluctuations in nutrient availability, and is flexible in the sugars that it metabolizes. The complexity of ppGpp metabolism in the firmicutes holds true for *S. mutans*: appropriate alarmone levels are maintained by an interplay between its three ppGpp synthetases, the monofunctional RelA protein and the bifunctional RelQ and RelP enzymes (114). The bacteria accumulate ppGpp by a *relA*-mediated pathway in response to isoleucine starvation, which is stimulated by mupirocin, a drug that inhibits isoleucine-tRNA synthetase (145). However, as for *E. faecalis*, an important role of *S. mutans* RelA may be to limit, through its hydrolase activity, the amount of ppGpp produced by RelP and RelQ (145).

To grow in the absence of branched-chain amino acids, *S. mutans* requires that RelP and RelQ maintain basal levels of ppGpp (115). CodY repression likely accounts for the poor growth of *relA* mutants in medium that lacks branched-chain amino acids, since the mutation of *codY* suppresses this growth defect. Like *L. monocytogenes*, *S. mutans* may utilize ppGpp to repress the CodY regulon (115). Thus, the interaction between ppGpp and the CodY regulator in *S. mutans* is further evidence for ppGpp control mechanisms that extend beyond the gammaproteobacterium paradigm. However, the molecular mechanism underlying CodY-dependent stringent control remains to be clarified for *S. mutans*, as its CodY appears to respond only to branched-chain amino acids (87) and not to GTP levels as in *L. monocytogenes*.

Expression profiling also indicates that ppGpp affects many loci associated with adaptation to the environment and the nutritional stress that *S. mutans* likely encounters in the oral cavity (145). Specifically, when starved for amino acids by mupirocin treatment, the bacteria alter the expression of genes involved in sugar metabolism, biofilm formation, and competence (145). However, some of these changes are *relA* independent, as judged by comparing the responses of WT and *relA* mutant cells (145). On the other hand, the *relA* mutant is defective for survival in medium containing high concentrations of carbohydrate, and it also dysregulates the catabolism of certain sugar substrates, such as mannose and inulin. The mutant also forms poor biofilms on hydroxyapatite; however, *relA* mutant biofilms do display increased acid tolerance, a pattern consistent with observations of *E. faecalis* (116). The intricacies of the interactions between the three ppGpp-metabolizing enzymes and their respective hydrolase and/or synthetase activities can be resolved by more detailed genetic and biochemical studies of *S. mutans* and other Gram-positive pathogens.

ALPHAPROTEOBACTERIA

Brucella spp.

The brucellae are Gram-negative alpha-2-proteobacteria that cause a disease known as brucellosis. In humans, this infection is characterized by a septicemic, febrile illness that is often persistent and sometimes fatal (76). *Brucella* can infect a wide variety of mammals ranging from sheep and goats (*B. melitensis*) to cattle (*B. abortus*) and hogs (*B. suis*), often causing abortion in pregnant females and sterility in males. Humans are exposed to *Brucella* during contact with contaminated products from livestock. As such, *Brucella* is a significant economic burden on the farm industry.

The ability of *Brucella* to cause disease requires bacterial replication and survival in host macrophages (76). Long-term survival in macrophages is marked by bacterial adaptation and modification of the vacuole into an ER-like compartment that is also suitable for propagation (41). Specifically, following uptake, *Brucella*-containing phagosomes avoid fusion with degradative lysosomes. This virulence strategy is mediated in part by the VirB T4SS. An acidic pH in the phagosome stimulates the full expression of VirB (41). Accordingly, it was proposed that decisions for vacuolar trafficking are not preprogrammed prior to entry; rather, they are made in response to conditions encountered within the vacuole. Their intracellular replication niche is thought to be nutrient limiting (107).

B. suis and *B. melitensis* each encode a 751-amino-acid RSH homologue designated *B. suis* Rsh (Rsh_{Bs}) and *B. melitensis* Rsh (Rsh_{Bm}). Each Rsh protein exhibits 36% identity to SpoT and 28% identity to RelA of *E. coli* K-12. These bifunctional enzymes are critical for intracellular survival, as *rsh* mutants of either pathogen exhibited intracellular growth defects in macrophages (59). In *B. melitensis*, Rsh is critical for persistence and adaptation in mice, as fewer rsh_{Bm} mutant bacteria than WT bacteria are recovered from the spleen at 4 weeks but not after 1 week of infection (59). Like its relatives, *B. abortus* encodes an Rsh enzyme (Rsh_{Ba}) critical for pathogenesis. Both replication and survival in mouse macrophage cultures require

Rsh_{Ba}. Likewise, fewer rsh_{Ba} mutant bacteria than WT bacteria are recovered from the spleens of mice 10 days after infection (104).

Although the signals triggering Rsh activity remain unknown, acidic vacuolar conditions may impact enzyme activity. In *B. abortus*, rsh_{Ba} transcription is induced by acid stress as assessed by RT-PCR analysis of broth cultures (104). Consistent with this model, *B. abortus* rsh_{Ba} mutants are more sensitive to acidic conditions in broth than WT bacteria. In contrast, Rsh is not critical for the acid resistance of *B. suis* and *B. melitensis* (59). Therefore, the *B. abortus* model cannot be applied to all species of *Brucella*.

While the accumulation of the ppGpp alarmone has not been observed directly, Rsh mediates a classical stringent response in *Brucella*. The *rsh* genes of *B. suis* and *B. melitensis* encode functional ppGpp synthetase enzymes, since the heterologous expression of either gene rescues the histidine auxotrophy of *relA* mutant *Sinorhizobium meliloti* (59). As discussed in detail below in the plant symbiont section, *S. meliloti* utilizes its bifunctional RelA enzyme to control a classical stringent response when growing in broth or in symbiosis with alfalfa (214). Similar functions of Rsh_{Bs} must be required during *B. suis* infection, as the expression of *S. meliloti relA* complements the intracellular growth defect of rsh_{Bs} mutants in macrophages (59).

An important aspect of *Brucella* pathogenesis is its ability to exert timely control over vacuolar trafficking events using the VirB T4SS. To ensure maximal expression, *Brucella* couples *virB* gene activation to ppGpp metabolism. The promoter activity of *virB* and accumulation of VirB proteins are maximal at the transition from the exponential phase to the stationary phase in broth (59). This starvation-dependent increase in levels of VirB requires ppGpp, as rsh_{Bm} mutant bacteria exhibit reduced levels of *virB* promoter activity at this transition, and VirB subunits are not detectable in either rsh_{Bs} or rsh_{Bm} mutant cultures. Additionally, WT *B. melitensis* cells that overexpress rsh_{Bm} in the exponential phase contain excess VirB. Together, these data suggest that in response to starvation, ppGpp induces VirB expression by increasing transcription at its promoter. Thus, the virulence defects of *rsh* mutant *Brucella* strains in mice may be partially attributed to their reduced levels of expression of the T4SS.

The role of ppGpp in VirB expression may provide insight into the phagosomal environment encountered by *Brucella* and other intravacuolar pathogens upon ingestion. In particular, *L. pneumophila* also relies on a T4SS-dependent mechanism to avoid degradative lysosomes and establish an ER-derived replication compartment. Perhaps, like *Brucella*, *L. pneumophila* also responds to intravacuolar stimuli to control its trafficking in host cells. Indeed, *L. pneumophila* requires its bifunctional synthetase/hydrolase, SpoT, to establish a replicative intracellular niche in macrophages (53).

EPSILONPROTEOBACTERIA

Campylobacter jejuni

Campylobacter jejuni is a major contributor to food-borne illness in humans. The colonization of the gastrointestinal tract with *C. jejuni* manifests as an intense inflammatory gastroenteritis termed campylobacteriosis. As a zoonotic pathogen, *C. jejuni* lives commensally in the gastrointestinal tract of avian species but causes significant illness when transmitted to susceptible humans. Thus, *C. jejuni* survives both outside and inside animals, where it tolerates fluctuations in osmolarity, temperature, and nutrient availability as well as the presence of antimicrobial agents such as bile salts and the host immune system. *C. jejuni* interacts intimately with host cells through mechanisms that promote adherence, transcytosis across the intestinal epithelium, and the invasion of nonphagocytic epithelial cells (64, 219). Following uptake by a distinct mechanism, *C. jejuni* resides intracellularly within a vacuole that subverts normal endosome trafficking (209). While it was initially thought that *C. jejuni* does not survive within host cells, it now appears that internalized bacteria maintain viability for at least 24 h. However, this survival likely requires that the bacteria differentiate to a distinct state, since *in vitro* culture is optimized by growing *C. jejuni* in an oxygen-limited environment (209). A physiological switch within host cells is thought to occur, such as a shift in respiration, which allows *C. jejuni* to adapt to its intracellular niche. Consistent with this, *C. jejuni* has evolved significant metabolic diversity that influences host colonization and tissue tropism (90).

As a microaerophilic bacterium, *C. jejuni* is relatively fragile during routine laboratory culture. Furthermore, the streamlined genome of *C. jejuni*, with a limited repertoire of regulatory genes, points to a host-restricted life-style (136). Nonetheless, because it also survives transmission between animal reservoirs and susceptible human hosts within food and water, *C. jejuni* must adapt to conditions outside animal hosts. Similar to adaptation to the vacuolar environment within intestinal epithelial cells, global changes in physiology, such as biofilm formation and differentiation into a VBNC form, may explain the surprising resilience of *C. jejuni* (193). In some manner, growth within a biofilm provides *C. jejuni* with the resources to tolerate conditions that are normally lethal to free-living cells, such as those in aquatic ecosystems and other intermediate reservoirs during transit between animal and human hosts (37).

Physiological changes that occur upon entry into stationary phase may contribute to the resilience of *C. jejuni* either in the environment, within a biofilm, or in host cell endosomes. Growth phase analysis of *C. jejuni* has identified a significant phenotypic switch upon entry into stationary phase. Its differentiation coincides with increased motility, the preferential utilization of specific amino acids, and an acetate switch mechanism (216). Moreover, in response to nutrient starvation, *C. jejuni* also shows both positive and negative changes in stress tolerance and pathogenicity (106). Taken together, these observations suggest that *C. jejuni* regulates the expression of survival and virulence factors at the onset of stationary phase. The *C. jejuni* genome encodes a relative paucity of regulatory elements, lacking alternative sigma factors such as RpoS and obvious cyclic di-GMP-metabolizing enzymes and harboring a limited number of two-component regulatory systems (151). This pattern is characteristic of host-restricted pathogens (136). *C. jejuni* does, however, encode a single bifunctional SpoT homologue, and a *spoT*-null mutant exhibits a $ppGpp^0$ phenotype (70). Together, these findings strongly suggest the capacity for a stringent response-mediated regulation of factors contributing to pathogenesis.

Expression profiling provided an early indication that the stringent response contributes to *C. jejuni* adaptation *in vivo*. Specifically, the *C. jejuni spoT* homologue was upregulated in a rabbit ileal-loop model (188), and a *spoT* mutant was significantly attenuated compared to the WT parental strain in competition under the same conditions (188). The *spoT* transcript is also upregulated in the presence of INT 407 intestinal epithelial cells, and the gene is potentially coregulated with other previously characterized virulence factors. These expression profiles, together with the Δ*spoT* mutant functional data described below (70), support the model that ppGpp mediates *C. jejuni*-host cell interactions.

Analysis of gene expression and host-related phenotypes of a Δ*spoT* (ppGpp^0) mutant likewise indicates that the stringent response is important to the adaptation of *C. jejuni* during interactions with host cells. The putative SpoT regulon, identified by microarray analysis, includes genes associated with nutritional adaptation, including phosphate uptake genes such as the *pstS* periplasmic phosphate binding protein. The expressions of numerous loci relating to redox balance and energy production are also affected by ppGpp levels. These include *nap*, encoding a periplasmic nitrate reductase; *nuo*, encoding the aerobic respiratory chain NADH dehydrogenase; and the operon composed of Cj0073c-Cj0075c, which includes genes with homology to an iron-sulfur oxidoreductase system. Furthermore, Cj0037, encoding a gene expressed more highly in high-O_2-adapted strains, also appears to be positively controlled by *spoT*. Taken together, these results are consistent with a *spoT*-dependent expression of genes associated with the adaptation of bacterial metabolism to changes in oxygen availability, such as the switch that was observed previously upon the adaptation of *C. jejuni* to the environment within host cells (209). Genes present on the putative virulence plasmid pVIR are also downregulated in the *spoT*-null strain, and stress response genes such as *groELS*, *dnaK*, *htrA*, and *clpB* are expressed at higher levels in the *spoT* mutant than in the WT strain (70). Whether ppGpp regulates each of these genes directly can now be investigated.

Consistent with the upregulation of *spoT* in the presence of epithelial cells, a Δ*spoT* mutant is defective for certain interactions with host cells, such as adherence, invasion, and intracellular survival in human epithelial cells. Phenotypic analyses also linked the *C. jejuni* stringent response to other aspects of pathogenesis. For instance, *C. jejuni* cells accumulate ppGpp upon a shift to nutrient-limited minimal medium in a *spoT*-dependent fashion (70). The *spoT* mutant also displays a stationary-phase survival defect, suggesting that some of the prototypical stringent response paradigms are conserved in *C. jejuni*. It is therefore surprising that the mutant tolerates numerous stresses as well as the WT. Nonetheless, the *spoT* mutant does exhibit key differences from the WT strain for phenotypes such as survival under low-CO_2/high-O_2 conditions. As *C. jejuni* is microaerophilic and capnophilic, the stringent response may regulate phenotypes particular to this pathogen and its life cycle.

Analysis of a *dksA*-deficient mutant also provides support for a role for ppGpp in controlling pathogenesis-related phenotypes in *C. jejuni* (220). Transcriptome and proteome comparisons of a *dksA* mutant to the WT showed changes in the expressions of amino acid- and iron metabolism-related genes. The *dksA* mutant is also impaired for the invasion of and induction of IL-8 secretion from epithelial cells *in vitro*, which further demonstrates the importance of the stringent response during the pathogenesis of *C. jejuni*.

Signal transduction pathways that control stress responses and biofilm formation also appear to be affected by ppGpp in *C. jejuni*. For example, compared to the WT, the *spoT* mutant exhibits markedly lower levels of polyphosphate upon entry into the stationary phase, linking ppGpp metabolism to polyphosphate in *C. jejuni* (40). Furthermore, a *ppk1* polyphosphate kinase mutant also accumulates less polyphosphate than the WT and is defective for osmotolerance and intracellular survival. Finally, both the *spoT* and the *ppk* mutants showed enhanced biofilm formation, suggesting that ppGpp and polyphosphate may be required to maintain a planktonic-growth life-style or promote biofilm dispersal in response to nutritional conditions in *C. jejuni* (40, 129). Biofilms have been proposed to contribute to the persistence of *C. jejuni* in aerobic environments, a trait that is surprising considering its fragility during planktonic growth in the laboratory (37). It follows that ppGpp and downstream regulatory networks contribute to the resilience of this pathogen.

Collectively, a SpoT-mediated stringent response is clearly important to the success of *C. jejuni* in environments encountered inside and outside animal hosts as well as during transmission to initiate zoonotic infection. Specific pathogenesis-related phenotypes, such as tolerance to aerobic atmospheres and low nutrient availability, appear to be *spoT* dependent in *C. jejuni*. Furthermore, the maintenance of ppGpp levels also appears to affect global shifts in bacterial physiology and life-style, which occur during biofilm formation. Thus, the dissemination of *C. jejuni* between commensal and susceptible hosts is potentially dependent on the stringent response pathway. Finally, gene expression analyses of *dksA* and ppGpp^0 *C. jejuni* mutants have demonstrated a perturbed regulation of metabolic genes. Therefore, the stringent response may also play a role in the metabolic shift that appears to occur upon adaptation to the endosome environment within host cells.

Helicobacter pylori

Up to 50% of the human population is thought to carry *Helicobacter pylori*, an epsilonproteobacterium that colonizes the gastric epithelium and leads to cellular changes that can result in ulcers and gastric cancer. In this harsh niche, *H. pylori* survives low pH, low iron and nutrient availability, and oxidative stress from phagocytic cells before entering the mucus layer, colonizing the epithelium, and interacting with host cells. During long-term colonization, the bacteria also subvert host immune responses. *H. pylori* adapts to the host environment despite a small genome and a minimal repertoire of regulatory elements. For example, *H. pylori* lacks alternative sigma factors such as RpoS and encodes few two-component regulatory systems. Furthermore, this pathogen was initially thought to lack a stringent response, as *H. pylori* cells did not accumulate ppGpp in response to pseudomonic acid-mediated isoleucine starvation (173) despite encoding a single bifunctional RelA/SpoT homologue (referred to as *spoT*).

H. pylori does exhibit a growth-phase-dependent regulation of virulence phenotypes, including the delivery of the effector

protein CagA and inducing the elongation of particular host cells during the transition into stationary phase (198). During this transition, the bacteria downregulate *spoT* and *gpp*, a gene encoding a guanosine pentaphosphate phosphohydrolase, as determined by microarray analysis. One potential untested interpretation is that the reduced SpoT hydrolase activity in the cell allows higher ppGpp levels to persist. It is also conceivable that, as a potential consequence of the lower GppA phosphohydrolase activity, a reduced conversion of pppGpp to ppGpp could extend the half-life of the alarmone. To date, ppGpp accumulation by *H. pylori* has been observed directly only in response to nutrient starvation and stress conditions (212). Nevertheless, the stationary-phase survival and morphology defects exhibited by Δ*spoT* ($ppGpp^0$) mutants (138) point to the importance of maintaining ppGpp levels during entry into stationary phase. At this transition, *H. pylori* also induces the expression of virulence factors such as flagellin and *napA*, encoding a homologue of Dps (DNA protection during starvation) that is thought to protect against oxidative stress damage (48). Therefore, the stringent response pathway likely coordinates virulence factor expression with growth phase.

A direct substantiation of a stringent response in *H. pylori* was the observation of a rapid accumulation of ppGpp upon nutrient downshift achieved by transferring cells to minimal MOPS (morpholinepropanesulfonic acid)-MGS medium (212). The activation and function of SpoT in *H. pylori* more closely resemble those of *E. coli* SpoT rather than those of RelA, as *H. pylori spoT* readily complements an *E. coli relA spoT* double mutant, more so than a single *relA* mutant (138). This attribute may explain the lack of stringent control in response to amino acid starvation previously reported; it also provides insight into the role of ppGpp in the response and adaptation to specific nutrient conditions. *H. pylori* requires serum for growth, and a *spoT* mutant grows to higher densities than the WT strain in serum-free medium, a trait mirroring the "relaxed" phenotype of *E. coli* mutants lacking ppGpp (224). SpoT function is also required to maintain the helical morphology of the bacteria in the absence of serum. Thus, *H. pylori* may require SpoT to limit its metabolic activity and growth appropriately under conditions of serum starvation, such as those that may be encountered in the microenvironment near gastric epithelial cells (224).

The ppGpp alarmone also influences the adaptation of *H. pylori* to challenges posed by the gastric environment during pathogenesis, including specific host-related phenotypes. An accumulation of ppGpp is induced during acid shock (212), and genetic studies indicated that SpoT function is required for the survival of acid exposure, stationary phase, and increased levels of oxygen (138). In addition to tolerance to low nutrient availability and high pH, the persistence of *H. pylori* during chronic infection of the gastric mucosa also depends on the avoidance of host innate immune components. For example, in macrophages, *H. pylori* interferes with the maturation of phagosomes and promotes the formation of large, more habitable, vacuoles called megasomes (8). Whereas Δ*spoT* mutants display WT levels of invasion, their ability to survive within macrophages is significantly reduced (224).

Whether a SpoT-mediated stringent response is required for the colonization of the gastric mucosa is unknown. It will be intriguing to learn whether the *H. pylori* stringent response modulates specific virulence factor genes such as *vacA*, urease, or the *cag* pathogenicity island. Reports of a growth-phase-dependent regulation of virulence traits (198) indicate that further analysis of the contribution of ppGpp to *H. pylori* colonization is warranted. Furthermore, the *H. pylori* requirement for polyphosphate for optimal colonization of mice (10, 195) suggests that, like in *C. jejuni*, the stringent response may intersect with polyphosphate metabolism to affect virulence and survival.

SPIROCHETES

Borrelia burgdorferi

Lyme disease, or borreliosis, is characterized by a systemic and sometimes relapsing or persistent infection initiated by the bite of an infected tick. As such, the zoonotic life-style of the *B. burgdorferi* spirochete involves both commensal relationships with insects and pathogenic interactions in susceptible human hosts. Throughout its life cycle, *B. burgdorferi* is exposed to many different environments. For example, *B. burgdorferi* must adapt to low-nutrient conditions present within arthropod hosts, which often go extended periods without feeding. Spirochetes must also tolerate unfavorable physicochemical conditions or evade the immune system during persistent infection of humans.

B. burgdorferi spirochetes can differentiate into cyst forms characterized by low metabolic activity and an altered surface antigen profile, promoting survival until conditions again support replication (28). Cyst formation in *B. burgdorferi* is stimulated by shifting spirochetes to unfavorable conditions, such as distilled water (28), extreme pH, high temperature, and peroxide (139). Serum starvation also induces differentiation into cysts, which revert to a spirochete morphology when serum is added to the medium (7). Accordingly, the encystation of *B. burgdorferi* in nutritionally demanding environments may be under regulatory control by ppGpp. Indeed, when spirochetes are cultured under conditions designed to mimic unfed ticks, their transcription profile has features of a stringent response (166). Cyst formation is also inhibited by tetracycline, suggesting that *de novo* protein synthesis is required for cyst development (7).

The minimal genome of *B. burgdorferi* harbors a single bifunctional *spoT* homologue (interchangeably called *B. burgdorferi rel* [rel_{Bbu}] and *spoT*) that is required for virulence in mice, indicating that *B. burgdorferi* pathogenesis is regulated by ppGpp (35). The alarmone likely contributes to nutritional adaptation, since during the growth of *B. burgdorferi* in medium with serum or in the presence of tick cells, the levels of both ppGpp and *spoT* (called rel_{Bbu}) mRNAs decrease (34). Conversely, *spoT* transcription is induced when spirochetes are cultured in medium supplemented with tick saliva (47). While this regulatory pattern is consistent with *spoT*-dependent ppGpp levels repressing growth when suitable nutrients are scarce, neither glucose nor amino acid starvation induces appreciable *spoT* expression by *B. burgdorferi* (35, 47). *B. burgdorferi* relies on serum as a source of fatty acids, which it cannot synthesize *de novo*. As a result, *B. burgdorferi* is sensitive to serum starvation (47), a condition that induces at least 20 proteins detectable by proteomic analysis. Therefore, it is con-

ceivable that a global regulatory mechanism mediated by *spoT* and ppGpp equips the spirochetes to adapt to fatty acid deprivation (7).

Beyond nuances under the conditions that trigger the stringent response of *Borrelia* spirochetes, the mechanisms of ppGpp-mediated regulation appear to have diverged from those of *E. coli*. Whereas the *B. burgdorferi* stringent response appears to contribute to adaptation to nutrient starvation and stationary phase (36), the stringent control of stable RNA synthesis does not correlate with ppGpp accumulation under starvation conditions in tick cells. Consistent with this difference, the region immediately downstream of the −10 hexamer of the 16S rRNA promoter region does not contain a GC discriminator motif characteristic of stringently controlled promoters in other bacteria (Fig. 3) (35). In addition, the accumulation of ppGpp does not affect *rpoS* expression in *B. burgdorferi* (36). Moreover, the RpoS sigma factor controls the expression of specific stress-related virulence determinants rather than global adaptation to stationary phase (38). Therefore, *B. burgdorferi* may have adapted the stringent response to suit needs specific to its unique life cycle (35), including the differential expression of specific proteins that promote adaptation while cycling between hosts (167). Nonetheless, the attenuation of *spoT* mutants in mice (35) underscores the contribution of ppGpp to *B. burgdorferi* fitness in the face of challenges encountered during infection.

PLANT PATHOGENS AND SYMBIONTS

Prokaryote-host cell interactions are not limited to those with animal cells or to interactions resulting in disease. Many bacteria form intimate relationships with plant hosts, with interactions ranging from symbiotic to commensal to pathogenic. Many of these interactions have a wide economic impact. Many factors important for plant cell interactions, such as quorum sensing (QS), are coordinated with growth phase and nutritional cues, often by the ppGpp alarmone. Well-characterized examples of such phenomena include those mediated by the single bifunctional SpoT homologues of genera such *Rhizobium*, *Sinorhizobium*, and *Agrobacterium*.

Nitrogen-fixing alphaproteobacteria, such as *Rhizobium etli* and *Sinorhizobium meliloti*, form symbiotic relationships with plant roots. In exchange for an environmentally and nutritionally suitable niche, the bacteria contribute nitrogenase activity that provides the plant with fixed nitrogen. *R. etli* forms nitrogen-fixing nodules on leguminous plants such as the common bean, and *Phaseolus vulgaris* and *S. meliloti* often colonize the roots of *Medicago* spp. such as alfalfa. These symbiotic bacteria are exposed to challenges such as high salinity and transient nutrient availability both prior to infection and during nodulation. In *R. etli* and *S. meliloti*, the bifunctional synthetase/hydrolase homologue, termed RelA, affects salt tolerance (25, 211). Furthermore, *relA* contributes to *R. etli* survival of heat shock, peroxide stress, and minimal medium (25) as well as nutritional competence (39).

In symbiotic rhizobia, ppGpp also affects phenotypes specifically related to interactions with the plant host during nodule formation. For example, an *R. etli relA* mutant fails to switch to a coccoid form, a morphology typical of WT nodule-residing bacteroids (25). Nodules produced in the host *P. vulgaris* by an *R. etli relA* mutant are smaller than those produced by the WT strain (39). Likewise, *S. meliloti relA* mutants produce few or no nodules, and the few bacteria that can be recovered from nodules harbor suppressor mutations in *rpoBC* (213, 214). Given that amino acid substitutions in the RpoBC subunits of *E. coli* RNA polymerase also suppress phenotypes normally activated by ppGpp (15), these genetic data implicate an alarmone in the regulation of *R. etli* nodulation. Any *relA* mutant-derived nodules formed by either symbiont are unable to fix nitrogen, and the *R. etli relA* mutant nodules exhibit lower nitrogenase activity than those formed by the WT strain (137). These *relA* mutant phenotypes indicate that ppGpp modulates metabolic pathways that are intimately related to its symbiotic relationship with its host. Specifically, the aberrant nodules may reflect a lower level of expression of *rpoN*-regulated genes that are required for nitrogen fixation (132), suggesting that ppGpp affects the expression of alternative sigma factors that promote interactions with the host.

During nodulation, the coordination of behavior between bacteria, as well as with the host, is critical. Communication ensures the correct temporal production of specific factors that contribute to host interactions, such as extracellular polymers. In *R. etli*, RelA positively controls the expression of the *cin*- and *rai*-encoded QS systems, thereby contributing to density-dependent adaptive responses related to symbiosis, such as nodulation and nitrogen fixation (137). Plant symbionts also communicate with host cells, initiating nodule formation by the secretion of lipochitooligosaccharide-based Nod factors. Interestingly, an *R. etli relA*-null mutant also produces Nod factors constitutively (39). The stringent response also coordinately regulates the production of specific effectors thought to stimulate nodule invasion, such as extracellular polysaccharides, as an *S. meliloti relA* mutant produces excess succinoglycan (214) and more readily forms biofilms (129).

Like symbionts, plant pathogens also utilize the stringent response to coordinate the expression of factors involved in host interactions. *Agrobacterium tumefaciens*, an alphaproteobacterium implicated in crown gall disease, relies on QS to induce the conjugal transfer of the virulence factor-encoding Ti plasmid. Once cells enter stationary phase, Ti plasmid conjugation is terminated by the AttM-mediated degradation of acyl homoserine lactone (AHL) quormones. *A. tumefaciens relA* mutants fail to induce the expression of *attM* upon entry into stationary phase (221). Additionally, the expression of the AldH succinic semialdehyde dehydrogenase, which also promotes quormone degradation through the succinic acid-dependent induction of AttM, appears to be negatively regulated by *relA* (206). These contrasting observations suggest a complex regulatory mechanism whereby RelA controls Ti plasmid transfer according to environmental conditions. Neighboring nonpathogenic bacteria that reside within the same tumor may provide a secondary source of AHL to modulate the virulence of plant pathogens. For example, coinhabitants of the genus *Novosphingobium* utilize a ppGpp-dependent pathway to produce AHL, which affects neighboring plant pathogens (69).

Nonalphaproteobacterial plant pathogens also exploit the stringent response to coordinate virulence. One example is the gammaproteobacterium *Erwinia carotovora* subsp. *atroseptica*, whose broad host range demands that the bacteria adapt to varied host environments. When nutrients become limiting, an

E. carotovora relA mutant is defective for motility, the production of the Pel and Prt exoenzyme virulence factors, and causing rot in potato tubers (207).

ppGpp^0 MUTANTS AS LIVE VACCINES

The striking virulence defects of ppGpp^0 mutant *Salmonella* and *Yersinia* strains in mice has motivated the application of these strains as live vaccines. Live, attenuated vaccines both confer long-lasting immunity and elicit niche-specific immune responses during infection (51, 165). A potential pitfall of using ppGpp^0 bacteria is the risk of residual pathogenicity. Nevertheless, work has shown that *S. enterica* serovar Typhimurium and *Y. pestis* mutants lacking the stringent response are highly efficacious at triggering immune responses and conferring protection against WT infection.

Oral and intraperitoneal inoculations of female BALB/c mice with ppGpp^0 mutant *S. enterica* serovar Typhimurium elicit a robust antibody response and confer protection against subsequent infection with WT bacteria (140). In these models, the LD_{50} of ppGpp^0 mutant bacteria is 10^5 times higher than that of WT bacteria irrespective of the oral or intraperitoneal inoculation route. The ppGpp^0 bacteria also colonize internal organs at numbers 2 to 3 logs lower than those of the WT. Both routes of infection trigger a significant serum and mucosal antibody response; however, intraperitoneal inoculation generates a more efficient systemic response than the oral route, which triggers a stronger mucosal response. The cell-mediated immune response of ppGpp^0-immunized mice is also robust, as inoculated mice subsequently exposed to WT bacterial lysates exhibited increased footpad swelling compared to their naïve counterparts. Finally, immunized mice were protected against WT challenge at 4 weeks postinoculation, as 95% of orally inoculated mice and 85% of intraperitoneally inoculated mice survived challenge with WT bacteria at CFU values 10^6-fold higher than the LD_{50} observed during infection of naïve mice (140). In comparison, 100% of nonimmunized control mice reached morbidity by 7 days postinfection. Taken together, these results strongly suggest that live vaccines consisting of ppGpp^0 mutant bacteria protect against *S. enterica* serovar Typhimurium infection.

In a murine model of bubonic plague, immunization with ppGpp^0 mutant *Y. pestis* induces a potent humoral immune response and provides full protection from subsequent bubonic challenge (190). Specifically, subcutaneous inoculation with ppGpp^0 mutant bacteria generates a robust serum antibody response to *Y. pestis* cell lysates. The mouse response to ppGpp^0 mutant *Y. pestis* is slightly biased toward a Th2 (antibody-mediated humoral) response rather than a Th1 (cell-mediated) response, as the levels of Th2-specific anti-*Y. pestis* antibodies were higher than those of Th1-specific antibodies. Accordingly, it is not surprising that ppGpp^0 mutant *Y. pestis* strains fail to induce the proinflammatory cytokines IFN-γ and TNF-α, as these hallmarks of a Th1 response are required to induce cell-mediated immunity during infection (190). Nevertheless, at 14 days postimmunization, 100% of mice were protected against subcutaneous (bubonic) challenge with WT *Y. pestis*, while 60% of mice were protected against intranasal (pneumonic) challenge. Hence, ppGpp^0 mutants are an effective *Y. pestis* live vaccine, providing excellent protection against the bubonic form and intermediate protection against pneumonic infection.

Indeed, the prospect of stringent response mutants as attenuated vaccines is promising, and work with *Salmonella* and *Yersinia* demonstrates their utility. It will be interesting to determine if ppGpp^0 mutants of *M. tuberculosis*, a pathogen that requires ppGpp for persistence in mice, also confer protection as a live vaccine (52). Whether such attenuated strains stimulate the appropriate arms of the immune system required to clear infection by each pathogen also warrants study. While preliminary work has no doubt provided evidence for the efficacy of ppGpp^0 strains in eliciting robust immune responses, further work is necessary to demonstrate the degree of attenuation, and, thus, the safety, of such stringent response-defective strains in humans.

CONCLUDING REMARKS

Investigations of bacteria grown in the laboratory in broth culture have generated a wealth of knowledge of bacterial pathogenesis. However, more recent studies emerging from the fields of cellular microbiology (bacterial-host cell interactions) and "social" microbiology (quorum sensing and biofilms) have significantly expanded our understanding of the pathogenic process. In particular, pathogen behavior is being examined under conditions that more closely resemble those encountered during infection as well as in the natural environment. Studies in these more complex settings have revealed that physiological adaptations to different growth conditions are intimately linked to the resilience and virulence of bacterial pathogens.

The distinct phenotypic profiles displayed by pathogens under different growth conditions indicate that changes in physiology can reflect a marked switch between two specialized cell types. Noteworthy examples include the differentiation between replicative and transmissive forms by facultative intracellular pathogens, such as *L. pneumophila*, and the shift between biofilm and free-swimming planktonic life-styles in pathogens like *P. aeruginosa*. The reciprocal pattern of expression of certain traits suggests that an activity that provides benefit during replication may decrease fitness when conditions deteriorate. As such, an efficient spatiotemporal regulation of factors that promote colonization or spread is critical.

The local nutrient supply is one factor that dictates which developmental and physiological programs confer benefit. The global design of the stringent response also allows bacteria to coordinate numerous factors that act in concert. Indeed, many pathogens rely on ppGpp to govern complex pathogenesis traits, such as constituents of the *Salmonella* SPI1 and SPI2 pathogenicity islands.

While many aspects of the stringent response paradigm discovered in classical studies of laboratory strains of *E. coli* hold true for bacterial pathogens, it has also become clear that some features of ppGpp-mediated gene regulation by pathogenic bacteria are distinct. For instance, it is generally observed that low ppGpp levels occur under nutrient-replete conditions and allow replication, whereas higher ppGpp levels induce growth arrest and new physiologies to increase tolerance to low-nutrient conditions and hostile environments. Thus, global shifts in physiology confer enhanced resilience and virulence to patho-

gens. In contrast to this general theme, a trend observed for pathogens that warrants further study is the observation that the appropriate control of pathogenesis-related phenotypes requires basal SpoT-dependent ppGpp pools but often not the RelA-dependent alarmone. An understanding of how the balance between SpoT hydrolase and synthetase activities is regulated, and the extent to which the hydrolase activity contributes to pathogenesis, will elucidate the contributions of SpoT to virulence. Furthermore, determining the degree to which the newly identified SAS enzymes such as RelP, RelQ, and RelV, some of which appear to maintain ppGpp levels during growth under nutrient-replete conditions, contribute to virulence- and survival-related phenotypes may also highlight potentially different roles for either basal or induced ppGpp pools in pathogenesis. Further studies of the diverse mechanisms of ppGpp metabolism, together with studies of specific virulence factors that are regulated by the stringent response, will continue to reveal new insights into transmission strategies and disease etiologies.

Finally, the conservation of mechanisms of ppGpp metabolism among bacteria, including significant pathogens from a variety of taxonomic divisions, together with the link of virulence and survival traits with the stringent response, warrants the consideration of this pathway as an antimicrobial target. RSH homologues are present in most bacterial pathogens that are not obligately intracellular. Furthermore, RelA- or SpoT-like enzymes have not yet been identified in humans. As discussed here, ppGpp-defective mutants have, so far, demonstrated potential as live vaccine strains. The body of work also demonstrates that the resilience of pathogens typically increases upon the induction of the stringent response, likely explaining the paradoxical success of organisms that are fragile when cultured under standard laboratory conditions. By this line of reasoning, antimicrobial agents that provoke the stringent response could inadvertently enhance the resilience of their target. Thus, studies evaluating antimicrobial tolerance mechanisms under stress conditions may be pertinent for an understanding of the emergence of resistance under selective pressure in a clinical setting. Numerous exciting and fruitful avenues of study of the link between virulence and the stringent response remain open. Likewise, regulatory pathways critical for carbon and nitrogen metabolism also impact virulence expression by a variety of pathogens (75, 158). Knowledge of how each pathogen's life-style serves its metabolic demands will both inform studies of pathogenesis and reveal new avenues for pathogen control.

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Zachary Dalebroux was born in Wisconsin and grew up in Madison, WI. He received his B.S. in Bacteriology from the University of Wisconsin—Madison in 2005. As an undergraduate research assistant with Dr. Juan de Pablo, he tested the effects of sugar-phosphate mixtures on liposome protection during lyophilization with the goal of developing effective formulas for protecting dehydrated biomaterials. In the laboratory of Dr. Kenneth Hammel, he investigated the mechanism by which brown rot fungi employ extracellular Fenton chemistry to generate hydroxyl radicals for biodegradation of lignocellulose. For his graduate studies at the University of Michigan, Ann Arbor, he joined the laboratory of Dr. Michele Swanson, focusing his research on mechanisms of bacterial gene regulation critical for pathogenesis. For his thesis, he studied the role of guanosine tetraphosphate (ppGpp) and DksA in controlling *Legionella pneumophila* transmission in macrophages. His work demonstrated that the bifunctional SpoT enzyme balances ppGpp pools to control both the replication and transmission of *L. pneumophila* in host cells. Subsequently, he determined that ppGpp cooperates and acts independently of DksA to control *L. pneumophila* virulence expression. After completing his Ph.D. in Microbiology and Immunology in 2010, he moved to the University of Washington, Seattle, for postdoctoral studies in the laboratory of Dr. Samuel Miller.

Sarah L. Svensson is a Ph.D. candidate in the Department of Microbiology and Immunology at the University of British Columbia. She was born and raised in Victoria, BC, Canada, and received her bachelor of science in biochemistry from the University of Victoria. While completing the Cooperative Education portion of her degree, she worked in laboratories across Canada on pathogens ranging from mycobacteria to parasites and was introduced to the stringent response while studying the regulation of the *E. coli* RelA ppGpp synthetase and the RelEB toxin-antitoxin system under Edward Ishiguro at the University of Victoria. She joined the laboratory of Erin Gaynor in Spring 2005 to study the pathogenesis of the food-borne pathogen *Campylobacter jejuni*, where she has continued her interest in the relationship between bacterial stress tolerance and pathogenesis. Specifically, she studies both environmental gene regulation by two-component regulatory systems and the mechanisms of biofilm formation in *C. jejuni* in the hopes of understanding the paradoxical success of an apparently fastidious bacterial pathogen.

Erin C. Gaynor is an Associate Professor and Canada Research Chair in the Department of Microbiology and Immunology at the University of British Columbia. Originally from California, she obtained her bachelor's degree from the University of California at San Diego (UCSD), majoring in Cell Biology/Biochemistry and Literature. Following graduation, she spent two years working as a research associate for Hybritech Incorporated, helping to develop monoclonal antibody technology for therapeutic applications. She then entered the UCSD Biology Department Ph.D. program and earned her graduate degree with Scott Emr, studying signals and molecules involved in Golgi-ER trafficking in *Saccharomyces cerevisiae*. She next combined her interests in molecular cell biology and human health by pursuing postdoctoral research in bacterial pathogenesis with Stanley Falkow at Stanford University. At Stanford, she initiated studies on the enteric pathogen *Campylobacter jejuni*, including those which led her, her colleagues, and her group at the University of British Columbia to identify and characterize the stringent response in both *C. jejuni* and the related human pathogen *Helicobacter pylori*. Her laboratory continues to explore the pathogenesis and stress survival mechanisms of *C. jejuni*, with the ultimate goal of unraveling how this prevalent pathogen causes such severe and widespread human disease.

Michele S. Swanson, a Professor in the Department of Microbiology and Immunology at the University of Michigan Medical School, was born and raised in the Midwest. She earned a B.S. in Biology from Yale, where she also played collegiate field hockey and softball. As a research technician at the Rockefeller University, she was introduced to the exciting world of experimental science in the laboratory of Samuel C. Silverstein, an expert in leukocyte cell biology who contributed to seminal studies of *Legionella pneumophila* growth in macrophages. She developed her love of genetics as a graduate student, using *Saccharomyces cerevisiae* as a tool to study gene expression with Marian Carlson at Columbia and Fred Winston at Harvard. After a brief hiatus devoted to her two children, she trained as a postdoctoral fellow with Ralph Isberg at Tufts and Howard Hughes Medical Institute (HHMI), where she developed cell biological methods to analyze the fate of *L. pneumophila* in macrophages. In addition to exploiting this pathogen as a genetic probe of macrophage function, her laboratory investigates how metabolic cues govern its virulence expression. At the University of Michigan, Dr. Swanson has had the privilege and pleasure of mentoring several gifted Ph.D. students; together, they developed the *L. pneumophila* paradigm described here.