JB Journals.ASM.org

The Bacterial Alarmone (p)ppGpp Activates the Type III Secretion System in *Erwinia amylovora*

Veronica Ancona,[a]* Jae Hoon Lee,[a] Tiyakhon Chatnaparat,[a] Jinrok Oh,[b] Jong-In Hong,[b] Youfu Zhao[a]

Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA[a]; Department of Chemistry, Seoul National University, Seoul, South Korea[b]

ABSTRACT

The hypersensitive response and pathogenicity (*hrp*) type III secretion system (T3SS) is a key pathogenicity factor in *Erwinia amylovora*. Previous studies have demonstrated that the T3SS in *E. amylovora* is transcriptionally regulated by a sigma factor cascade. In this study, the role of the bacterial alarmone ppGpp in activating the T3SS and virulence of *E. amylovora* was investigated using ppGpp mutants generated by Red recombinase cloning. The virulence of a ppGpp-deficient mutant (ppGpp^0) as well as a *dksA* mutant of *E. amylovora* was completely impaired, and bacterial growth was significantly reduced, suggesting that ppGpp is required for full virulence of *E. amylovora*. Expression of T3SS genes was greatly downregulated in the ppGpp^0 and *dksA* mutants. Western blotting showed that accumulations of the HrpA protein in the ppGpp^0 and *dksA* mutants were about 10 and 4%, respectively, of that in the wild-type strain. Furthermore, higher levels of ppGpp resulted in a reduced cell size of *E. amylovora*. Moreover, serine hydroxamate and α-methylglucoside, which induce amino acid and carbon starvation, respectively, activated *hrpA* and *hrpL* promoter activities in *hrp*-inducing minimal medium. These results demonstrated that ppGpp and DksA play central roles in *E. amylovora* virulence and indicated that *E. amylovora* utilizes ppGpp as an internal messenger to sense environmental/nutritional stimuli for regulation of the T3SS and virulence.

IMPORTANCE

The type III secretion system (T3SS) is a key pathogenicity factor in Gram-negative bacteria. Fully elucidating how the T3SS is activated is crucial for comprehensively understanding the function of the T3SS, bacterial pathogenesis, and survival under stress conditions. In this study, we present the first evidence that the bacterial alarmone ppGpp-mediated stringent response activates the T3SS through a sigma factor cascade, indicating that ppGpp acts as an internal messenger to sense environmental/nutritional stimuli for the regulation of the T3SS and virulence in plant-pathogenic bacteria. Furthermore, the recovery of an *spoT* null mutant, which displayed very unique phenotypes, suggested that small proteins containing a single ppGpp hydrolase domain are functional.

Erwinia amylovora causes a devastating fire blight disease of apples and pears, which results in severe economic losses to growers around the world (1, 2). *E. amylovora* is closely related to members of the *Enterobacteriaceae* family, including many important human pathogens, such as *Escherichia coli*, *Yersinia pestis*, and *Salmonella enterica* (3). Studies have revealed that the hypersensitive response and pathogenicity (*hrp*) type III secretion system (T3SS) is a major pathogenicity factor in *E. amylovora* (4–7). The *hrp* T3SS genes are carried on a pathogenicity island (8), and the alternative sigma factor HrpL, a member of the ECF subfamily of sigma factors, serves as the master regulator to control the expression of the structural and effector genes by binding to a consensus sequence known as the *hrp* box (9–13). In turn, expression of *hrpL* is positively regulated by the sigma 54 (σ^{54}) protein RpoN, its modulation protein YhbH, and HrpS, a member of the NtrC family of σ^{54} enhancer binding proteins (EBPs) (4, 9, 11). However, the molecular mechanism that triggers the T3SS or activates the sigma factor cascade in *E. amylovora* remains unknown.

Upon initiating plant infection, plant-pathogenic bacteria undergo tremendous stresses, especially limiting nutrient stress and oxidative stress. T3SS genes are believed to be expressed rapidly under conditions such as limited nutrition (minimal medium), low pH, and relatively low temperature and are induced *in planta* or by iron but repressed in rich media (14–16). These observations suggest that nutrient limitation and/or oxidative stress may be one of the primary factors that activate the sigma factor cascade and trigger the expression of the T3SS. However, the exact environmental/host signal(s) remains elusive.

As one of the global regulatory systems in bacteria, the stringent response often results in swift and massive transcriptional reprogramming in response to various nutrient limitation conditions (17, 18). During the stringent response, bacterial cells accumulate high levels of the linear nucleotide second messengers, i.e., guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) [collectively known as (p)ppGpp; referred to here as ppGpp] (19). In bacteria, the RelA-SpoT homologue (RSH) proteins are responsible for (p)ppGpp biosynthesis and degradation in response to nutrient starvation, e.g., lack of amino acids, phosphates, fatty acids, carbon, or iron, similar to the conditions activating the T3SS (17, 20). In *E. coli*, RelA is a ribosome-

Received 5 December 2014 **Accepted** 2 February 2015

Accepted manuscript posted online 9 February 2015

Citation Ancona V, Lee JH, Chatnaparat T, Oh J, Hong J-I, Zhao Y. 2015. The bacterial alarmone (p)ppGpp activates the type III secretion system in *Erwinia amylovora*. J Bacteriol 197:1433–1443. doi:10.1128/JB.02551-14.

Editor: I. B. Zhulin

Address correspondence to Youfu Zhao, zhao888@illinois.edu.

* Present address: Veronica Ancona, Texas A&M University-Kingsville Citrus Center, Weslaco, Texas, USA.

doi:10.1128/JB.02551-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. amylovora strains		
Ea1189	Wild type; isolated from apple	72
Δ*relA* mutant	*relA*::Cm; Cm^r insertional mutant of *relA* of Ea1189; Cm^r	This study
Δ*spoT* mutant	*spoT*::Cm; Cm^r insertional mutant of *spoT* of Ea1189; Cm^r	This study
Δ*dksA* mutant	*dksA*::Cm; Cm^r insertional mutant of *dksA* of Ea1189; Cm^r	This study
Δ*relA/spoT* mutant	*relA*::Cm *spoT*::Km; Km^r insertional mutant of *spoT* into Δ*relA* mutant	This study
Δ*relA/dksA* mutant	*relA*::Cm *dksA*::Km; Km^r insertional mutant of *dksA* into Δ*relA* mutant	This study
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL nupG* λ	Invitrogen, CA
Plasmids		
pKD46	Ap^r P_{BAD} *gam bet exo* pSC101 oriTS	42
pKD32	Cm^r FRT *cat* FRT tL3 oriR6Kγ *bla rgnB*	42
pkD13	Km^r FRT *kan* FRT tL3 oriR6Kγ *bla rgnB*	42
pWSK29	Ap^r; cloning vector; low copy number	73
pRelA	3.0-kb SacI-KpnI fragment including the *relA* gene in pWSK29	This study
pSpoT	2.8-kb SacI-KpnI fragment including the *spoT* gene in pWSK29	This study
pDksA	1.0-kb SacI-KpnI fragment including the *dksA* gene in pWSK29	This study
pFPV25	Ap^r; GFP-based promoter trap vector containing promoterless *gfpmut3a* gene	74
pZW2(HrpL)	608-bp KpnI-XbaI DNA fragment containing promoter sequence of *hrpL* gene of Ea1189 in pFPV25	75
pHrpA-GFP	708-bp EcoRI-BamHI DNA fragment containing promoter sequence of *hrpA* gene in pFPV25	40
pHrpA-His6	803-bp DNA fragment containing promoter sequence of *hrpA* gene and C-terminal His tag coding sequence in pWSK29	This study
pKH91	ori15A gfp_{uv} *bla* Ap^r *tet* Tc^r	76

associated monofunctional protein that synthesizes ppGpp by using ATP and GTP in response to amino acid starvation and is sensed by the presence of uncharged tRNA molecules in the A site of a ribosome (21, 22). In contrast, the cytoplasmic SpoT protein has both ppGpp synthase and hydrolase activities and is activated in response to a lack of fatty acids, carbon, phosphorus, or iron, as well as hyperosmotic shock and oxidative stress (22, 23).

In *E. coli*, the ppGpp-mediated stringent response redirects the global transcriptional capacity of a cell from genes responsible for growth and reproduction toward those responsible for survival (17). Interactions among RNA polymerase (RNAP), ppGpp, and its partner transcription factor DksA result in downregulation of highly expressed stable RNA, DNA replication, ribosome and protein synthesis, and simultaneous upregulation of stress and starvation genes as well as virulence genes (20, 24, 25). In general, ppGpp directly interacts with the interface of the β′ and ω subunits of RNAP to orchestrate fine-tuning of cellular processes through direct inhibition and activation of genes (26–28), whereas the DksA protein, which binds to the RNAP secondary channel, greatly enhances the effect of ppGpp by modulating the direct interaction between RNAP and ppGpp (18, 28). In addition, ppGpp inhibits RNAP binding to σ^{70}-dependent stringent promoters, thus indirectly allowing RNAP to bind to alternative sigma factors, such as RpoN, and promoting the expression of alternative sigma factor-dependent genes (19, 29). Importantly, inhibition of σ^{70} binding by ppGpp is transient and reversible, thus enabling rapid and reversible control of stress response genes (17).

In *S. enterica*, accumulated ppGpp induces HilA, a master regulator of *Salmonella* pathogenicity island 1 (SPI1). Furthermore, ppGpp directly interacts with SlyA, a transcriptional activator of pathogenicity island 2 (SPI2), to facilitate the intracellular virulence program of *S. enterica* (30, 31). In *E. coli*, accumulation of ppGpp activates *LEE* gene expression and increases bacterial adherence (32). In plant-associated pseudomonads and rhizobia, ppGpp affects epiphytic fitness, biocontrol activity, biofilm formation, and hydrogen peroxide and antibiotic tolerance, as well as nodulation (33–36). In plant-pathogenic bacteria, ppGpp is required for cell wall-degrading enzyme production, quorum sensing signal degradation, and Ti plasmid transfer (37–39). However, it remains unknown whether ppGpp regulates the T3SS and virulence in plant-pathogenic bacteria.

The goal of this study was to determine whether ppGpp regulates T3SS gene expression and virulence in *E. amylovora*. Our results demonstrate that ppGpp and DksA play central roles in *E. amylovora* virulence and suggest that *E. amylovora* utilizes ppGpp as an internal messenger to sense environmental/nutritional signals for regulation of the T3SS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. LB broth was used for routine growth of *E. amylovora* and *E. coli* strains. An *hrp*-inducing minimum medium (HMM) [1g $(NH_4)_2SO_4$, 0.246 g $MgCl_2 \cdot 6H_2O$, 0.1 g NaCl, 8.708 g K_2HPO_4, 6.804 g KH_2PO_4] with 10 mM galactose as the carbon source was used to induce T3SS gene expression (4, 40). MBMA minimal medium [3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 ml glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$] supplemented with 1% sorbitol (7, 41) was also used. When required, antibiotics were used at the following concentrations: 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} ampicillin, and 10 μg ml^{-1} chloramphenicol. Primer sequences used for mutant construction, mutant confirmation, quantitative real-time PCR (qRT-PCR), and cloning are available upon request.

Generation of single and double mutants by λ Red recombinase cloning. *E. amylovora* mutant strains were generated using the λ phage recombinase method as described previously (6, 7, 42). Briefly, overnight cultures of *E. amylovora* strains harboring pKD46 were inoculated into LB broth containing 0.1% arabinose and grown to exponential phase (optical density at 600 nm [OD_{600}] = 0.8). Cells were harvested, made electrocompetent, and stored at −80°C. These cells were electroporated with recombination fragments containing a *cat* or *kan* gene with its own promoter flanked by a 50-nucleotide homology region from the target gene(s). Recombination fragments were obtained by PCR amplification from the pKD32 or pKD13 plasmid, respectively. To confirm *relA*, *spoT*, and *dksA* mutations, PCR amplifications from the internal *cat* or *kan* primers to the external region of the target genes were performed. The coding regions of the *relA*, *spoT*, and *dksA* genes were absent from the corresponding mutant strains, except for the first and last 50 nucleotides. Double mutants were generated by using single mutants as the background.

Construction of plasmids. For complementation of the mutant strains, the genomic regions containing the promoter and gene sequences of the *relA*, *spoT*, and *dksA* genes were PCR amplified, gel purified, cut with KpnI and SacI, and ligated into the pWSK29 plasmid digested with the same enzymes. Standard molecular procedures were performed (43). Plasmid verification was performed by sequencing at the UIUC Core Sequencing Facility. The resulting plasmids were designated pRelA, pSpoT, and pDksA and transformed by electroporation into the *relA*, *spoT*, and *dksA* single and double mutant strains. For Western blot assay, the *hrpA* gene with a six-His tag coding sequence at the C terminus was cloned into the pWSK29 plasmid by use of KpnI and EcoRI restriction sites. The resulting plasmid was verified by sequencing and designated pHrpA-6His.

ppGpp quantification. ppGpp measurements were performed as previously described (44, 45), with some modifications. Briefly, overnight cultures of wild-type (WT) and mutant strains were washed three times with HMM and inoculated into 50 ml of HMM to a final OD_{600} of 0.2. Cells were incubated for 2 h with shaking at 18°C. Cultures were harvested by centrifugation, and 3 ml of 100% methanol was added to the pellets and mixed by vortexing for 50 s. To remove cell debris, suspensions were centrifuged and supernatants were freeze-dried at −50°C overnight. After drying, samples were dissolved in double-distilled water (ddH_2O) and mixed with a fluorescent chemosensor [pyrene (Py) plus bis(Zn^{2+}-dipicolylamine) (DPA) (PyDPA)] to a final concentration of 10 μM, and fluorescence was measured in a microplate reader with excitation at 365 nm and emission at 470 nm. ppGpp concentrations were calculated by comparison to a standard curve created with purified ppGpp (TriLink Biotechnologies Inc., CA). This experiment was performed twice.

Epifluorescence microscopy. Cell sizes of *E. amylovora* strains in rich and minimal media were determined by epifluorescence microscopy. Overnight cultures of bacterial strains constitutively expressing green fluorescent protein (GFP) were harvested, washed, and transferred to LB broth or HMM. Following 4 h of incubation, 3 μl of bacterial suspension was mixed with 5 μl of Aqua-Polymount (Polysciences, Warrington, PA), mounted on a coverslip, and immediately observed under an Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany), using a fluorescein isothiocyanate (FITC) filter set with absorbance at 490 to 494 nm and emission at 517 nm. Images were captured with an AxioCam MPc digital camera. ImageJ software was used to analyze the cell lengths of at least 200 individual cells from 10 different images.

Virulence and bacterial growth assays. Virulence assays on apple trees were performed as described previously (46). Briefly, overnight cultures of *E. amylovora* WT and mutant strains were harvested by centrifugation and suspended in 0.5× phosphate-buffered saline (PBS). Cell suspensions were adjusted to an OD_{600} of 0.1 in PBS and inoculated onto seven actively growing cv. 'Gala' apple shoots by pricking the tip with a sterile needle and pipetting 5 μl of bacterial inoculum onto the tip. Symptom development was recorded at 7 days postinoculation (dpi), and the experiment was performed at least two times.

For virulence and bacterial growth assays, immature pear fruits (*Pyrus communis* L. cv. 'Bartlett') were surface sterilized with 10% bleach, pricked with a sterile needle, and inoculated with 2 μl of a 100× dilution of bacterial suspension at an OD_{600} of 0.1 in PBS (12, 13). The tissue surrounding the inoculation site was excised with a no. 4 cork borer and homogenized in 1 ml of 0.5× PBS. Bacterial growth within the pear tissue was monitored at 1, 2, and 3 dpi by dilution plating on LB medium with appropriate antibiotics. For each time point and strain tested, fruits were assayed in triplicate. Symptom development was recorded at 4 and 8 days postinoculation, and the experiment was performed three times.

Hypersensitive response (HR) assay. Overnight cultures of *E. amylovora* WT, mutant, and complementation strains were harvested by centrifugation and suspended in 0.5× PBS to an OD_{600} of 0.1. Bacterial suspensions were infiltrated into tobacco leaves (*Nicotiana tabacum*) by use of a needleless syringe. HR symptoms were recorded at 24 h postinfiltration, and the experiment was repeated three times.

RNA isolation. Bacterial strains grown overnight in LB medium with appropriate antibiotics were harvested by centrifugation and washed three times before being inoculated into 5 ml of HMM, to a final OD_{600} of 0.2. After 3 h of incubation at 18°C in HMM, 2 ml of RNAprotect reagent (Qiagen) was added to 1 ml of bacterial cell culture, mixed by vortexing, and incubated at room temperature for 5 min. Cells were harvested by centrifugation, and RNA was extracted using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase I treatment was performed with a Turbo DNA-free kit (Ambion, Austin, TX), and RNA was quantified using a NanoDrop ND100 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For *in vivo* conditions, overnight cultures of bacterial strains were harvested by centrifugation, washed three times, and resuspended in PBS. Immature pear fruits were cut in half and inoculated with bacterial suspensions. After 3 h of incubation at 28°C in a moist chamber, bacterial cells were collected by washing pear surfaces with RNAprotect reagent (Qiagen) mixed 2:1 with water, and RNA was extracted as described above.

qRT-PCR. One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. One microliter of cDNA was used as the template for qPCR, using a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). Power SYBR green PCR master mix (Applied Biosystems) was used to detect the expression of selected genes amplified with primers designed using Primer3 software. Amplifications were carried out by incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curve analysis was performed after the program was completed to confirm the amplification specificity. Three technical replicates were performed for each biological sample. Relative gene expression was calculated by the relative quantification ($\Delta\Delta C_T$) method, using the *rpoD* gene as an endogenous control.

Western blotting. *E. amylovora* cells grown in HMM at 18°C for 6 h were harvested, and equal amounts of cell lysates were separated in sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and blocked with 5% milk in PBS. To detect HrpA-6His, membranes were probed with rabbit anti-His antibodies (GeneScript, Piscataway, NJ) that were diluted to 1.0 μg/ml with PBS containing 0.1% Tween 20 (PBST). Immunoblots were then developed with horseradish peroxidase-linked anti-rabbit IgG antibodies (Amersham Biosciences), diluted 1:10,000 in PBST, followed by enhanced chemiluminescence reagents (Pierce). Images of the resulting blots were acquired using an ImageQuant LAS 4010 charge-coupled device (CCD) camera (GE Healthcare).

Flow cytometry analysis. Bacterial strains containing promoter-GFP fusion plasmids were grown overnight in LB medium, washed three times with HMM, and inoculated into HMM containing 10 mM galactose to a final OD_{600} of 0.2 (40, 47). DL-Serine hydroxamate (SHX) and α-methylglucoside (αMG) were added to cells, to final concentrations of 0.1 mM and 0.5%, respectively. GFP intensities were measured by flow cytometry (BD Biosciences, San Jose, CA) after incubation at 18°C for 18 h. Flow

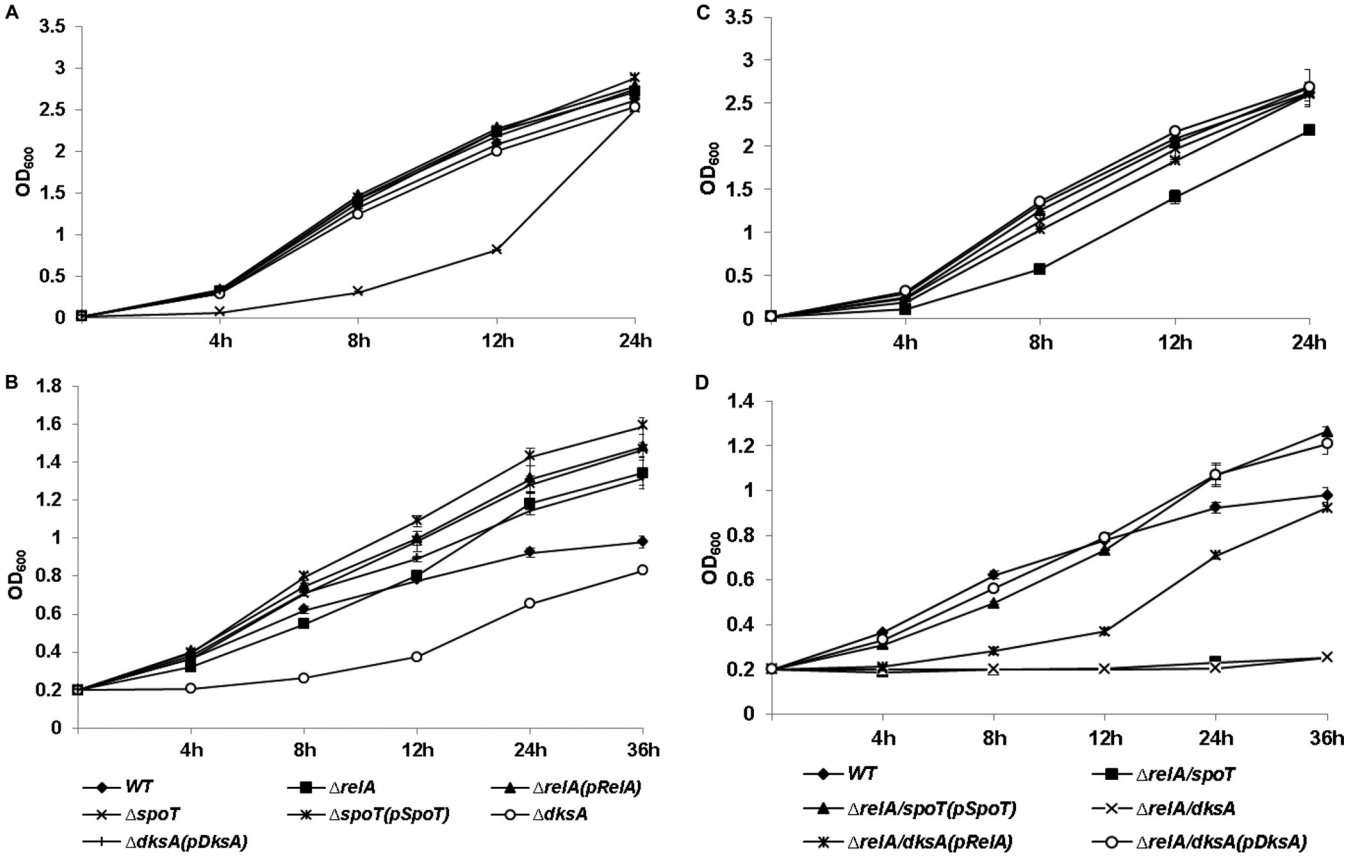

FIG 1 Growth of *Erwinia amylovora* is affected by DksA and ppGpp. The graphs show the growth of the *E. amylovora* WT strain, the *relA*, *spoT*, and *dksA* single mutants, and their corresponding complementation strains in LB (A) and MBMA (B) media and the growth of the *E. amylovora* WT strain, the *relA/spoT* ($ppGpp^0$) and *relA/dksA* double mutants, and their corresponding complementation strains in LB (C) and MBMA (D) media. The experiments were repeated at least two times with similar results.

cytometry was performed on a BD LSRII 10 parameter multilaser analyzer (BD Bioscience, San Jose, CA). Data were collected for a total of 100,000 events and analyzed statistically by gating using the flow cytometry software FCS Express V3 (De Novo Software, Los Angeles, CA). The geometric mean was calculated for each sample. Each treatment was performed in triplicate, and each experiment was repeated three times.

RESULTS

Growth of *Erwinia amylovora* is regulated by ppGpp and DksA. Based on the genome sequence of *Erwinia amylovora* (48), two annotated genes were found to encode RelA-SpoT homologue (RSH) proteins, i.e., *Eam_2706* (*relA*) and *Eam_0043* (*spoT*), whereas *Eam_0793* encodes DksA. During mutant construction, growth of the *spoT* mutant was observed to be very slow in rich medium, which is consistent with observations in *E. coli*, where the *spoT* deletion mutant is lethal due to accumulation of high levels of ppGpp (23). An *spoT* deletion mutant has also been reported for *Pseudomonas fluorescens* (35).

Growth rates of the WT and five mutant strains in rich (LB) and minimal (MBMA) media were determined. In LB medium, growth rates of the WT and four mutants (the *relA*, *dksA*, *relA/spoT*, and *relA/dksA* mutants) were similar, with the *spoT* mutant exhibiting slower growth at the beginning but reaching a level similar to that of the WT at 24 h (Fig. 1A and C). When the *spoT* gene was provided in *trans*, slow growth of the *spoT* mutant was restored (Fig. 1A). In contrast, growth rates of the *spoT* and *relA* mutants were similar to that of the WT in MBMA medium (Fig. 1B), but growth of the *dksA* mutant was much reduced in MBMA medium, and its growth could be complemented by expressing the *dksA* gene in *trans* (Fig. 1B). Furthermore, both the *relA/spoT* and *relA/dksA* mutants were unable to grow in MBMA medium (Fig. 1D). Complementation of the *relA/spoT* mutant with the *spoT* gene in *trans* recovered its growth, whereas complementation with the *relA* gene was unsuccessful, possibly due to ppGpp accumulation. Complementation of the *relA/dksA* mutant with the *dksA* gene in *trans* recovered its growth, whereas complementation with the *relA* gene in *trans* partially recovered its growth (Fig. 1D). These results suggest that DksA and ppGpp play roles in regulating cell growth under both nutrient-rich and nutrient-limited conditions.

Intracellular ppGpp levels in *E. amylovora* WT and mutant strains were quantified in HMM by using the selective and sensitive fluorescent chemosensor PyDPA for ppGpp as reported previously (44, 45). PyPDA contains pyrene (Py) and bis(Zn^{2+}-dipicolylamine) (DPA), the latter of which is well known for its strong binding to pyrophosphate groups in water (45). We found that the ppGpp level was slightly increased (10% higher) in the *spoT* mutant, while the ppGpp level was about 7 times lower in the *relA* mutant than in the WT. Accumulation of ppGpp in the *dksA* mutant was about half that in the WT (Fig. 2). Furthermore, ppGpp

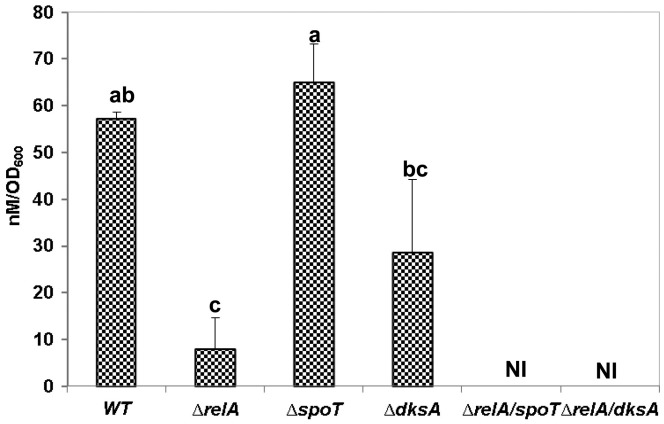

FIG 2 ppGpp measurement. Intracellular ppGpp levels in *Erwinia amylovora* WT and *relA*, *spoT*, *dksA*, *relA/spoT*, and *relA/dksA* mutant strains were quantified in HMM by using the fluorescent chemosensor PyDPA as reported previously (44, 45). One-way analysis of variance (ANOVA) and Student's *t* test ($P = 0.05$) were used to analyze the data. Values marked with the same letter were not significantly different ($P < 0.05$). NI, not included in statistical analysis. This experiment was performed twice.

levels in the *relA/dksA* and *relA/spoT* mutants (the latter is referred to as ppGpp^0 from this point on) were undetectable (Fig. 2). These findings indicate that both RelA and SpoT are required for ppGpp synthesis in HMM and also suggest that RelA plays a major role in ppGpp synthesis.

Mutations in *relA* and *spoT* of *Erwinia amylovora* result in altered cell lengths. When *E. coli* ppGpp mutant cells were grown under isoleucine starvation conditions, they were found to be considerably longer than WT cells (49, 50). The cell sizes/lengths of *E. amylovora* WT and mutant strains constitutively expressing GFP and grown in LB medium or HMM were observed by epifluorescence microscopy (Fig. 3A and B). When cells were grown in nutrient-rich LB medium, the average length of WT cells was about 2.23 μm, whereas the average lengths of the *relA* and ppGpp^0 mutant cells were slightly or much longer, reaching 2.65 μm and 3.87 μm, respectively. In contrast, the average length of the *spoT* mutant cells was 1.94 μm, which is slightly shorter than that of the WT (Fig. 3). However, the lengths of the majority of the *spoT* mutant and WT cells were the same (Fig. 3B). When cells were grown in nutrient-limited HMM, they were shorter than those grown in LB medium, except for the *spoT* mutant (Fig. 3A and B). While the average lengths of the *relA* and ppGpp^0 mutant cells reached 2.37 μm and 3.47 μm, respectively, the average lengths of WT and *spoT* mutant cells were about 1.37 and 2.17 μm, respec-

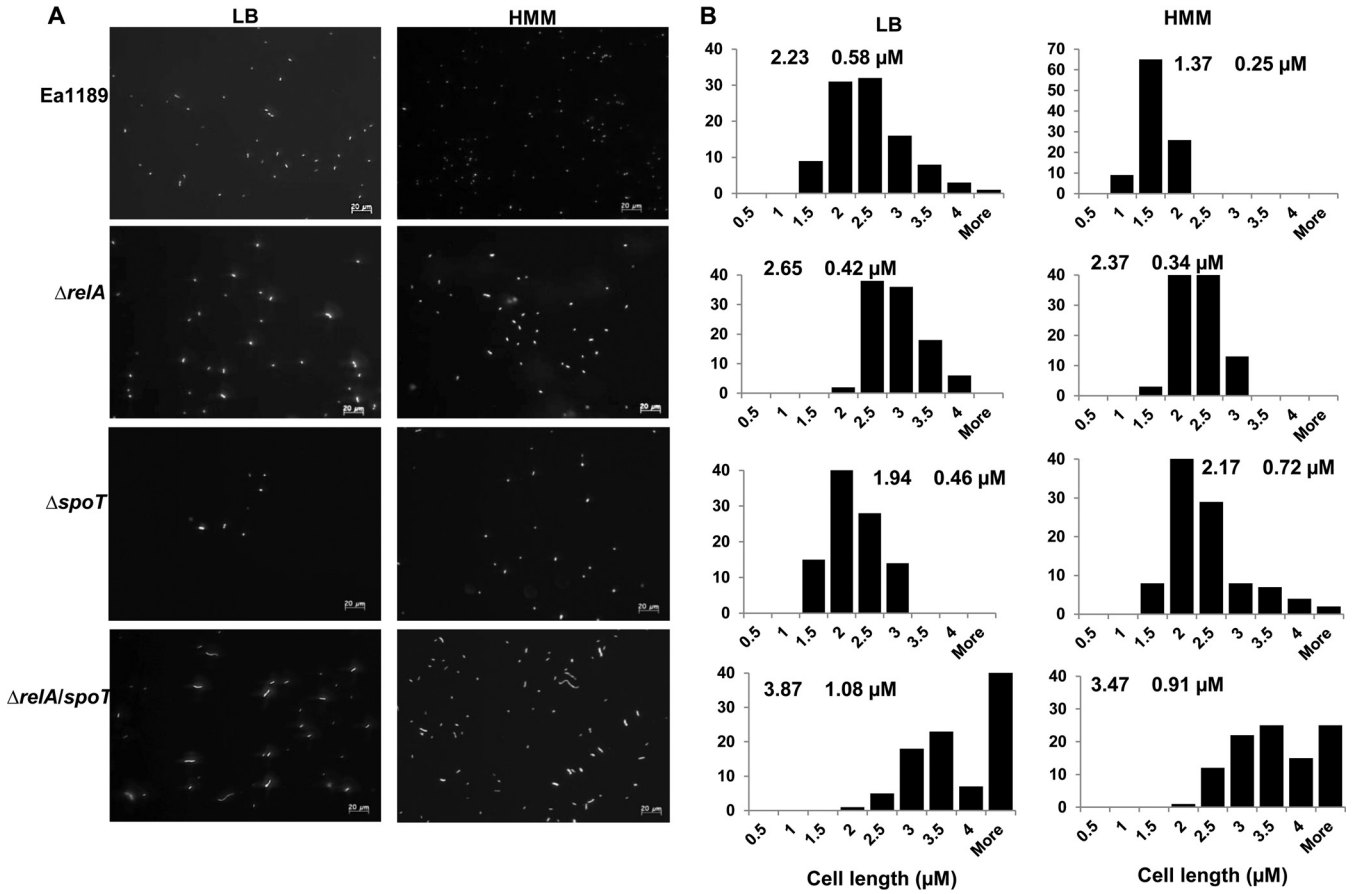

FIG 3 ppGpp controls cell size in *Erwinia amylovora*. (A) Epifluorescence microscopy images of *E. amylovora* WT and *relA*, *spoT*, and *relA/spoT* (ppGpp^0) mutant strains constitutively expressing GFP and grown in LB medium or HMM for 4 h. Magnification, ×200. (B) Distributions of sizes and average cell lengths of WT and *relA*, *spoT*, and *relA/spoT* (ppGpp^0) mutant strains constitutively expressing GFP and grown in LB medium or HMM. The experiments were repeated at least two times with similar results.

TABLE 2 Comparison of disease severities with *Erwinia amylovora* strain Ea1189, ppGpp mutants, and complementation strains

Strain	No. of shoots infected/no. of shoots inoculated	Length of necrosis (cm) (mean ± SD)[a]
Ea1189	7/7	30.2 ± 5.6[a]
Δ*relA* mutant	2/7	10 ± 2.8[c]
Δ*relA*(pRelA) mutant	7/7	30.8 ± 3.2[a]
Δ*spoT* mutant	6/7	19 ± 1.6[b]
Δ*spoT*(pSpoT) mutant	6/7	29 ± 3.1[a]
Δ*dksA* mutant	0/7	—[NI]
Δ*dksA*(pDksA) mutant	7/7	32.2 ± 5.08[a]
Δ*relA/spoT* mutant	0/7	—[NI]
Δ*relA/spoT*(pSpoT) mutant	3/7	1.16 ± 0.28[d]
Δ*relA/dksA* mutant	0/7	—[NI]
Δ*relA/dksA*(pRelA) mutant	0/7	—[NI]
Δ*relA/dksA*(pDksA) mutant	2/8	3.25 ± 1.06[c,d]

[a] Average necrosis length for 7 or 8 inoculated apple shoots (cv. 'Gala') at 7 days postinoculation. —, no disease detected. The experiment was repeated with similar results. One-way ANOVA and Student's *t* test ($P = 0.05$) were used to analyze the data. Values marked with the same letter were not significantly different ($P < 0.05$). NI, not included in statistical analysis.

tively (Fig. 3). However, the lengths of the majority of the *spoT* mutant cells were the same in both media and were also identical to those of the *relA* mutant cells in HMM (Fig. 3B). These findings indicate that the reduced lengths of WT cells and increased lengths of *relA* and ppGpp^0 mutant cells might be due to ppGpp accumulation in these cells, thus suggesting that the ppGpp-mediated stringent response controls the cell size of *E. amylovora*, and also suggesting that *E. amylovora* might require certain levels of ppGpp to control cell size *in vitro*.

Both DksA and ppGpp are required for virulence, elicitation of the hypersensitive response (HR), and bacterial growth. To determine the role of ppGpp/DksA in *E. amylovora* pathogenesis, virulence assays were performed on apple shoots and immature pear fruits. Necrosis around the point of inoculation was visible at 3 dpi and moved quickly into the apple shoots, and the length of necrotic diseased shoots reached more than 30 cm after 7 days for the WT strain (Table 2). While disease symptoms were not visible for the *dksA*, *relA/dksA*, and ppGpp^0 mutant strains, disease severity was strongly reduced for the *relA* and *spoT* mutants, with average lengths of necrotic shoots of 10 and 19 cm, respectively. Complementation of the *relA*, *spoT*, and *dksA* mutants with their respective genes restored disease severity to the WT level, while complementation of the *relA/dksA* and ppGpp^0 double mutants with a single gene rescued the ability to cause disease to the level of a single mutant (Table 2). Similarly, the *dksA*, *relA/dksA*, and ppGpp^0 mutant strains were nonpathogenic on immature pear fruits, while the *relA* and *spoT* mutants caused disease similarly to the WT strain (Fig. 4A). Complementation of these mutant strains resulted in disease recovery similar to that described above for apple shoots (Fig. 4B).

When infiltrated into tobacco leaves, the WT, the *relA* and *spoT* mutants, and the *relA*, *spoT*, and *dksA* complementation strains elicited typical HR cell death in tobacco leaves (Fig. 4C and D). However, no HR was observed for the *dksA*, *relA/dksA*, and ppGpp^0 mutant strains (Fig. 4C and D), indicating that both DksA and ppGpp are required for eliciting HR in tobacco.

To determine whether disease symptoms were correlated with

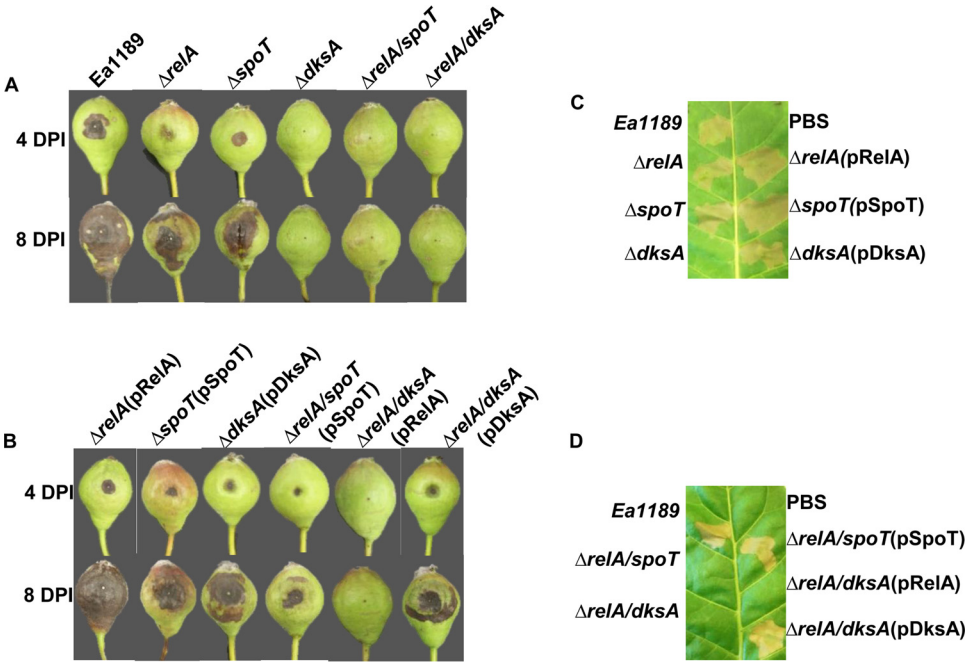

FIG 4 Pathogenicity and HR assays. (A and B) Symptoms caused by the WT strain, the *relA*, *spoT*, *dksA*, *relA/spoT* (ppGpp^0), and *relA/dksA* mutants (A), and their complementation strains (B) on immature pear fruits. Immature pears (cv. 'Bartlett') were surface sterilized, pricked with a sterile needle, and inoculated with 2 μl of bacterial suspension. Symptoms were recorded and photos were taken at 4 and 8 dpi. (C and D) HR assay on tobacco leaves. The *E. amylovora* WT strain, the *relA*, *spoT*, *dksA*, *relA/dksA*, and ppGpp^0 mutants (C), and their complementation strains (D) were allowed to infiltrate into 8-week-old tobacco leaves at a concentration of 10^8 CFU ml^{-1}. PBS was used as a negative control. Photographs were taken at 24 h postinfiltration.

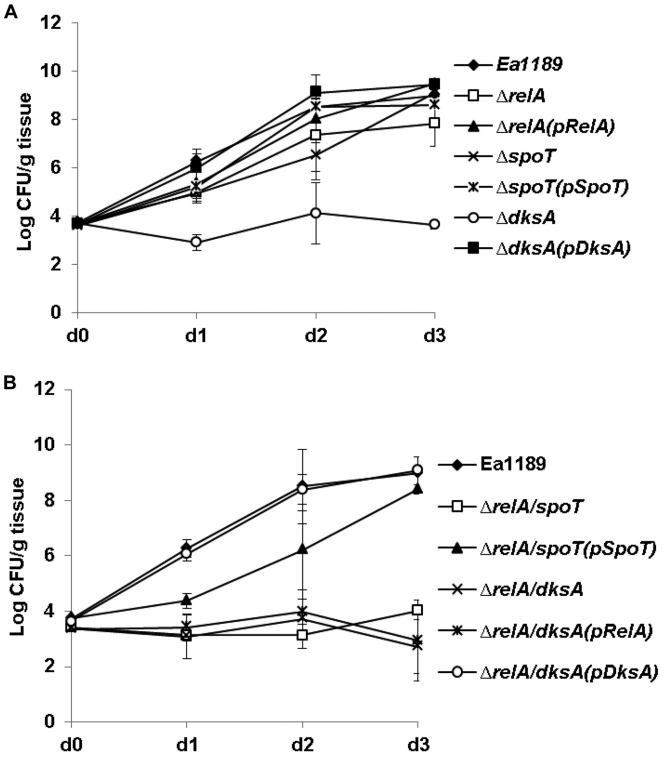

FIG 5 Both DksA and ppGpp are required for bacterial growth *in planta*. (A) Growth of the *E. amylovora* WT strain, the *relA*, *spoT*, and *dksA* single mutants, and their corresponding complementation strains in immature pears. (B) Growth of the *E. amylovora* WT strain, the *relA/dksA* and *relA/spoT* (ppGpp^0) double mutants, and their corresponding complementation strains in immature pears. Immature pears (cv. 'Bartlett') were surface sterilized, pricked with a sterile needle, and inoculated with 2 μl of bacterial suspension. Tissue surrounding the inoculation site was excised with a no. 4 cork borer and homogenized in 1 ml of 0.5× PBS. Bacterial growth within the pear tissue was monitored at 1, 2, and 3 days postinoculation by dilution plating on LB medium with appropriate antibiotics. d, day.

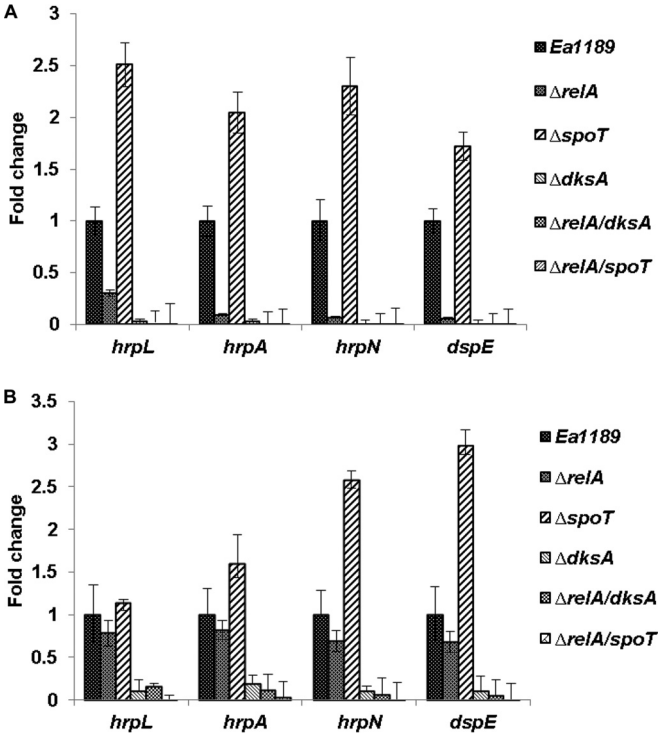

FIG 6 Both DksA and ppGpp activate T3SS gene expression in *Erwinia amylovora*. (A) Expression of T3SS regulatory and effector genes (*hrpL*, *hrpA*, *hrpN*, and *dspE*) in the *relA*, *spoT*, *dksA*, *relA/dksA*, and *relA/spoT* (ppGpp^0) mutant strains compared to the WT strain in HMM, as determined by qRT-PCR. (B) Expression of T3SS regulatory and effector genes (*hrpL*, *hrpA*, *hrpN*, and *dspE*) in the *relA*, *spoT*, *dksA*, *relA/dksA*, and ppGpp^0 mutant strains compared to the WT on immature pear fruits. Relative gene expression of selected T3SS genes was calculated by the $2^{-\Delta\Delta CT}$ method, utilizing the *rpoD* gene as an endogenous control. Fold changes are the means of results for three replicates. Each experiment was performed at least two times with similar results. Error bars indicated standard deviations.

bacterial growth, growth of the five mutants on immature pears was compared to that of the WT. At 1 and 2 dpi, bacterial growth of the *relA* and *spoT* mutants, as well as the *relA*, *spoT*, and *dksA* complementation strains, was slightly reduced compared to that of the WT strain, but growth of these strains was similar to that of the WT at 3 dpi (Fig. 5A). In contrast, bacterial growth of the *dksA*, *relA/dksA*, and ppGpp^0 mutant strains as well as the *relA/dksA* mutant complemented with the *relA* gene did not increase at all from 1 to 3 dpi, representing 100- to 1,000-fold less growth than that of the WT (Fig. 5A and B). At 3 dpi, the ppGpp^0 mutant complemented with the *spoT* gene and the *relA/dksA* mutant complemented with the *dksA* gene had restored growth on immature pears, and the bacterial growth was similar to that of the WT (Fig. 5B). These findings indicate that ppGpp and DksA are required for virulence, HR elicitation, and bacterial growth of *E. amylovora* and also suggest that both RelA and SpoT might be involved in ppGpp biosynthesis *in planta*.

Both DksA and ppGpp are required for T3SS gene expression. Since both DksA and ppGpp are required for virulence and HR, the effects of DksA and ppGpp on T3SS gene expression were determined using qRT-PCR. We found that expression of T3SS regulatory and effector genes in HMM, including *hrpL*, *hrpA*, *hrpN*, and *dspE*, was abolished in the *dksA*, *relA/dksA*, and ppGpp^0 mutants (Fig. 6A). In the *relA* mutant, expression of *hrpL*, *hrpA*, *hrpN*, and *dspE* was about 2- to 5-fold lower than that in the WT. In contrast, expression of *hrpL*, *hrpA*, *hrpN*, and *dspE* in the *spoT* mutant was 1.7- to 2.5-fold higher than that in the WT (Fig. 6A). Similarly, expression of T3SS genes in the *dksA*, *relA/dksA*, and ppGpp^0 mutants was strongly downregulated on immature pears, but expression levels were similar in the *relA* mutant and the WT (Fig. 6B). While expression of *hrpN* and *dspE* was upregulated >2-fold in the *spoT* mutant, the *hrpL* and *hrpA* genes in the *spoT* mutant were not differentially expressed compared to those of the WT on immature pears (Fig. 6B). These results indicate that both ppGpp and DksA are required for T3SS gene expression.

Moreover, an abundance of HrpA protein in the WT and four mutants grown in HMM was detected by Western blotting (Fig. 7). Only 4 and 10% protein signals were detected for the *dksA* and ppGpp^0 mutants, respectively, but about 96 and 89% protein signals were detected for the *relA* and *spoT* mutants, respectively, compared to the signal of the WT strain (Fig. 7). These results indicate that both ppGpp and DksA are required for protein accumulation.

RelA is mainly responsible for T3SS activation *in vitro*. To assess the roles of RelA and SpoT in T3SS gene expression in response to different starvation signals, promoter activities of the

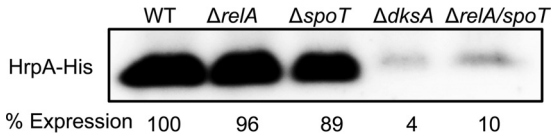

FIG 7 Accumulation of HrpA protein is controlled by DksA and ppGpp. The HrpA-His6 protein in the WT and *relA*, *spoT*, *dksA*, and *relA/spoT* (ppGpp^0) mutant strains was detected by Western blotting using an anti-histidine protein antibody after growth in HMM at 18°C for 6 h. Relative protein abundances were calculated by using ImageJ software, utilizing the average pixel value of the signals and considering the abundance of the WT sample to be 100%.

hrpL and *hrpA* genes were measured by flow cytometry analysis of bacterial strains grown in HMM supplemented with serine hydroxamate (SHX) or the nonmetabolizable glucose analog α-methylglucoside (αMG), both of which induce the stringent response (51, 52). The GFP intensities from both the *hrpL* and *hrpA* promoters in the WT and the *relA* mutant exhibited little change in HMM plus SHX or αMG compared to those in HMM alone (Table 3). In contrast, the GFP intensities from both the *hrpL* and *hrpA* promoters in the *spoT* mutant were higher in HMM plus SHX and 2- to 4-fold higher in HMM plus αMG compared to those in HMM alone (Table 3). These findings indicate that RelA is mainly responsible for activating T3SS gene expression *in vitro* and might respond to both SHX- and αMG-induced stresses in HMM. These results also suggest that SpoT does not respond to these starvation signals or that the hydrolase activity of SpoT is dominant under *in vitro* conditions.

DISCUSSION

E. amylovora utilizes the *hrp* T3SS to deliver effector proteins into host plants to suppress host defense, acquire nutrients, and cause disease (1, 2). In the regulatory networks of *E. amylovora*, a sigma factor cascade (RpoN-HrpL) quickly activates the T3SS in response to inducing signals, including limited nutrients and oxidative stress (4, 9, 11). These signals are similar to those sensed by RSH proteins, which are responsible for bacterial alarmone (p)ppGpp biosynthesis and degradation. In this study, we demonstrated that the DksA/ppGpp-mediated stringent response is essential for T3SS gene expression and virulence in *E. amylovora* through activation of the sigma factor cascade. These findings suggest that signals triggering ppGpp biosynthesis are most likely to be responsible for activation of the T3SS and also indicate that activation of the sigma factor cascade by the ppGpp-mediated stringent response might allow the pathogen to integrate diverse host/environmental signals encountered during the infection process (4).

It has been reported that the null mutation of the *spoT* gene in the *relA*$^+$ background in *E. coli* is lethal, suggesting that high levels of ppGpp may be toxic (23, 53–55). On the other hand, an *spoT* mutant of *E. coli* was first reported and later found to contain a secondary spontaneous point mutation in the *relA* gene (H354Y), which reduces RelA activity to about 20% of the wild-type level (56, 57). In this study, an *spoT* null mutant was generated in *E. amylovora*, as reported for *P. fluorescens*, both of which accumulated only slightly higher levels of ppGpp (35). To exclude the possibility of any secondary mutations in the *relA* gene in the *spoT* null mutant, the *relA* gene was sequenced, and no mutations were found (data not shown). Bioinformatic analysis revealed that many bacterial genomes encode additional single-domain, ppGpp-synthesizing or -hydrolyzing RSHs (58, 59). A gene (*Eam_3399*) annotated a hypothetical gene in *E. amylovora* (48) encodes a small RSH protein (paSpo) containing a single hydrolase domain (58). A similar RSH protein (pbcSpo2), also containing a single hydrolase domain, is encoded in the genome of *P. fluorescens* (35, 58). Although these small RSH proteins have not been characterized functionally (59), the recovery of an *spoT* single deletion mutant for both *E. amylovora* and *P. fluorescens* indirectly suggests that both paSpo and pbcSpo2 might be functional as ppGpp hydrolases, and this might also explain why deletion of the *spoT* gene is not lethal (59).

Characterization of the *spoT* null mutants of both *E. amylovora* and *P. fluorescens* yielded very interesting results. For *P. fluorescens*, despite higher levels of ppGpp, the *spoT* mutant showed an attenuated level of antibiotic activity, like the *relA* mutant, which produced almost undetectable levels of ppGpp (35). In this study, despite higher levels of ppGpp, the length of the *spoT* mutant cells was much longer than that of the WT cells in HMM. However, the lengths of the majority of the *spoT* mutant cells were the same in both media and were identical to those observed for the WT in LB medium and for the *relA* mutant in HMM (Fig. 3B). Growth rates of the *relA* and *spoT* mutants were slightly reduced compared to that of the WT *in planta*, suggesting that accumulation of ppGpp, which may depend on both RelA and SpoT within plants, is required for bacterial multiplication in a plant environment. Furthermore, both *relA* and *spoT* mutants caused reduced disease symptoms in apple shoots, suggesting that both RelA and SpoT are required for full virulence of *E. amylovora in planta*. An earlier

TABLE 3 Promoter activities of *hrpL* and *hrpA* genes in *Erwinia amylovora* WT and ppGpp mutant strains

Strain	Plasmid (gene)[a]	GFP intensity (geometric mean ± SD)[b]		
		HMM	HMM + 0.1 mM SHX	HMM + 0.5% αMG
Ea1189	pFPV25	1.34 ± 0.021^{e}	1.35 ± 0.007^{e}	1.36 ± 0.014^{d}
	pZW2 (*hrpL*)	1.58 ± 0.035^{c,d}	1.54 ± 0.007^{c,d}	1.52 ± 0.014^{c}
Δ*relA* mutant	pZW2 (*hrpL*)	1.47 ± 0.007^{c,d}	1.42 ± 0.021^{d}	1.41 ± 0.022^{c}
Δ*spoT* mutant	pZW2 (*hrpL*)	2.3 ± 0.028^{b}	2.64 ± 0.233^{b}	5.15 ± 0.234^{b}
Ea1189	pHrpA-GFP	1.72 ± 0.049^{c}	1.93 ± 0.035^{c}	1.98 ± 0.036^{c}
Δ*relA* mutant	pHrpA-GFP	1.42 ± 0.021^{d}	1.49 ± 0.14^{c,d}	1.44 ± 0.15^{c}
Δ*spoT* mutant	pHrpA-GFP	3.55 ± 0.355^{a}	5.98 ± 0.63^{a}	13.94 ± 0.96^{a}

[a] Promoter-GFP fusion plasmid.
[b] Bacteria were grown in HMM for 18 h, with or without addition of serine hydroxamate (SHX) or α-methylglucoside (αMG). One-way ANOVA and Student's *t* test ($P = 0.05$) were used to analyze the data. GFP intensity values within a treatment marked with the same letter were not significantly different ($P < 0.05$).

report also found that low levels of ppGpp are sufficient for full virulence in *Enterococcus faecalis* (60). Although we still lack plausible explanations for the nonlinear relationship between ppGpp levels and various observed phenotypes, it is possible that quantitative differences in the intracellular ppGpp level determine the precise expression of genes controlling various phenotypes in different environments, as reported recently (61). These findings also suggest that *E. amylovora* might require certain levels of ppGpp to cause disease *in vivo* and to control cell size *in vitro*.

It is well known that both DksA and ppGpp bind to RNAP and that DksA enhances the effect of ppGpp by modulating the direct interaction between RNAP and ppGpp (18, 26–28). Although growth of the *dksA* and ppGpp^0 mutants was normal in rich medium, growth of the *dksA* and ppGpp^0 mutants on immature pears and in HMM was impaired, and these mutants were unable to cause disease or elicit HR, suggesting that both DksA and ppGpp strongly influence the growth of *E. amylovora in planta* and in HMM. In addition, many studies have reported that nutrient availability adversely affects cell size (49, 62–67). Upon inoculation of *Pseudomonas syringae* onto a bean leaf surface, the length of these cells was rapidly reduced, suggesting that the leaf surface is a habitat with limited nutrients (66). Similarly, ppGpp mutant cells of *E. coli* were considerably longer than WT cells when cultured under starvation conditions (49). It has also been reported that cells of smaller sizes become increasingly resistant to abiotic stresses, including osmotic and oxidative stresses, thus enhancing their ability to survive under harsh environmental conditions (33, 36, 68–70). This may provide an explanation for why *dksA* and ppGpp^0 mutants fail to grow in HMM, suggesting that *E. amylovora* might require both ppGpp and DksA to coregulate cell size and resistance to environmental stresses, thus contributing to overall survival (59). It is interesting that the ppGpp level in the *dksA* mutant was reduced, suggesting that a positive-feedback regulation may exist for the biosynthesis of ppGpp, as reported previously (71). It is also possible that a growth defect of the *dksA* mutant in HMM contributes to reduced ppGpp accumulation. Furthermore, it is tempting to speculate that DksA may control gene expression independently of ppGpp, since DksA acts as a transcriptional factor (59). These findings suggest that both DksA and ppGpp are required for virulence, bacterial growth, and T3SS gene expression, and thus survival, in *E. amylovora*.

Although we observed a linear relationship between ppGpp levels and T3SS gene expression *in vitro*, Western blotting showed that HrpA protein accumulations in the *spoT* and *relA* mutants were not significantly different and were about 5 to 10% less than that in the WT. This result was consistent with the virulence assay data showing that both *relA* and *spoT* mutants caused reduced disease on apple shoots. The discrepancy between T3SS gene expression, protein accumulation, and disease-causing ability may be due to the T3SS in *E. amylovora* also being regulated at the posttranscriptional or translational level, as reported for other plant-pathogenic bacteria. In addition, both SHX and αMG could induce the stringent response in *E. coli* by mimicking amino acid and carbon starvation, respectively (17, 51, 52). Interestingly, when either SHX or αMG was added to HMM, promoter activities of *hrpL* and *hrpA* were strongly induced only in the *spoT* mutant, not in the WT and the *relA* mutant, suggesting that RelA is mainly responsible for activating T3SS gene expression *in vitro* and might respond to both SHX- and αMG-induced stresses. Initially, it appears that our results contrast with the notion that SpoT responds to carbon starvation. However, our results are consistent with those of a previous report indicating that amino acids and fructose act synergistically in activating the T3SS in *P. syringae* (14). Since HMM contains galactose, a sugar used to induce the T3SS (15), it is possible that αMG acts synergistically with galactose to activate T3SS expression. Furthermore, these findings also suggest that the hydrolase activity of SpoT is dominant and that RelA may act as a main ppGpp synthase under *in vitro* conditions. However, it will be interesting to investigate why only the *spoT* mutant responds to both inducers.

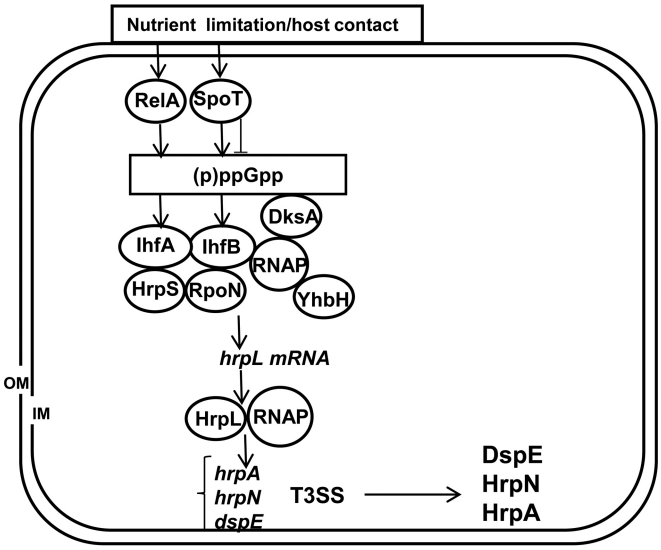

FIG 8 Working model illustrating the role of ppGpp in *Erwinia amylovora* in response to plant and environmental stimuli. This model is based on findings obtained in this study as well as those reported in previous studies (4, 5, 8, 11). Symbols: ↓, positive effect; ⊥, negative effect; IhfA and -B, integration host factors α and β; RNAP, RNA polymerase; OM, outer membrane; IM, inner membrane.

Based on our results and previously reported data, the following model is proposed for how *E. amylovora* incorporates host and environmental signals in regulating T3SS gene expression (Fig. 8). Upon arrival on a plant surface, *E. amylovora* cells experience stress conditions, such as limited nutrients and oxidative stress, which trigger the activation of the RelA/SpoT system, leading to the accumulation of ppGpp. Both DksA and ppGpp directly bind to RNAP, indirectly promoting binding to alternative sigma factors, such as RpoN and HrpL. RpoN, along with HrpS and YhbH, binds to the *hrpL* promoter to trigger *hrpL* transcription (4). HrpL then recognizes a conserved "*hrp* box" at the promoter regions of HrpL-dependent operons or genes, leading to the expression of other T3SS structural and effector genes. However, the exact signals sensed by RelA and SpoT in the plant environment are still not clear. In the future, research should focus on investigating the global effects of ppGpp both *in vitro* and *in planta*, as well as the signals that activate ppGpp accumulation and the T3SS. Given that the ppGpp^0 strain is unable to survive and cause disease, further research on targeting ppGpp in *E. amylovora* for development of control strategies is warranted.

ACKNOWLEDGMENTS

We thank Raymond Zielinski at the University of Illinois for his help with epifluorescence microscopy.

This project was supported by Agriculture and Food Research Initiative competitive grant 2010-65110-20497 from the USDA National Institute of Food and Agriculture and by USDA-Hatch/Multistate Project grants ILLU-802-913 and ILLU-802-396 (to Y.Z.).

REFERENCES

1. **Khan MA, Zhao YF, Korban SS.** 2012. Molecular mechanisms of pathogenesis and resistance to the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight disease in Rosaceae. Plant Mol Biol Rep **30:**247–260. http://dx.doi.org/10.1007/s11105-011-0334-1.
2. **Zhao YF.** 2014. Genomics of *Erwinia amylovora* and related species associated with pome fruit trees, p 1–36. *In* Gross DC, Lichens-Park A, Kole C (ed), Genomics of plant-associated bacteria. Springer, New York, NY.
3. **Zhao YF, Qi M.** 2011. Comparative genomics of *Erwinia amylovora* and related *Erwinia* species—what do we learn? Genes **2:**627–639. http://dx.doi.org/10.3390/genes2030627.
4. **Ancona V, Li WT, Zhao YF.** 2014. Alternative sigma factor RpoN and its modulator protein YhbH are indispensable for *Erwinia amylovora* virulence. Mol Plant Pathol **15:**58–66. http://dx.doi.org/10.1111/mpp.12065.
5. **Li W, Ancona V, Zhao YF.** 2014. Co-regulation of polysaccharide production, motility, and expression of type III secretion genes by EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora*. Mol Gen Genomics **289:**63–75. http://dx.doi.org/10.1007/s00438-013-0790-4.
6. **Zhao Y, Sundin GW, Wang D.** 2009. Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora*. Can J Microbiol **55:**457–464. http://dx.doi.org/10.1139/W08-147.
7. **Zhao YF, Wang D, Nakka S, Sundin GW, Korban SS.** 2009. Systems level analysis of two-component signal transduction systems in *Erwinia amylovora*: role in virulence, regulation of amylovoran biosynthesis and swarming motility. BMC Genomics **10:**245. http://dx.doi.org/10.1186/1471-2164-10-245.
8. **Oh CS, Beer SV.** 2005. Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. FEMS Lett **253:**185–192. http://dx.doi.org/10.1016/j.femsle.2005.09.051.
9. **McNally RR, Toth IK, Cock PJ, Pritchard L, Hedley PE, Morris JA, Zhao YF, Sundin GW.** 2012. Genetic characterization of the HrpL regulon of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. Mol Plant Pathol **13:**160–173. http://dx.doi.org/10.1111/j.1364-3703.2011.00738.x.
10. **Nissinen RM, Ytterberg AJ, Bogdanove AJ, van Wijk K, Beer SV.** 2007. Analyses of the secretomes of *Erwinia amylovora* and selected *hrp* mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. Mol Plant Pathol **8:**55–67. http://dx.doi.org/10.1111/j.1364-3703.2006.00370.x.
11. **Wei Z, Kim JF, Beer SV.** 2000. Regulation of hrp genes and type III protein secretion in *Erwinia amylovora* by HrpX/HrpY, a novel two-component system, and HrpS. Mol Plant Microbe Interact **13:**1251–1262. http://dx.doi.org/10.1094/MPMI.2000.13.11.1251.
12. **Zhao YF, Blumer SE, Sundin GW.** 2005. Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. J Bacteriol **187:**8088–8103. http://dx.doi.org/10.1128/JB.187.23.8088-8103.2005.
13. **Zhao YF, He SY, Sundin GW.** 2006. The *Erwinia amylovora avrRpt2*$_{EA}$ gene contributes to virulence on pear and AvrRpt2$_{EA}$ is recognized by *Arabidopsis* RPS2 when expressed in *Pseudomonas syringae*. Mol Plant Microbe Interact **19:**644–654. http://dx.doi.org/10.1094/MPMI-19-0644.
14. **Anderson JC, Wan Y, Kim YM, Pasa-Tolic L, Metz TO, Peck SC.** 2014. Decreased abundance of type III secretion system-inducing signals in *Arabidopsis* mkp1 enhances resistance against *Pseudomonas syringae*. Proc Natl Acad Sci U S A **111:**6846–6851. http://dx.doi.org/10.1073/pnas.1403248111.
15. **Huynh TV, Dahlbeck D, Staskawicz BJ.** 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science **245:**1374–1377. http://dx.doi.org/10.1126/science.2781284.
16. **Wei ZM, Sneath BJ, Beer SV.** 1992. Expression of *Erwinia amylovora* hrp genes in response to environmental stimuli. J Bacteriol **174:**1875–1882.
17. **Dalebroux ZD, Swanson MS.** 2012. ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol **10:**203–212. http://dx.doi.org/10.1038/nrmicro2720.
18. **Kalia D, Merey G, Nakayama S, Zheng Y, Zhou J, Luo Y, Guo M, Roembke BT, Sintim HO.** 2013. Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. Chem Soc Rev **42:**305–341. http://dx.doi.org/10.1039/c2cs35206k.
19. **Potrykus K, Cashel M.** 2008. (p)ppGpp: still magical? Annu Rev Microbiol **62:**35–51. http://dx.doi.org/10.1146/annurev.micro.62.081307.162903.
20. **Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS.** 2010. ppGpp conjures bacterial virulence. Microbiol Mol Biol Rev **74:**171–199. http://dx.doi.org/10.1128/MMBR.00046-09.
21. **Haseltine WA, Block R.** 1973. Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proc Natl Acad Sci U S A **70:**1564–1568. http://dx.doi.org/10.1073/pnas.70.5.1564.
22. **Magnusson LU, Farewell A, Nyström T.** 2005. ppGpp: a global regulator in *Escherichia coli*. Trends Microbiol **13:**236–242. http://dx.doi.org/10.1016/j.tim.2005.03.008.
23. **Xiao H, Kalman M, Ikehara K, Zemel S, Glaser G, Cashel M.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J Biol Chem **266:**5980–5990.
24. **Barker MM, Gaal T, Gourse RL.** 2001. Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. J Mol Biol **305:**689–702. http://dx.doi.org/10.1006/jmbi.2000.4328.
25. **Maciąg-Dorszyńska M, Szalewska-Pałasz A, Węgrzyn G.** 2013. Different effects of ppGpp on *Escherichia coli* DNA replication in vivo and in vitro. FEBS Open Biol **3:**161–164. http://dx.doi.org/10.1016/j.fob.2013.03.001.
26. **Ross W, Vrentas CE, Sanchez-Vazquez P, Gaal T, Gourse RL.** 2013. The magic spot: a ppGpp binding site on *E. coli* RNA polymerase responsible for regulation of transcription initiation. Mol Cell **50:**420–429. http://dx.doi.org/10.1016/j.molcel.2013.03.021.
27. **Zuo Y, Wang Y, Steitz TA.** 2013. The mechanism of *E. coli* RNA polymerase regulation by ppGpp is suggested by the structure of their complex. Mol Cell **50:**430–436. http://dx.doi.org/10.1016/j.molcel.2013.03.020.
28. **Mechold U, Potrykus K, Murphy H, Murakami KS, Cashel M.** 2013. Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. Nucleic Acids Res **41:**6175–6189. http://dx.doi.org/10.1093/nar/gkt302.
29. **Szalewska-Palasz A, Johansson LUM, Bernardo LMD, Skärfstad E, Stec E, Brännström K, Shingler V.** 2007. Properties of RNA polymerase bypass mutants: implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on sigma54. J Biol Chem **282:**18046–18056. http://dx.doi.org/10.1074/jbc.M610181200.
30. **Pizarro-Cerdá J, Tedin K.** 2004. The bacterial signal molecule, ppGpp, regulates Salmonella virulence gene expression. Mol Microbiol **52:**1827–1844. http://dx.doi.org/10.1111/j.1365-2958.2004.04122.x.
31. **Ramachandran VK, Shearer N, Thompson A.** 2014. The primary transcriptome of *Salmonella enterica* serovar Typhimurium and its dependence on ppGpp during late stationary phase. PLoS One **9:**e92690. http://dx.doi.org/10.1371/journal.pone.0092690.
32. **Nakanishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, Kuhara S, Sugimoto N, Tobe T.** 2006. ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. Mol Microbiol **61:**194–205. http://dx.doi.org/10.1111/j.1365-2958.2006.05217.x.
33. **Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D.** 2013. The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. J Bacteriol **195:**2011–2020. http://dx.doi.org/10.1128/JB.02061-12.
34. **Moris M, Braeken K, Schoeters E, Verreth C, Beullens S, Vanderleyden J, Michiels J.** 2005. Effective symbiosis between *Rhizobium etli* and *Phaseolus vulgaris* requires the alarmone ppGpp. J Bacteriol **187:**5460–5469. http://dx.doi.org/10.1128/JB.187.15.5460-5469.2005.
35. **Takeuchi K, Yamada K, Haas D.** 2012. ppGpp controlled by the Gac/Rsm regulatory pathway sustains biocontrol activity in *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact **25:**1440–1449. http://dx.doi.org/10.1094/MPMI-02-12-0034-R.
36. **Vercruysse M, Fauvart M, Jans A, Beullens S, Braeken K, Cloots L, Engelen K, Marchal K, Michiels J.** 2011. Stress response regulators identified through genome-wide transcriptome analysis of the (p)ppGpp-dependent response in *Rhizobium etli*. Genome Biol **12:**R17. http://dx.doi.org/10.1186/gb-2011-12-2-r17.
37. **Bowden SD, Eyres A, Chung JCS, Monson RE, Thompson A, Salmond GPC, Spring DR, Welch M.** 2013. Virulence in *Pectobacterium atrosepticum* is regulated by a coincidence circuit involving quorum sensing and the stress alarmone, (p)ppGpp. Mol Microbiol **90:**457–471. http://dx.doi.org/10.1111/mmi.12369.

38. **Wang J, Gardiol N, Burr T, Salmond GPC, Welch M.** 2007. RelA-dependent (p)ppGpp production controls exoenzyme synthesis in *Erwinia carotovora* subsp. *atroseptica*. J Bacteriol **189:**7643–7652. http://dx.doi.org/10.1128/JB.00920-07.
39. **Zhang HB, Wang C, Zhang LH.** 2004. The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. Mol Microbiol **52:**1389–1401. http://dx.doi.org/10.1111/j.1365-2958.2004.04061.x.
40. **Yang F, Korban SS, Pusey L, Elofsson M, Sundin GW, Zhao YF.** 2014. Small molecule inhibitors suppress expression of both type III secretion and amylovoran biosynthesis genes in *Erwinia amylovora*. Mol Plant Pathol **15:**44–57. http://dx.doi.org/10.1111/mpp.12064.
41. **Bellemann P, Bereswill S, Berger S, Geider K.** 1994. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. Int J Biol Macromol **16:**290–296. http://dx.doi.org/10.1016/0141-8130(94)90058-2.
42. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
43. **Sambrook J, Russell D.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
44. **Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee HW, Hong JI, Hartland EL, Stinear TP, Howden BP.** 2010. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog **6:**e1000944. http://dx.doi.org/10.1371/journal.ppat.1000944.
45. **Rhee HW, Lee CR, Cho SH, Song MR, Cashel M, Choy HE, Seok YJ, Hong JI.** 2008. Selective fluorescent chemosensor for the bacterial alarmone (p)ppGpp. J Am Chem Soc **130:**784–785. http://dx.doi.org/10.1021/ja0759139.
46. **Wang D, Korban SS, Pusey PL, Zhao YF.** 2011. Characterization of the RcsC sensor kinase from *Erwinia amylovora* and other enterobacteria. Phytopathology **101:**710–717. http://dx.doi.org/10.1094/PHYTO-09-10-0258.
47. **Wang D, Korban SS, Zhao YF.** 2009. The Rcs phosphorelay system is essential for pathogenicity in *Erwinia amylovora*. Mol Plant Pathol **10:**277–290. http://dx.doi.org/10.1111/j.1364-3703.2008.00531.x.
48. **Smits THM, Rezzonico F, Kamber T, Blom J, Goesmann A, Frey JE, Duffy B.** 2010. Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. Mol Plant Microbe Interact **23:**384–393. http://dx.doi.org/10.1094/MPMI-23-4-0384.
49. **Traxler MF, Summers SM, Nguyen HT, Zacharia VM, Hightower GA, Smith JT, Conway T.** 2008. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol Microbiol **68:**1128–1148. http://dx.doi.org/10.1111/j.1365-2958.2008.06229.x.
50. **Potrykus K, Murphy H, Philippe N, Cashel M.** 2011. ppGpp is the major source of growth rate control in *E. coli*. Environ Microbiol **13:**563–575. http://dx.doi.org/10.1111/j.1462-2920.2010.02357.x.
51. **Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ.** 2008. Transcription profiling of the stringent response in *Escherichia coli*. J Bacteriol **190:**1084–1096. http://dx.doi.org/10.1128/JB.01092-07.
52. **Maisonneuve E, Castra-Camargo M, Gerdes K.** 2013. (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. Cell **154:**1140–1150. http://dx.doi.org/10.1016/j.cell.2013.07.048.
53. **Weiss LA, Stallings CL.** 2013. Essential roles for *Mycobacterium tuberculosis* Rel beyond the production of (p)ppGpp. J Bacteriol **195:**5629–5638. http://dx.doi.org/10.1128/JB.00759-13.
54. **Hernandez VJ, Bremer H.** 1993. Characterization of RNA and DNA synthesis in *Escherichia coli* strains devoid of ppGpp. J Biol Chem **268:**10851–10862.
55. **Sarubbi E, Rudd KE, Cashel M.** 1988. Basal ppGpp level adjustment shown by new *spoT* mutants affect steady state growth rates and *rrnA* ribosomal promoter regulation in *Escherichia coli*. Mol Gen Genet **213:**214–222. http://dx.doi.org/10.1007/BF00339584.
56. **Shachrai I, Zaslaver A, Alon U, Dekel E.** 2010. Cost of unneeded proteins in E. coli is reduced after several generations in exponential growth. Mol Cell **38:**758—767. http://dx.doi.org/10.1016/j.molcel.2010.04.015. (Erratum, **42:**401, 2011.)
57. **Gropp M, Strausz Y, Gross M, Glaser G.** 2001. Regulation of *Escherichia coli* RelA requires oligomerization of the C-terminal domain. J Bacteriol **183:**570–579. http://dx.doi.org/10.1128/JB.183.2.570-579.2001.
58. **Atkinson GC, Tenson T, Hauryliuk V.** 2011. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PLoS One **6:**e23479. http://dx.doi.org/10.1371/journal.pone.0023479.
59. **Gaca AO, Colomer-Winter C, Lemos JA.** 20 January 2015. Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. J Bacteriol http://dx.doi.org/10.1128/JB.02577-14.
60. **Gaca AO, Kajfasz JK, Miller JH, Liu K, Wang JD, Abranches J, Lemos JA.** 2013. Basal levels of (p)ppGpp in *Enterococcus faecalis*: the magic beyond the stringent response. mBio **4:**e00646-13. http://dx.doi.org/10.1128/mBio.00646-13.
61. **Traxler MF, Zacharia VM, Marquardt S, Summers SM, Nguyen H, Stark SE, Conway T.** 2011. Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the 'feast to famine' gradient in *Escherichia coli*. Mol Microbiol **79:**830–845. http://dx.doi.org/10.1111/j.1365-2958.2010.07498.x.
62. **Ojha AK, Mukherjee TK, Chatterji D.** 2000. High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes the morphology of the bacterium. Infect Immun **68:**4084–4091. http://dx.doi.org/10.1128/IAI.68.7.4084-4091.2000.
63. **Abranches J, Martinez AR, Kajfasz JK, Chávez V, Garsin DA, Lemos JA.** 2009. The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. J Bacteriol **191:**2248–2256. http://dx.doi.org/10.1128/JB.01726-08.
64. **Bugrysheva JV, Bryksin AV, Godfrey HP, Cabello FC.** 2005. *Borrelia burgdorferi rel* is responsible for generation of guanosine-3′-diphosphate-5′-triphosphate and growth control. Infect Immun **73:**4972–4981. http://dx.doi.org/10.1128/IAI.73.8.4972-4981.2005.
65. **Hill NS, Buske PJ, Shi Y, Levin PA.** 2013. A moonlighting enzyme links *Escherichia coli* cell size with central metabolism. PLoS Genet **9:**e1003663. http://dx.doi.org/10.1371/journal.pgen.1003663.
66. **Monier JM, Lindow SE.** 2003. *Pseudomonas syringae* responds to the environment on leaves by cell size reduction. Phytopathology **93:**1209–1216. http://dx.doi.org/10.1094/PHYTO.2003.93.10.1209.
67. **Yao Z, Davis RM, Kishony R, Kahne D, Ruiz N.** 2012. Regulation of cell size in response to nutrient availability by fatty acid biosynthesis in *Escherichia coli*. Proc Natl Acad Sci U S A **109:**E2561–E2568. http://dx.doi.org/10.1073/pnas.1209742109.
68. **Steinberger RE, Allen AR, Hansa HG, Holden PA.** 2002. Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa* unsaturates biofilms. Microb Ecol **43:**416–423. http://dx.doi.org/10.1007/s00248-001-1063-z.
69. **Jubair M, Morris JG, Ali A.** 2012. Survival of *Vibrio cholerae* in nutrient-poor environments is associated with a novel "persister" phenotype. PLoS One **7:**e45187. http://dx.doi.org/10.1371/journal.pone.0045187.
70. **Crompton MJ, Dunstan RH, Macdonald MM, Gottfries J, von Eiff C, Roberts TK.** 2014. Small changes in environmental parameters lead to alterations in antibiotic resistance, cell morphology and membrane fatty acid composition in *Staphylococcus lugdunensis*. PLoS One **9:**e92296. http://dx.doi.org/10.1371/journal.pone.0092296.
71. **Shyp V, Tankov S, Ermakov A, Kudrin P, English BP, Ehrenberg M, Tenson T, Elf J, Hauryliuk V.** 2012. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. EMBO Rep **13:**835–839. http://dx.doi.org/10.1038/embor.2012.106.
72. **Burse A, Weingart H, Ullrich MS.** 2004. NorM, an *Erwinia amylovora* multidrug efflux pump involved in *in vitro* competition with other epiphytic bacteria. Appl Environ Microbiol **70:**693–703. http://dx.doi.org/10.1128/AEM.70.2.693-703.2004.
73. **Wang RF, Kushner SR.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199. http://dx.doi.org/10.1016/0378-1119(91)90366-J.
74. **Valdivia RH, Falkow S.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011. http://dx.doi.org/10.1126/science.277.5334.2007.
75. **Wang D, Korban SS, Zhao YF.** 2010. Molecular signature of differential virulence in natural isolates of *Erwinia amylovora*. Phytopathology **100:**192–198. http://dx.doi.org/10.1094/PHYTO-100-2-0192.
76. **Mellies JL, Barron AMS, Haack KR, Korson AS, Oldridge DA.** 2006. The global regulator Ler is necessary for enteropathogenic *Escherichia coli* colonization of *Caenorhabditis elegans*. Infect Immun **74:**64–72. http://dx.doi.org/10.1128/IAI.74.1.64-72.2006.