The Stringent Response Mediated by (p)ppGpp Is Required for Virulence of *Pseudomonas syringae* pv. *tomato* and Its Survival on Tomato

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The hypersensitive response and pathogenicity (*hrp*) type III secretion system (T3SS) is a key pathogenicity factor in *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000). In this study, the role of the second messenger (p)ppGpp on virulence and survival of DC3000 was investigated. Results have demonstrated that (p)ppGpp-deficient mutant (ppGpp⁰) of DC3000 exhibited lower levels of expression of the T3SS and genes of other virulence traits, such as coronatine toxin. The ppGpp⁰ mutant of DC3000 was greatly impaired in causing disease and in growth in planta. Furthermore, (p)ppGpp was required for swarming motility, pyoverdine production, the oxidative stress response, as well as γ -amino butyric acid utilization. Screening of amino acids, major signals in activation of ppGpp biosynthesis, revealed that promoter activities of the *avrPto* gene could be either activated or suppressed by various amino acids in a ppGpp-dependent or -independent manner. Moreover, the ppGpp⁰ mutant exhibited increased cell size and decreased survival on plant surfaces. Altogether, these findings indicate that ppGpp acts as an internal signal that regulates the T3SS as well as other virulence factors in pseudomonads and suggest that bacterial pathogens utilize intracellular messengers to sense environmental and nutritional signals for rapid, precise, and reversible control of their pathogenesis and survival.

The stringent response is one of the most important global regulator systems that bacteria utilize to orchestrate fine-tuning of their cellular processes in response to changing environmental conditions, especially of limited nutrient conditions. The hallmark of the stringent response is the accumulation of linear nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp, hereafter referred to as ppGpp, and is also referred to as bacterial alarmone (Potrykus and Cashel 2008). ppGpp is one of several nucleotide second messengers produced in response to environmental stresses to support survival of an organism (Dalebroux and Swanson 2012; Dalebroux et al. 2010). Proteins responsible for ppGpp synthesis and degradation, i.e., RelA/SpoT homolog

(RSH) proteins, have been identified in almost all bacterial genomes examined (Atkinson et al. 2011). Under amino acid-starvation conditions, uncharged tRNAs that bind to the ribosomal A site would activate ribosome-associated monofunctional RelA to synthesize ppGpp using ATP and GTP in *Escherichia coli* (Haseltine and Block 1973; Magnusson et al. 2005). The bifunctional SpoT, containing both synthase and hydrolase activities, is activated in the absence of fatty acid, carbon, phosphorous, and iron, as well as in response to hyperosmotic shock and oxidative stresses (Magnusson et al. 2005; Xiao et al. 1991). In addition, single domain ppGpp-synthesizing or -hydrolyzing RSHs have been found in different bacteria (Atkinson et al. 2011).

During the stringent response, direct interactions between RNA polymerase (RNAP), ppGpp, and a partner transcription factor, DksA, would lead to downregulation of highly expressed stable RNA, DNA replication, ribosome and protein synthesis, and simultaneous upregulation of stress, starvation, and virulence gene expression (Barker et al. 2001; Dalebroux and Swanson 2012; Dalebroux et al. 2010). Therefore, ppGpp acts as a global regulator of cellular transcription reprogramming. ppGpp binds directly to RNAP in *E. coli* to inhibit transcription of genes controlling cell growth and cell division and to activate transcription of amino acid biosynthesis genes (Dalebroux and Swanson 2012; Dalebroux et al. 2010; Potrykus and Cashel 2008). As a result of ppGpp's direct inhibition of RNAP binding to σ^{70} -dependent stringent promoters, such as rRNA promoters, increased availability of core RNAP to alternative σ -factors such as RpoN (σ^N), leads to higher levels of expression of alternative σ -dependent genes (Bernardo et al. 2006; Costanzo et al. 2008; Dalebroux et al. 2010; Gummesson et al. 2009; Szalewska-Palasz et al. 2007).

Following global transcriptome analysis, it has been reported that ppGpp regulates expression of hundreds of genes involved in amino acid biosynthesis, DNA synthesis, fatty acid metabolism, flagella production, universal stress response, survival, and virulence (Aberg et al. 2006; Bremer and Ehrenberg 1995; Chang et al. 2002; Eichel et al. 1999; Hernandez and Bremer 1993; Johansson et al. 2000; Maciąg-Dorszyńska et al. 2013). In plant-associated bacteria, ppGpp influences epiphytic fitness and biocontrol activity in *Pseudomonas fluorescens* (*P. protegens*), biofilm formation in *P. chlororaphis*, hydrogen peroxide and antibiotic tolerance in *P. aeruginosa*, and nodulation in *Rhizobium etli* (Moris et al. 2005; Selin et al. 2010; Takeuchi et al. 2012). In plant-pathogenic bacteria, ppGpp is required for cell-wall degrading enzyme production in *Pectobacterium atrosepticum*

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and quorum sensing signal degradation in *Agrobacterium tumefaciens* (Bowden et al. 2013; Wang et al. 2007; Zhang et al. 2004).

P. syringae pv. *tomato* DC3000 (herein called DC3000), the causal agent of bacterial speck of tomato and *Arabidopsis thaliana*, is an extracellular hemibiotrophic pathogen. It takes up nutrients from living host tissues, multiplies in the apoplast without apparent host cell death, and reaches high population during late stages of pathogenesis when host cells die and infected tissues exhibit extensive necrosis (Moore et al. 1989; Wang et al. 2002; Whalen et al. 1991). In contrast to other pseudomonads, DC3000 is a relatively weak epiphyte and can only survive on leaf surfaces for a short period of time (Boureau et al. 2002; Manceau and Horvais 1997). As a highly aggressive pathogen, small populations of leaf-associated DC3000 are capable of causing disease symptoms (Xin and He 2013). During a compatible interaction, nutritional status of the host, such as high levels of nitrogen (N), as well as favorable environmental conditions often lead to increased disease severity (Xin and He 2013).

DC3000 has been used as a model for studying molecular mechanisms of bacterial pathogenesis as well as for understanding plant-microbe interactions (Xin and He 2013). A functional hypersensitive response and pathogenicity (*hrp*) type III secretion system (T3SS) is essential for DC3000 to colonize host plants (Alfano and Collmer 2004; Collmer et al. 2000). The T3SS in DC3000 is regulated by the master regulator HrpL, which, in turn, is activated by RpoN along with the enhancer binding proteins HrpR and HrpS (Alarcón-Chaidez et al. 2003; Tang et al. 2006). Other regulators of T3SS include Lon protease (Bretz et al. 2002; Lan et al. 2007) and HrpV, which negatively control HrpR and HrpS activities, respectively (Jovanovic et al. 2011; Ortiz-Martín et al. 2010; Wei et al. 2005). Furthermore, CorR, a response regulator of coronatine toxin, also activates *hrpL* expression in DC3000 (Peñaloza-Vazquez et al. 2000; Sreedharan et al. 2006). The polyketide coronatine, a virulence factor of DC3000, plays a critical role in opening stomata and in suppressing plant defense responses (Brooks et al. 2004; Uppalapati et al. 2007; Zhao et al. 2003).

In general, earlier studies have reported that the T3SS is rapidly expressed in response to limited nutrients, including low nitrogen and rich plant-derived sugars such as sucrose, fructose, and mannitol, as well as physical contact between host and pathogen (Huynh et al. 1989; Kim et al. 2010; Rahme et al. 1992; Rico and Preston 2008). In contrast, T3SS genes are suppressed in nutrient-rich media containing complex nitrogen sources or certain organic acids, including amino acids (Huynh et al. 1989; Rahme et al. 1992). However, the exact signals that activate the T3SS remain unknown. Moreover, it is also not clear whether or not ppGpp regulates the T3SS or controls virulence in pseudomonads or both. In this study, the role of the bacterial alarmone ppGpp on expression of the T3SS in response to amino acids, virulence, and survival in DC3000 has been investigated.

RESULTS

The RelA-SpoT system in DC3000.

Based on bioinformatics analysis of the DC3000 genome sequence, three genes (*PSPTO_1694*, *PSPTO_007*, and *PSPTO_0856*) encoding RSH proteins have been identified (Buell et al. 2003). Following a blast search, *PSPTO_1694* (RelA) and *PSPTO_0073* (SpoT), corresponding to long RSH proteins, are found to be conserved among all sequenced pseudomonads. Another hypothetical protein with unknown function, *PSPTO_0856* (fpRel), is found to contain a single ppGpp synthase domain, belonging to the small alarmone synthetases subgroup (Atkinson et al. 2011). A blast search has also revealed that fpRel is not conserved among sequenced pseudomonads and is only present in a few pseudomonads, including *P. syringae* pv. *maculicola* ES4326. In

contrast, most pseudomonads, including *P. syringae* pv. *syringae* B728a, contain another small RSH protein, *pbcSpo2*, having a single hydrolase domain and belonging to the small alarmone hydrolases subgroup (Atkinson et al. 2011).

To study the role of ppGpp-mediated stringent response in the virulence of DC3000, the following mutants of DC3000, *relA*, *relA/spoT*, and *relA/spoT/fpRel*, were generated using overlap extension mutagenesis. However, attempts to generate a single *spoT* mutant were deemed unsuccessful, as SpoT was the only ppGpp hydrolase present in DC3000 and accumulation of high levels of ppGpp in cells was found to be lethal, as previously reported for *E. coli* (Xiao et al. 1991). In contrast, a single *spoT* deletion mutant in both B728a and *P. fluorescens* was recently reported, possibly due to the presence of *pbcSpo2* (T. Chatnaparat, Z. Li, S. S. Korban, and Y. F. Zhao unpublished; Takeuchi et al. 2012).

When intracellular ppGpp levels were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS), it was found that ppGpp in the DC3000 strain was 10.03 nmol per milliliter at optical density at 600 nm (OD₆₀₀) when grown in *hrp*-inducing minimal medium (HMM) for 30 min (Table 1). Levels of ppGpp in the *relA* mutant were about one third of that in the wild-type (WT) strain; while ppGpp was undetectable in both *relA/spoT* double and *relA/spoT/fpRel* triple mutants (Table 1). This could be attributed to limitations in the detection methodology used (<2 nM/ml at OD₆₀₀) rather than complete absence of ppGpp in the *relA/spoT* double mutant. These findings suggested that under HMM conditions, RelA plays a major role in ppGpp synthesis.

Full virulence of DC3000 on tomato requires ppGpp-mediated stringent response.

To determine the role of the ppGpp-mediated stringent response in virulence of DC3000 on tomato plants, DC3000 WT, *relA*, *relA/spoT*, and *relA/spoT/fpRel* mutants were spray-inoculated onto tomato plants. The WT strain exhibited chlorotic and necrotic lesions (typical disease symptoms) on leaves of tomato plants 7 days postinoculation (dpi) (Fig. 1A). Most leaves inoculated with *relA* and *relA/spoT* mutants also exhibited typical disease symptoms. In contrast, inoculation of tomato leaves with the *relA/spoT/fpRel* mutant (thereafter referred to as ppGpp⁰) yielded only sporadic leaf spots, suggesting that ppGpp contributes to virulence of DC3000 (Fig. 1A). Virulence of the ppGpp⁰ mutant could be partially restored by complementation with *relA* or *fpRel* genes in trans (Fig. 1B). These findings suggested that ppGpp synthesized by RelA, SpoT, and fpRel is required for full virulence of DC3000.

To determine whether or not reduced virulence was correlated with bacterial growth in tomato plants, growth of the *relA*, *relA/spoT*, and ppGpp⁰ mutants of DC3000 in tomato leaves was compared with that of the WT. Bacterial growth of WT and the *relA* mutant was similar, reaching approximately 10⁷ CFU/g 6 dpi (Fig. 2A), whereas growth of the *relA/spoT* mutant was about 100-fold lower than that of the WT strain at 3 and 6 dpi (Fig. 2A). Furthermore, growth of the ppGpp⁰ mutant was approximately 1,000-fold lower than that of the WT strain (Fig. 2B). Bacterial growth of the complementation strains of mutants was partially restored in tomato when compared with that of the mutant strains by 6 dpi (Fig. 2A and B). To rule out the likelihood that slow growth of the mutants in plants was due to bacterial growth defects, growth of mutants in M9 minimal medium and in King's medium B (KB) rich medium were determined. In all strains grown in KB and M9 media, no differences were found (Supplementary Fig. S1) (results for strains grown in HMM medium are provided in Table 2). These results indicated that, although similar symptoms were observed in both the *relA/spoT* mutant and the WT, bacterial growth was significantly reduced in the mutant, thus suggesting that a certain

level of ppGpp might be required for growth of bacteria in plants. In addition, these results suggested that fpRel is functional and contributes to ppGpp accumulation in cells.

Expression of T3SS and coronatine genes in DC3000 requires ppGpp-mediated stringent response.

As growth and virulence of the ppGpp⁰ mutant was strongly affected in tomato plants, the role of ppGpp in the regulation of T3SS, effectors, and of other virulence genes was investigated. When promoter activities of the effector *avrPto* gene were determined in HMM medium by flow cytometry for WT and its mutants carrying *avrPto* promoter fused to a promoterless green fluorescent protein (GFP) (Table 1), the geometric means of GFP intensities in WT, *relA*, *relA/spoT*, and ppGpp⁰ mutants were 112.11, 62.91, 22.0, and 9.41, respectively, relative to the vector control (GFP value of about 2.45) in HMM for 3 h (Table 1). When cells were grown in HMM for 5 h, the geometric means of GFP intensities in WT, *relA*, and *relA/spoT* mutants were increased to 330, 367, and 229, respectively; whereas the

geometric mean of GFP intensity in ppGpp⁰ mutant (42) was about eightfold lower than that of WT (Table 1). These results indicated that ppGpp is required for *avrPto* gene expression.

When expression of T3SS-related genes was determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), levels of expression of *hrpL* and *hrpZ* in the ppGpp⁰ mutant were more than three- and 10-fold lower than those of the WT, respectively; whereas expression of *hrpR* and *hrpS* was about twofold lower than those of the WT in HMM at 3 h postincubation (Fig. 3A). In the *relA/spoT* mutant, expression of *hrpS* and *hrpZ* was about twofold lower than that of the WT, while expression of *hrpR* and *hrpL* was only slightly decreased. Furthermore, levels of expression of *hrpR*, *hrpS*, and *hrpZ* in the *relA* mutant were similar to those of WT, although *hrpL* expression was slightly up-regulated when compared with that of the WT (Fig. 3A). These results indicated that ppGpp is required for activating T3SS-related genes in DC3000.

Mutation of the *hrpL* gene inhibited production of coronatine in DC3000 (Sreedharan et al. 2006). Expression of selected *cor* genes, including *corR* (coronatine regulatory gene) and *cfl* (coronamic acid biosynthesis gene), in both WT and ppGpp mutants in HMM medium was then determined. Expression of both *corR* and *cfl* in the ppGpp⁰ mutant was down-regulated more than fourfold that of the WT (Fig. 3B). Moreover, expression of *corR* and *cfl* genes in the *relA/spoT* mutant was also about twofold lower than that of the WT, while expression of both coronatine genes in the *relA* mutant was not significantly different from those of the WT (Fig. 3B). These results suggested that ppGpp also influences coronatine gene expression in DC3000.

GABA metabolism in DC3000 is regulated by ppGpp-mediated stringent response.

It has been reported that the nonprotein amino acid γ -amino butyric acid (GABA) is highly abundant in tomato apoplasts and that DC3000 is capable of utilizing GABA as a sole carbon and nitrogen source (Park et al. 2010; Rico and Preston 2008).

Table 1. Levels of ppGpp and green fluorescent protein (GFP) intensity in wild type (WT) and mutant strains

Strains	ppGpp concentration ^a	GFP intensity ^b	
		3 h	5 h
DC3000	10.03 $\pm$ 2.55	112.11 $\pm$ 5.73	330 $\pm$ 9.64
$\Delta relA$	3.21 $\pm$ 1.13	62.91 $\pm$ 2.59	367.78 $\pm$ 4.86
$\Delta relA/spoT$	ND	22.0 $\pm$ 1.16	229.04 $\pm$ 7.85
$\Delta relA/spoT/fpRel$	ND	9.41 $\pm$ 0.14	42 $\pm$ 2.59

^a Measured as nmol per milliliter at optical density at 600 nm (OD₆₀₀). Data are presented as averages of four replicates $\pm$ standard deviation. ND = not detected due to being below the detection limit (2 nM/ml at OD₆₀₀) in liquid chromatography-tandem mass spectrometry.

^b GFP intensity was measured by flow cytometry in WT and mutant strains containing the *avrPto* promoter-GFP reporter construct at time points indicated.

Fig. 1. Pathogenicity of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) wild type (WT), ppGpp mutants, and complementation strains. **A**, Disease symptoms caused by DC3000 WT, *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutants in susceptible tomato. **B**, Symptoms caused by the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) complementation strains in susceptible tomato. Pictures were taken 7 days postinoculation. The experiment was repeated at least three times and similar results were obtained.

Fig. 2. Growth of *Pseudomonas syringae* pv. tomato DC3000 (DC3000), ppGpp mutants, and complementation strains. **A**, Bacterial growth of DC3000 wild type (WT) and *relA* and *relA/spoT* mutants, along with their complementation strains in susceptible tomato. **B**, Bacterial growth of DC3000 WT, the *relA/spoT/fpRel* (ppGpp⁰) mutant, and complementation strains in susceptible tomato. Bacterial growth was monitored at 0, 1, 3, and 6 days postinfiltration. The vertical bars correspond to standard deviations of means.

Expression of *gabT* (encoding GABA transaminase) genes, including *gabT1*, *gabT2*, and *gabT3* in WT and mutants, grown in modified mannitol-glutamate (MG) medium, revealed that all three genes in the ppGpp⁰ mutant were about two to fivefold lower than those of the WT (Fig. 3B). In the *relA/spoT* mutant, it was found that expression of only *gabT2* was about threefold lower than that of the WT. However, expression of all three *gabT* genes in the *relA* mutant was similar to that of the WT (Fig. 3B). These results suggested that ppGpp controls GABA gene expression in DC3000. Moreover, when DC3000 and its ppGpp mutants were grown in a minimal medium containing 10 mM GABA as the sole carbon or nitrogen source (Fig. 3C), growth of the WT and the *relA* mutant was similar, reaching an OD₆₀₀ of about 0.1 at 24 h postincubation. In contrast, the ppGpp⁰ and *relA/spoT* double mutants failed to grow in GABA (Fig. 3C). Complementation of mutants with both *relA* and *fpRel* genes in trans partially restored bacterial growth, except for the triple mutant complemented with *fpRel* (Fig. 3C). Therefore, it is likely that a certain level of ppGpp is required for growth of bacteria on GABA, as *fpRel* may only play a minor role in synthesis of ppGpp. These findings suggested that ppGpp is required for GABA metabolism in DC3000.

Amino acids influence T3SS gene expression either through ppGpp or independently.

Amino acids are known to either affect T3SS gene expression or trigger plant defense responses (Huynh et al. 1989; Rahme et al. 1992; Stauber et al. 2012; Xiao et al. 1992). To assess the influence of amino acids on T3SS expression, the *avrPto* promoter activities in both the WT and the ppGpp⁰ mutant, grown in HMM supplemented with either an individual amino acid or GABA, were determined by flow cytometry. Based on means of GFP intensities in the ppGpp⁰ mutant when compared with those of the WT, amino acids were classified into four groups (Table 2). Group I, including aspartic acid, glutamic acid, asparagine, and GABA, strongly induced *avrPto* promoter activities in both the WT and the ppGpp⁰ mutant as compared with

Table 2. Green fluorescent protein (GFP) intensity and growth of wild type (WT) and ppGpp⁰ mutant strains grown in *hrp*-inducing minimal medium (HMM) supplemented with different amino acids

Media ^a	GFP intensity ^b		Growth		Growth of <i>rpoN</i> ^c
	DC3000	<i>ΔrelA/spoT/fpRel</i>	DC3000	<i>ΔrelA/spoT/fpRel</i>	
HMM	112.11 ± 5.73	9.41 ± 0.14	0.12	0.11	...
Aspartic Acid	688.26 ± 38.22	785.80 ± 28.19	0.35	0.48	–
Glutamic Acid	618.83 ± 13.75	278.24 ± 12.15	0.80	0.81	+
Asparagine	589.66 ± 22.99	245.02 ± 2.93	0.28	0.28	+
GABA	406.12 ± 4.48	387.57 ± 9.45	0.66	0.48	...
Glycine	335.72 ± 8.14	36.02 ± 4.07	0.17	0.21	...
Glutamine	327.50 ± 0.41	28.16 ± 28.40	0.70	0.90	+
Lysine	293.29 ± 11.02	22.02 ± 0.11	0.21	0.16	+
Threonine	250.10 ± 0.04	20.00 ± 0.12	0.15	0.12	W
Arginine	221.94 ± 1.17	40.02 ± 1.32	0.18	0.16	+
Serine	349.52 ± 34.17	7.25 ± 0.08	0.62	0.79	W
Proline	311.88 ± 25.29	9.62 ± 0.06	0.81	0.27	–
Leucine	242.39 ± 7.33	10.68 ± 0.31	0.23	0.13	W
Histidine	232.78 ± 11.67	7.68 ± 0.04	0.22	0.08	–
Phenylalanine	228.72 ± 2.36	8.11 ± 0.03	0.24	0.07	–
Alanine	163.16 ± 0.46	7.79 ± 0.17	0.32	0.07	W
Methionine	127.04 ± 3.93	7.63 ± 0.09	0.13	0.05	–
Isoleucine	96.47 ± 1.95	8.04 ± 0.01	0.05	0.05	W
Cysteine	22.64 ± 0.86	17.60 ± 0.62	0.06	0.04	...
Valine	12.04 ± 0.08	9.21 ± 0.01	0.07	0.05	...

^a Each of the amino acids and GABA were used at 10 mM. Cultures (3 ml) were shaken (250 rpm, 22°C) for 3 h.

^b GFP intensities of the WT and mutant strains containing the *avrPto* promoter-GFP reporter construct were measured by flow cytometry.

^c For *RpoN* mutant, + = growth; W = weak; – = lack of growth on plates containing specific amino acid, data obtained from Hendrickson et al. (2000a). Ellipses (...) indicate not determined.

those grown in HMM alone. Group II, consisting of glycine, glutamine, lysine, threonine, and arginine, exhibited slightly elevated *avrPto* promoter activities in the ppGpp⁰ mutant compared with cells grown in HMM alone; however, the *avrPto* promoter activity was strongly enhanced in the WT. Group III, including serine, proline, leucine, histidine, alanine, and phenylalanine, influenced *avrPto* promoter activities only in the WT. Finally, Group IV, consisting of methionine, isoleucine, cysteine, and valine, either had no effects or suppressed *avrPto* promoter activities in the WT only. These results indicated that amino acids could either activate or suppress T3SS expression in either ppGpp-dependent or -independent manners. Furthermore, bacterial growth of the WT and the ppGpp⁰ mutant was influenced by adding amino acids into the HMM medium (Table 2). The ppGpp⁰ mutant but not the WT was unable to grow in HMM supplemented with proline, histidine, phenylalanine, alanine, and methionine; whereas, both the WT and the ppGpp⁰ mutant were unable to grow in HMM supplemented with isoleucine, cysteine, and valine (Table 2). These results indicated that ppGpp serves as a signal that influences amino acid utilization in DC3000.

Swarming motility, pyoverdine production, and resistance to hydrogen peroxide are dependent on ppGpp-mediated stringent response.

The ppGpp⁰ mutant was found to be unable to swarm, while the *relA* and *relA/spoT* mutants showed reduced swarming motilities relative to those of the WT (Fig. 4A and B), thus

suggesting that ppGpp controls swarming motility in DC3000. Complementation of the ppGpp⁰ strain by expressing either *relA* or *fpRel* in trans partially restored swarming motility, whereas complementation of the *relA/spoT* mutant by expressing *relA* and *spoT* in trans resulted in reduced swarming motility (Fig. 4A and B). When the effects of ppGpp on pyoverdine production in DC3000 and its mutants were determined, it was found that pyoverdine production was reduced by three- to 10-fold in the *relA*, *relA/spoT*, and ppGpp⁰ mutants as compared with those of the WT under low-iron conditions (Fig. 5A). Complementation of mutants with *relA* and *fpRel* but not of *spoT* in trans restored pyoverdine levels in ppGpp mutant strains (Fig. 5A). These results suggested that ppGpp affects pyoverdine production, likely in response to availability of iron.

It has been reported that ppGpp is involved in hydrogen peroxide tolerance in *Pseudomonas* spp. (Khakimova et al. 2013). Following exposure to 1 mM H₂O₂ for 15 min, it was observed that only 0.69% of the ppGpp⁰ mutant survived compared with 29.5% of the WT (Fig. 5B). Survival of the *relA/spoT* mutant was about 19.5%, while survival of the *relA* mutant was not significantly different (31%) from that of the WT. Complementation of the ppGpp⁰ mutant by expressing *relA* and *fpRel* in trans partially recovered its survival (Fig. 5B). However, presence of *spoT* in trans in the *relA/spoT* mutant led to higher sensitivity to H₂O₂ when compared with the *relA/spoT* mutant (Fig. 5B). These results indicated that ppGpp is required for resistance to hydrogen peroxide in DC3000.

Fig. 3. Virulence gene expression of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) and ppGpp mutants. **A**, Expression of type III secretion system genes in the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutant strains as compared with DC3000 wild type (WT) strain grown in *hrp*-inducing minimal medium 3 h postinoculation determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). **B**, Expression of *corR*, *cfl*, *gabT1*, *gabT2*, and *gabT3* in the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutant strains as compared with DC3000 WT strain by qRT-PCR. The *rpoD* gene was used as a control. Vertical bars represent the standard deviations of mean ratio. Similar results were obtained in repeated independent experiments. One-way analysis of variance (ANOVA) and the Student-Newmans-Kleus test ($P = 0.05$) was used to analyze qRT-PCR data. For each gene tested in WT and mutants, fold changes marked with the same letter were not significantly different ($P < 0.05$). **C**, Growth of DC3000 WT, ppGpp mutants, and complementation strains in minimal medium supplemented with 10 mM GABA at 28°C for 24 h. Vertical bars correspond to standard deviations. One-way ANOVA and the Student-Newmans-Kleus test ($P = 0.05$) were used to analyze growth data. Changes marked with the same letter were not significantly different ($P < 0.05$).

Cell size and survival of DC3000 on tomato is controlled by ppGpp.

It has been reported that bacterial cells protect themselves from a wide range of adverse conditions by adjusting their cell sizes, thereby promoting survival under these adverse conditions (Dalebroux and Swanson 2012; Potrykus et al. 2011). Using epifluorescence microscopy, cell lengths of DC3000 WT and its ppGpp mutants constitutively expressing GFP were determined in both KB and HMM medium. In KB medium, no significant differences were found for bacterial cell lengths for both the WT and its ppGpp mutants, ranging from 2.75 to 2.89 (data not shown). When cells were grown in HMM, lengths of WT cells were significantly shortened to 1.59 μm (Fig. 6A and B). Mean cell lengths for *relA* and *relA/spoT* mutants were about 2.17 μm , slightly shorter than those grown in KB, whereas cell length of the ppGpp⁰ mutant of DC3000 was 2.59 μm (Fig. 6). These results indicated that ppGpp controls cell size under nutrient limited conditions in DC3000.

When confocal microscopy was used in a time-course study to assess whether or not ppGpp influenced bacterial cell survival on either host or nonhost plant leaf surfaces, it was found that, at 24 h postinoculation (hpi), WT viable cells (green in color) were detected in small groups along epidermal cell surfaces (Fig. 7A). At 48 hpi, most WT cells were found to be dead (red in color) on leaf surfaces but there were small numbers of viable cells (Fig. 7B). At 96 hpi, abundant small-sized viable

WT cells were observed as large aggregates along newly formed lesions (Fig. 7C). In contrast, most ppGpp⁰ mutant cells were found to be dead on leaf surfaces at each time point (Fig. 7D, E, and F). On nonhost tobacco leaf surfaces, some WT cells were found to be viable, while most ppGpp⁰ mutant cells were found to be dead at 24 h (Fig. 7G and H). These results suggested that ppGpp is required for survival of DC3000 on both host and nonhost plants.

DISCUSSION

In DC3000, T3SS is quickly activated by a sigma factor cascade (RpoN and HrpL) when in contact with plants or in HMM medium (Alarcón-Chaidez et al. 2003; Ferreira et al. 2006; Hendrickson et al. 2000a; Jovanovic et al. 2011; Rahme et al. 1992; Tang et al. 2006). The proposed signals for activating T3SS, which include low nutrients and oxidative stress, are similar to those signals sensed by RSH proteins. In this study, it was demonstrated that bacteria deficient in their ability to produce ppGpp exhibit lower levels of expression of T3SS and of other virulence factors. Furthermore, it was found that ppGpp controls both cell size and survival both in vitro and on plant surfaces and is required for various stress responses. These findings indicate that ppGpp serves as a critical internal signal that coordinates expression of virulence factors in DC3000 in response to plant stimuli. Moreover, this suggests that bacterial pathogens utilize intracellular

Fig. 4. ppGpp controls swarming motility. **A**, Swarming motility of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) wild type (WT), the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutants, and their complementation strains on swarming plates (King's medium B containing 0.4% agar) 24 h postinoculation (hpi). The experiments were repeated at least three times with similar results. **B**, Swarming diameter of DC3000 WT, the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutant strains, and their complementation strains 24 hpi. One-way analysis of variance and the Student-Newmans-Kleus test ($P = 0.05$) was used to analyze the data. For WT, mutants, and complementation strains, changes marked with the same letter were not significantly different ($P < 0.05$).

messengers to sense environmental and nutritional signals for rapid, precise, and reversible control of pathogenesis and survival.

Unlike most proteobacteria, which contain only RelA/SpoT, a novel small ppGpp synthase, fpRel, is present in the genome of DC3000. Although levels of ppGpp produced in *relA/spoT* and *relA/spoT/fpRel* (ppGpp⁰) mutants have been found to be undetectable by LC-MS/MS, the *relA/spoT* mutant was found to be capable of causing disease while the ppGpp⁰ mutant was not. This indirectly suggests that fpRel is functional and that it is required for ppGpp synthesis in DC3000. Unfortunately, we could not generate a *spoT* null mutant in DC3000, as it has been reported that the *E. coli spoT* mutant is lethal due to accumulation of high levels of ppGpp (Xiao et al. 1991). This finding is in contrast with what has been observed in *P. syringae* B728a,

in which a *spoT* mutant was successfully generated, possibly due to the presence of the *pbcSpo2* gene encoding a small ppGpp hydrolase (T. Chatnaparat, Z. Li, S. S. Korban, and Y. F. Zhao unpublished). On the other hand, mutation of the *relA* gene of DC3000 did not affect bacterial growth in planta while both *relA/spoT* and ppGpp⁰ mutants exhibited gradually reduced growth in planta. This indicates that low levels of ppGpp in the *relA* mutant could be produced by both SpoT and fpRel, which may be sufficient to contribute to both virulence and growth of DC3000.

In this study, cell size (length) and survival as well as resistance to stress of DC3000 and the ppGpp mutants were found to be correlated with levels of ppGpp produced in cells (Figs. 6 and 7; Table 1). WT cells exhibited short cell lengths both in HMM medium and in planta and survived well on leaf surfaces, whereas ppGpp⁰ mutant cells were longer but could not survive on leaf surfaces, thus indicating that plant leaf surfaces offer a nutrient-limited habitat. These results were consistent with previous reports found in both *P. syringae* and in *E. coli* (Monier and Lindow 2003b; Traxler et al. 2008), further suggesting that ppGpp controls bacterial cell size and survival under nutrient-limited conditions. Earlier, it has been reported that

Fig. 5. ppGpp regulates pyoverdine production and oxidative stress tolerance. **A**, Pyoverdine production by *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) wild type (WT), the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutants, and their complementation strains grown in mannitol-glutamate (MG) medium at 28°C for 24 h. Pyoverdine levels were determined by measuring absorbance at 405 nm (A_{405}) of cell-free culture supernatants in MG medium normalized by the absorbance at 600 nm (A_{600}) of the bacterial culture. Data were presented as relative fluorescence levels (A_{405}/A_{600}). The experiments were repeated three times with similar results. **B**, Survival of DC3000 WT, the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutants, and their complementation strains after exposure to 1 mM H₂O₂ for 15 min. Similar results were obtained in repeated independent experiments. The vertical bars represent the standard deviation of the mean. One-way analysis of variance and the Student-Newmans-Kleus test ($P = 0.05$) were used to analyze the data. For WT, mutants, and complementation strains, changes marked with the same letter were not significantly different ($P < 0.05$).

Fig. 6. ppGpp controls cell size in medium. **A**, Microscopy images of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) wild type (WT) and the *relA*, *relA/spoT*, and *relA/spoT/fpRel* (ppGpp⁰) mutant strains constitutively expressing green fluorescent protein (GFP) observed by epifluorescence microscope after growth in hrp-inducing minimal medium (HMM) medium for 4 h. Pictures were taken at 200 × magnification with a scale bar of 20 μm. **B**, Distribution of bacterial sizes and average bacterial cell lengths of DC3000 WT and the *relA*, *relA/spoT*, and ppGpp⁰ mutant strains constitutively expressing GFP in HMM media.

smaller-sized cells were more resistant to various stress conditions (Crompton et al. 2014; Hase 1999; Jubair et al. 2012; Monier and Lindow 2003b; Steinberger et al. 2002) and that ppGpp controlled antibiotic tolerance and oxidative and osmotic stresses (Khakimova et al. 2013; Vercruysse et al. 2011) as well as motility and siderophore production (Magnusson et al. 2007; Takeuchi et al. 2012; Vogt et al. 2011; Wang et al. 2007). In DC3000, it was found that ppGpp is required for swarming motility, pyoverdine production, and oxidative stress responses. These results suggested that DC3000 might require ppGpp accumulation to coregulate cell size and resistance to environmental stresses on tomato, thus contributing to overall survival.

DC3000 employs both T3SS and phytotoxin coronatine to cause disease (Elizabeth and Bender 2007; Melotto et al. 2008; Uppalapati et al. 2007; Zhao et al. 2003). Several regulatory proteins, including HrpL and RpoN, control expression of T3SS and coronatine genes (Chatterjee et al. 2003; Hendrickson et al. 2000b; Sreedharan et al. 2006). In this study, it has been demonstrated that transcription of *hrpL*, *hrpRS*, *hrpZ*, and *avrPto* as well as of *corR* and *cfl* was under positive regulation of ppGpp. Moreover, these regulatory effects correlated with levels of ppGpp in cells, suggesting that ppGpp is required for activating T3SS and coronatine gene expression. These findings were consistent with previous reports indicating that activation of *hrpL* transcription occurred early on in the infection process

(Sreedharan et al. 2006). Therefore, ppGpp levels in cells are critically important for stimulating early induction of T3SS and of coronatine genes. As *corR* and *hrpL* regulate each other (Sreedharan et al. 2006), the effects of ppGpp on coronatine gene expression in DC3000 are likely to be mediated by ppGpp through the RpoN-HrpL sigma factor cascade. In addition, recent studies have shown that ppGpp is involved in the Gac/Rsm signal transduction pathway and that mutation of *GacA* in DC3000 suppresses expression of *rpoN*, *hrpRS*, *hrpL*, and coronatine genes (*corR* and *corS*) (Chatterjee et al. 2003; Takeuchi et al. 2012). Therefore, it is likely that ppGpp may interact with the Gac/Rsm system to regulate virulence factors in DC3000. However, this deserves further investigation.

The most abundant amino acids in tomato apoplasts are GABA and aspartic and glutamic acids, which could be used by DC3000 as carbon and nitrogen sources (Rico and Preston 2008). In addition, genes involved in GABA metabolism of B728a are up-regulated in planta, thus indicating that GABA is being utilized as a nutrient source (Yu et al. 2013). In this study, both *relA/spoT* and ppGpp⁰ mutants failed to grow in a medium containing only GABA as a sole carbon or nitrogen source. Furthermore, expression of three *gabT* genes encoding GABA transaminase in DC3000 was under positive regulation of ppGpp and this correlated well with ppGpp levels. It has been reported that *gabT2* plays a major role in GABA utilization in DC3000 (Park et al. 2010). This is consistent with our findings that expression of

Fig. 7. ppGpp mediates survival on plants. **A**, Microscopy images of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) (A to C) and the *relA/spoT/fpRel* (ppGpp⁰) mutant strain (D to F) constitutively expressing green fluorescent protein (GFP) observed by confocal laser scanning microscopy at 24, 48, and 96 h following spray inoculation on tomato. **B**, Microscopy images of DC3000 (G) and the ppGpp⁰ mutant strain (H) constitutively expressing GFP and observed by confocal laser scanning microscopy 24 h following spray inoculation on tobacco leaf surface. GFP-labeled bacterial cells were green, dead bacterial cells stained with propidium iodide were red, and leaf cells were either red or black in color. White arrows indicate GFP-labeled bacteria. Pictures were taken at 200 $\times$ magnification with a scale bar of 20 μ m.

gabT2 in the *relA/spoT* and ppGpp⁰ plants was significantly reduced. Furthermore, higher levels of GABA in plants can suppress T3SS in DC3000 and, also, quash virulence in *Agrobacterium tumefaciens* (Chevrot et al. 2006; Park et al. 2010; Wang et al. 2006), suggesting that ppGpp may be required for GABA utilization to suppress plant defense responses mediated by GABA and to promote bacterial growth in planta.

Amino acids are known to affect T3SS gene expression (Huynh et al. 1989; Rahme et al. 1992; Stauber et al. 2012; Xiao et al. 1992). Our results have revealed that amino acids can either activate or repress T3SS expression in both ppGpp-dependent and -independent manners (Table 2). GABA, aspartic acid, glutamic acid, and asparagine have strongly activated expression of T3SS in HMM and promoted growth of both the WT and the ppGpp⁰ strain. This finding is consistent with a previous report indicating that aspartic and pyroglutamic acids in *Arabidopsis* along with fructose or mannitol but not alone strongly induced *avrPto* expression in DC3000 (Anderson et al. 2014). However, our results were in contrast with other previous findings indicating that, in *P. syringae* pv. *glycinea*, both aspartic and glutamic acids inhibit T3SS expression (Huynh et al. 1989). At first glance, it appears that our GABA results are in contrast with those reported previously, i.e., GABA suppresses T3SS in DC3000 (Park et al. 2010). However, this discrepancy may be attributed to the different media used in these studies (HMM versus MG), as the latter medium contains glutamate, which, along with GABA, may contribute to different T3SS expression, but this needs to be further explored.

Based on the above and previous findings, the following model is proposed to illustrate how DC3000 perceives host and environmental signals and coordinates virulence and survival through second-messenger ppGpp (Fig. 8). A plant phyllosphere has long been deemed as a stress environment, as nutrients are limited. Upon contact with plant surfaces, it is likely that the *relA*, *spoT*, and *fpRel* genes are immediately activated when DC3000 cells sense the limited-nutrient environment, iron availability, and oxidative stress conditions, thereby triggering the ppGpp-mediated stringent response. In turn, the stringent response indirectly promotes alternative sigma factor activities, including RpoN and HrpL, leading to expression of T3SS and of other virulence factors as well as of stress-related genes. It is known that the genome of DC3000 contains 15 sigma factors, including 10 extracytoplasmic function (ECF) sigma factors (Oguiza et al.

2005). These ECF sigma factors are involved in regulating T3SS gene expression (HrpL), iron transport systems (PvdS, PSPTO1203, PSPTO1209, PSPTO1286, and PSPTO0444), outer membrane modifications (SigX and AlgU), as well as unknown functions (PSPTO1043 and PSPTO4224) (Markel et al. 2011, 2013; Oguiza et al. 2005). In addition, the DC3000 genome encodes over 12% of the genes required for rapid adaptation to diverse environments during epiphytic growth and pathogenesis (Buell et al. 2003). Together with other global regulatory systems, such as the Gac/Rsm system, ppGpp-mediated stringent response coordinately regulates nutrient acquisition through different bacterial enhancer binding proteins, such as NtrC, as well as various stress responses, including siderophore production, tolerance to oxidative stress, cell size, and survival. These findings suggest that signals triggering ppGpp biosynthesis are most likely to be responsible for activation of T3SS in DC3000. Therefore, future studies should identify these signals, which, possibly, are multifaceted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and recombinant techniques.

Bacterial strains and plasmids used in this study are listed in Table 3. *P. syringae* pv. *tomato* strains were cultured on KB or MG medium at 28°C (Park et al. 2010) or in HMM at 22°C (Huynh et al. 1989). *E. coli* strains were routinely grown in Luria Bertani medium at 37°C. Bacterial growth was monitored by measuring absorbance of cell suspensions at 600 nm. All primers used in this study are listed in Supplementary Table S1. Antibiotics were added to medium at the following concentrations (μg/ml): kanamycin, 50; rifampicin, 100; tetracycline, 15; and spectinomycin, 100. All DNA manipulations, including DNA isolation, plasmid extraction, restriction enzyme digestion, ligation, and gel electrophoresis were performed as described previously (Sambrook et al. 1989).

Construction of *relA*, *relA/spoT*, and *relA/spoT/fpRel* mutants.

Deletion mutants of *relA*, *relA/spoT*, and *relA/spoT/fpRel* were generated using splice overlap extension mutagenesis (Heckman and Pease 2007). Briefly, 1-kb upstream and downstream fragments of *relA*, *spoT*, and *fpRel* in DC3000 were amplified using primers specific to these genes. These primers also contained an

Fig. 8. A working model illustrating the role of ppGpp in *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) 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 (Chatterjee et al. 2003; Peñaloza-Vázquez et al. 2000; Sreedharan et al. 2006; Takeuchi et al. 2012; Tang et al. 2006). The down arrows indicate positive effects and the intersecting lines a negative effect; bold lines indicate results from this study. σ^{54} = RpoN and CorRS = regulators of coronatine.

extension sequence complementary to a kanamycin resistance cassette flanked with FLP recombination target (FRT) sites from pKD13. The two PCR fragments contained ends overlapping with those of the kanamycin-resistance cassette (Datsenko and Wanner 2000). The two amplicons were then ligated with the kanamycin-resistance cassette, using overlap extension PCR. The final fragment was then cloned into the pTok2 vector digested with *Sma*I, yielding pTok2::*ΔrelA*, pTok2::*ΔspoT*, and pTok2::*ΔfpRel*, respectively. Each of these plasmids was electroporated into DC3000, and transformants were plated on KB plates containing kanamycin. Those kanamycin-resistant but tetracycline-sensitive mutants were selected. Gene disruption was confirmed using PCR, using primers specific to sequences flanking each of the genes. To generate markerless mutant strains, plasmid pFLP2-Ω expressing an FLP recombinase, was electroporated into the mutant strains. Transformants were plated on KB plates containing spectinomycin to allow for FLP recombinase-mediated recombination between the FRT sites, thus resulting in loss of the kanamycin-resistance cassette. The pFLP2-Ω plasmid was cured from these mutants by replica-planting on KB plates. Markerless deletions were confirmed by PCR. To generate a *relA/spoT* double mutant, plasmid pTok2::*ΔspoT* was transferred to the *relA* markerless mutant strain via conjugation. For a *relA/spoT/fpRel* triple mutant, the plasmid pTok2::*ΔfpRel* was transferred to the *relA/spoT* markerless mutant strain.

Mutant complementation.

For complementation of mutants, a 3.0-kb fragment containing the native promoter and the *relA* gene, a 2.9-kb fragment containing the native promoter and the *spoT* and *rpoZ* genes, and a 2.4-kb fragment containing the native promoter and *fpRel* gene were amplified using the primer pairs RelAcom-F(*Bam*HI) and RelAcom-R (*Bam*HI), SpoTcom-F(*Bam*HI) and SpoTcom-R (*Bam*HI), and FpRelAcom-F(*Sal*I) and FpRelAcom-R(*Sal*I), respectively. PCR fragments were digested with corresponding restriction enzymes and were cloned into the pVSP61 vector to yield plasmids pRelA, pSpoT, and pFpRel. These were then introduced into corresponding marker-less mutant strains by electroporation.

Nucleotide extraction and ppGpp measurement by LC-MS/MS.

Bacterial strains were grown in 100 ml of KB to an OD₆₀₀ of approximately 1. Cells were harvested by centrifugation at 4,000 rpm, using an Eppendorf S5810 R centrifuge, for 15 min and were washed with 5 ml of HMM medium. Cell pellets were resuspended in 100 ml of HMM medium and were incubated with shaking at 250 rpm at 22°C for 30 min. Subsequently, bacterial cells were pelleted, and cells were immediately suspended in 2 ml of 100% cold methanol, were vortexed for 50 s, were frozen in liquid nitrogen, and were allowed to thaw on ice. Cell suspensions were then centrifuged at 4,000 rpm for 10 min at 4°C. Supernatants were transferred to a new tube and were stored on ice. Then, 2 ml of 100% cold methanol was added to the pellet. The freeze-thaw cycle was repeated, and the methanol extract was combined and, then, was filtered using a 0.22-μm pore size filter (Millipore, Bedford, MA, U.S.A.). The filtrate was freeze-dried and resuspended in 200 μl of distilled water for LC-MS/MS analysis. Pure ppGpp, purchased from TriLink Biotech Inc. (San Diego, CA, U.S.A.), was used as a positive control.

Nucleotide-enriched samples were analyzed in a 5500 QTRAP LC-MS/MS system (AB Sciex) using a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.), including a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent SB-Aq column (4.6 × 50 mm, 5μm) with a mobile phase A (10 mM ammonia formate in water)

and a mobile phase B (methanol). The flow rate was 0.3 ml/min. The linear gradient was as follows: 0 to 4 min, 99% A; 4.1 to 8.0 min, 5% A; and 8.1 to 13.0 min, 99% A. The autosampler was set at 5°C. The injection volume was 10 μl. Mass spectra were acquired under positive electrospray ionization with an ion spray voltage of +5,500 V. The source temperature was 450°C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 55 psi, respectively. Multiple reaction monitoring was used for quantification of ppGpp (m/z 604.1→m/z 152.0). The final ppGpp concentration was normalized to OD₆₀₀.

Table 3. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic ^a	Reference or source
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	Wild type	
<i>ΔrelA</i>	Km ^r , <i>relA</i> ::Kan, DC3000 derivative	This study
<i>ΔrelA/spoT</i>	Km ^r , <i>relA</i> and <i>spoT</i> ::Kan, DC3000 derivative	This study
<i>ΔrelA/spoT/fpRel</i>	Km ^r , <i>relA</i> , <i>spoT</i> and <i>fpRel</i> ::Kan, DC3000 derivative	This study
<i>Escherichia coli</i> DH10B	F ⁻ <i>mcrA</i> <i>Δ</i> (<i>mrr-hsdRMS-mcrBC</i>) <i>Φ80lacZΔM15 ΔlacX74 recA1 endA1 araΔ139 Δ</i> (<i>ara, leu</i>)7697 <i>galU galK λ-rpsL</i> (<i>Str</i> R) <i>nupG</i>	Invitrogen
DH5α	F ⁻ <i>Φ80lacZΔM15 Δ</i> (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (<i>rk</i> -, <i>mk</i> +) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i>	Invitrogen
Plasmids		
pTok2	ColE1 replicon, suicide plasmid, Tc ^r	Kitten and Willis 1996
pKD13	FRT-Kan-FRT, oriR6K; Ap ^r , Km ^r	Datsenko and Wanner 2000
pVSP61	Broad-host-range plasmid with replicons of pVS1 and pACYC184, Km ^r	Loper and Lindow 1994
pFLP2-omega	Suicide vector encoding flp recombinase (the omega fragment conferring spectinomycin resistance had been added into pFLP2); Sp ^r	R. Scott, unpublished data
pKT-trp	<i>gfp</i> marker gene driven by the <i>trp</i> promoter from <i>Salmonella typhimurium</i> cloned into the stable plasmid vector pPROBE-KT	Monier and Lindow 2003a
pAvrPto-gfp	<i>avrPto</i> effector promoter fused to GFP	Xiao et al. 2004
pTok2:: <i>ΔrelA</i>	<i>ΔrelA</i> ::Kan from overlapping polymerase chain reaction (PCR) cloned into pTok2; Tc ^r , Km ^r	This study
pTok2:: <i>ΔspoT</i>	<i>ΔspoT</i> ::Kan from overlapping PCR cloned into pTok2; Tc ^r , Km ^r	This study
pTok2:: <i>ΔfpRel</i>	<i>ΔfpRel</i> ::Kan from overlapping PCR cloned into pTok2; Tc ^r , Km ^r	This study
pRelA	<i>relA</i> gene with native promoter cloned into pVSP61 Km ^r	This study
pSpoT	<i>spoT</i> gene with native promoter cloned into pVSP61 Km ^r	This study
pFpRel	<i>spoT</i> gene with native promoter cloned into pVSP61 Km ^r	This study

^a Km^r, Tc^r, Ap^r, and Sp^r correspond to kanamycin, tetracycline, ampicillin and spectinomycin resistance, respectively.

RNA isolation and qRT-PCR.

Bacterial strains grown overnight at 28°C in KB medium were harvested by centrifugation, were washed with phosphate buffered saline (PBS), were reinoculated in either HMM or modified MG medium (replacing mannitol and L-glutamic acid in MG medium with 10 mM GABA), and were grown for 3 h. An RNA protect reagent (Qiagen, Hilden, Germany) was added to bacterial cells and total RNA was extracted using the RNeasy Mini BacteriaProtect kit (Qiagen). DNA was removed using the Turbo DNA-free kit (Ambion, Austin, TX, U.S.A.). RNA samples were quantified using a Nano-Drop ND-100 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, U.S.A.). cDNA was synthesized from 1 µg of RNA using SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and random hexamers. qRT-PCR was performed using 100 ng of cDNA and a Power SYBR Green PCR Master Mix on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.), following the manufacturer's specifications. Melting curve analysis was used to verify amplification of a single product. Gene expression was calculated using the relative quantification ($\Delta\Delta C_t$ [cycle threshold]) method. The C_t values of each gene were normalized to C_t values of the housekeeping sigma factor gene *rpoD*.

Flow cytometry analysis.

Bacterial cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, U.S.A.) as describe previously (Wang et al. 2009; Zhao et al. 2009). The WT and mutant strains containing the pAvrPto-GFP plasmid were grown overnight in KB and were harvested, washed, and resuspended in either HMM or HMM supplemented with 10 mM of amino acids. Bacteria cells were incubated in an appropriate medium for either 3 or 5 h at 18°C. Subsequently, bacterial cells were diluted in PBS for flow cytometry assay. Flow cytometry was performed using an LSR II 10-parameter multilaser analyzer (BD Biosciences). Data were collected for a total of 100,000 events and were analyzed statistically by gating, using flow cytometry software FCS Express V3 (De Novo Software, Los Angeles). A geometric mean was calculated for each sample, and the experiment was repeated three times.

Swarming motility, pyoverdine production, oxidative stress, and GABA utilization assay.

For the swarming motility assay, cells were grown overnight in KB and were harvested and washed in PBS. Cells were resuspended in PBS to a final concentration of $OD_{600} = 0.3$. A total of 10 µl was spotted onto the center of swarming plates (0.4% KB agar), and plates were incubated for 24 h at room temperature. Motility of bacterial cells was visually examined.

For pyoverdine production, bacterial cells of overnight cultures in KB were washed, were resuspended to an OD_{600} of 0.05 in MG medium, and were incubated along with shaking at 28°C for 24 h. Pyoverdine from supernatants was measured at OD_{405} , and were normalized by OD_{600} (Imperi et al. 2009).

For oxidative stress assay, bacterial cells were grown in KB medium, were washed, and were resuspended in PBS buffer with or without 1 mM H_2O_2 to a final concentration of approximately 1.0×10^8 CFU per milliliter (Yu et al. 2013). We selected this concentration of H_2O_2 so that more than 99% of the triple mutant would not survive. Bacterial suspensions were then incubated at room temperature for 15 min. Subsequently, serial dilutions were plated on KB agar to enumerate surviving cells. Percent survival was calculated by dividing H_2O_2 treated versus untreated cells.

For the GABA utilization assay, bacterial cells of overnight cultures in KB were washed and resuspended to an OD_{600} of 0.02 in modified MG medium with 10 mM GABA. Cells were grown overnight at 28°C, and growth was monitored by measuring OD_{600} .

Virulence assay and bacterial growth in tomato.

Virulence of DC3000 and its mutants was assessed on tomato (*Lycopersicon esculentum*), following spray inoculation. Briefly, bacterial cells were grown overnight in KB broth, were harvested by centrifugation, and were resuspended in PBS buffer with bacterial cells adjusted to an OD_{600} of 0.1 (approximately 10^8 CFU per milliliter). Bacterial cells were sprayed onto tomato plants (approximately 4 weeks old) until leaf surfaces were uniformly wet. For the first 24 h following inoculation, plants were covered with plastic tents to maintain high humidity and moisture. Inoculated plants were maintained in a greenhouse at 25°C for 7 days. Symptoms were recorded at 7 dpi. The experiments were repeated at least three times with similar results. For bacterial growth, four leaves were randomly collected from each plant at a given time point. Leaves were weighed separately and were macerated in PBS, using a mortar and pestle. Serial dilutions were made in PBS and were plated on KB agar medium amended with appropriate antibiotics. Cell numbers were calculated after enumeration of colonies on plates incubated at 28°C for 48 h.

Determination of cell lengths by epifluorescence microscopy.

Lengths of *P. syringae* cells were determined by epifluorescence microscopy. Bacterial strains constitutively expressing GFP were grown in KB to mid exponential phase ($OD_{600} = 0.7$) and were harvested, washed, and reinoculated at $OD_{600} = 0.1$ in either KB broth or in HMM. After 4 h, 3 µl of bacterial suspension was mixed with 5 µl of Aqua-Polymount (Polysciences, Warrington, PA, U.S.A.), was mounted between cover slips, and was immediately observed under an Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany) using FITC filter sets (absorbance: 490 to 494 nm; emission: 517 nm). Images were captured with an AxioCam MPc digital camera (Carl Zeiss). At least 200 individual cells from 10 different images were measured using ImageJ software available online at the National Institutes of Health.

Determination of cell viability by confocal microscopy.

Bacterial cell viability on leaf surfaces was observed using a confocal laser scanning microscope (Zeiss). Suspensions of bacterial strains harboring pKT-trp or pAvrPto-GFP in PBS buffer ($OD_{600} = 0.1$) were sprayed onto tomato (host) and tobacco (nonhost) leaves that were incubated under controlled conditions at 22°C. Plants were held under plastic tents for 24 hpi to maintain high humidity and moisture. Two plants were inoculated simultaneously, and leaves were randomly sampled from plants at each time point. Leaf discs (12 mm diameter) were mounted between cover slips (22 × 50-1) in Aqua-Polymount (Polysciences) and were mixed with 5 µl of propidium iodide (10 µg/ml). Samples were kept in the dark at room temperature for 15 min and were observed by an LSM 700 laser scanning microscope (Zeiss), using a 20× objective. At least six leaf discs were evaluated per strain at each time point. Images were obtained by mixing the signals recorded in two excitation lines (488-nm laser for GFP fluorescence and 568-nm laser for chloroplastic autofluorescence and propidium iodide emission). At least 10 randomly selected fields were examined for viability of bacterial cells on leaf surfaces.

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