The alternative sigma factor AlgT, but not alginate synthesis, promotes *in planta* multiplication of *Pseudomonas syringae* pv. *glycinea*

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The phytopathogen *Pseudomonas syringae* pv. *glycinea* produces the exopolysaccharide (EPS) alginate, which is thought to function in epiphytic fitness and virulence. A key regulator for alginate biosynthesis in *Pseudomonas aeruginosa* and *P. syringae* is the alternative sigma factor AlgT (σ^{22}). In this study, the contribution of alginate synthesis and AlgT to *in planta* epiphytic fitness and virulence of *P. syringae* was examined. Alginate biosynthesis mutants were generated for the *P. syringae* pv. *glycinea* strains PG4180 and PG4180.muc, representing a comprehensive set of alginate- and AlgT-positive or -negative derivatives. Analysis of *in vitro* and *in planta* phenotypes revealed that AlgT strongly promoted *in planta* growth, survival and symptom development, but decreased the ability to grow *in vitro*. In contrast, alginate biosynthesis had only marginal impact. Quantitative *in vitro* and *in planta* gene expression analyses for alginate biosynthesis and *algT* were carried out at two temperatures in AlgT-negative and -positive backgrounds. *algT* as well as *algD* gene expression was AlgT-dependent, plant-inducible and temperature-dependent, with higher expression at 18 compared to 28 °C; however, no temperature dependence was observed *in vitro*. Our data suggest that AlgT may act as a global regulator for virulence and *in planta* fitness traits of *P. syringae* independent of its role in EPS biosynthesis.

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INTRODUCTION

Virulence of phytopathogenic bacteria is often associated with production of exopolysaccharides (EPSs), which mediate multiple functions, including water absorption, accumulation of minerals and nutrients, and protection from hydrophobic and toxic macromolecules (Denny, 1995). EPSs are carbohydrate polymers that are secreted by a wide variety of bacteria and form loosely associated extracellular slime or remain closely attached to cells in the form of capsules (Whitfield, 1988). *Pseudomonas syringae* pv. *glycinea* PG4180 infects soybean plants (*Glycine max*) and causes bacterial blight disease. The infection process involves epiphytic colonization of leaf surfaces, establishment of infection sites via entry into the plant apoplast, multiplication within host tissue and development of disease symptoms (Alfano & Collmer, 1996; Hirano & Upper, 2000). *P. syringae* and other plant-pathogenic bacteria may cause 'cold-weather' diseases and many genes studied with respect to temperature exhibit increased transcription at temperatures well below the respective growth optima (Ullrich *et al.*, 2000). This includes virulence determinants such as those directing bacteria-to-plant gene transfer, plant cell-wall-degrading enzymes,

phytotoxins, ice nucleation activity, the type III protein secretion machinery and EPS production (Smirnova *et al.*, 2001). *P. syringae* pv. *glycinea* has been demonstrated to produce at least two EPSs: (i) the polyfructan levan and (ii) alginate, a copolymer of O-acetylated β -(1,4) linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Gross & Rudolph, 1987a; Hettwer *et al.*, 1998; Osman *et al.*, 1986).

P. syringae alginate production has been associated with increased epiphytic fitness, virulence, resistance to desiccation and toxic molecules, dissemination *in planta* (Rudolph *et al.*, 1994; Yu *et al.*, 1999) and the induction of water-soaked lesions on infected leaves (Fett & Dunn, 1989). A positive correlation between virulence of *P. syringae* and the quantity of alginate produced *in planta* has been demonstrated (Gross & Rudolph, 1987b; Osman *et al.*, 1986). The biosynthetic route to alginate in *P. syringae* is similar to that established for *P. aeruginosa*, where alginate synthesis has been studied extensively and where it functions as a virulence factor in strains infecting the lungs of cystic fibrosis patients (Fett *et al.*, 1992; Fialho *et al.*, 1990; Peñaloza-Vázquez *et al.*, 2004, 1997; Pier, 1998; Yu *et al.*, 1999). Genes encoding enzymes of alginate biosynthesis in *P. aeruginosa* and *P. syringae* are organized in an operon (*algD*–*algA*), with the exception of *algC*

Abbreviation: EPS, exopolysaccharide.

(Chitnis & Ohman, 1993; Peñaloza-Vázquez *et al.*, 2004, 1997; Zielinski *et al.*, 1991). The first gene of the operon, *algD*, encodes a GDP-mannose dehydrogenase, whose activity is the kinetic control point and is indispensable for the alginate pathway (Tatnell *et al.*, 1994). In *P. syringae*, *algD* is expressed during colonization of susceptible and resistant plant hosts (Keith *et al.*, 2003). The alginate biosynthetic operon in *P. aeruginosa* is controlled by several two-component systems and by the alternative sigma factor, AlgT (Mathee *et al.*, 2002). AlgT of *P. aeruginosa* acts as a global stress response sigma factor that activates transcription of the main heat-shock sigma factor RpoH, as well as different virulence determinants or toxic factors (Firoved *et al.*, 2002; Firoved & Deretic, 2003; Schurr & Deretic, 1997). AlgT in *P. syringae* (σ^{22}) is encoded by the first gene of the *algT-mucAB* operon, which activates alginate biosynthesis by activating its own transcription and that of *algD* (Keith & Bender, 1999; Schenk *et al.*, 2006).

All wild-type *P. aeruginosa* strains have the genetic capacity to synthesize alginate, but normally produce only small amounts of this polymer (Anastassiou *et al.*, 1987; Lam *et al.*, 1980; Pier *et al.*, 1986). Like *P. aeruginosa*, most phytopathogenic strains of *P. syringae*, including PG4180, are normally non-mucoid *in vitro* (Kidambi *et al.*, 1995; Schenk *et al.*, 2006). The loss of alginate production in *P. syringae* PG4180 is caused by a naturally occurring

nonsense mutation in the *algT* gene, which by chance reverted in the alginate-producing derivative PG4180.muc (Schenk *et al.*, 2006).

For *P. syringae*, the results of several previous studies have invited speculation on potential functions for alginate *in planta*. However, a comprehensive *in planta* study, which directly compares alginate and *algT* mutants, has not been reported thus far. In the present study, we investigated the contribution of the *P. syringae* EPS alginate and the sigma factor AlgT to *in vitro* and *in planta* growth, survival and virulence. Additionally, the impact of temperature on *in vitro* and *in planta* gene expression was analysed for *algT* and *algD* in strains producing or lacking the AlgT sigma factor.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was maintained and grown at 37 °C on Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). *P. syringae* was routinely maintained at 28 °C on mannitol–glutamate (MG) medium (Keane *et al.*, 1970). For liquid cultures at 18 or 28 °C, bacteria were grown in 200 ml Hoitink–Sinden minimal medium (HSC) (Palmer & Bender, 1993) in 1 l Erlenmeyer flasks. Bacterial growth was continuously monitored by measuring OD₆₀₀. Antibiotics were added to the media at the following concentrations (µg ml⁻¹): ampicillin, 50; kanamycin, 25; tetracycline, 25; gentamicin, 2.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook <i>et al.</i> (1989)
SM10	<i>thi-1</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacY</i> <i>supE</i> <i>recA</i> ::RP4-2-Tc::Mu; Km ^r	Hoang <i>et al.</i> (1998)
<i>Pseudomonas syringae</i> pv. <i>glycinia</i>		
PG4180.muc	Mucoid wild-type, <i>algT</i> ⁺	Schenk <i>et al.</i> (2006)
PG4180	Wild-type producing negligible amounts of alginate due to a point mutation in <i>algT</i>	Schenk <i>et al.</i> (2006)
PG4180.muc.alg	<i>algD</i> mutant derived from PG4180.muc; <i>algT</i> ⁺	This study
PG4180.alg	<i>algD</i> mutant derived from PG4180; <i>algT</i> ⁻	This study
Plasmids		
pGEM-T Easy	Ap ^r	Promega
pRK2013	Km ^r ; helper plasmid	Figurski & Helinski (1979)
pEX18Tc	Tc ^r , <i>oriT</i> ⁺ <i>sacB</i> ⁺ ; gene replacement vector	Hoang <i>et al.</i> (1998)
pPS858	Ap ^r Gm ^r ; FRT-cassette vector, containing <i>gfp</i>	Hoang <i>et al.</i> (1998)
pFLP2	Ap ^r , <i>oriT</i> ⁺ <i>sacB</i> ⁺ ; Flp recombinase-producing plasmid	Hoang <i>et al.</i> (1998)
pGEM_algD_left#3	Ap ^r ; contains a 1386 bp <i>Bam</i> HI– <i>Xba</i> I PCR fragment (located upstream of <i>algD</i>)	This study
pGEM_algD_right#3	Ap ^r ; contains a 2275 bp <i>Xba</i> I– <i>Bam</i> HI PCR fragment (located downstream of <i>algD</i>)	This study
pGEM_algD3_both	Ap ^r ; 2322 bp <i>Xba</i> I– <i>Sac</i> I fragment of pGEM_algD_right#3 cloned into <i>Xba</i> I/ <i>Sac</i> I-linearized pGEM_algD_left#3	This study
pGEM_algD3_GmGFP	Ap ^r Gm ^r ; 1833 bp FRT-cassette fragment of pPS858 cloned into <i>Xba</i> I-linearized pGEM_algD3_both	This study
pEX18Tc_algD3_GmGFP	Tc ^r Gm ^r ; 5950 bp <i>Bam</i> HI fragment of pGEM_algD3_GmGFP cloned into <i>Bam</i> HI-linearized pEX18Tc	This study

*Ap, Ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

Molecular genetic techniques. Small-scale isolation of plasmid DNA, restriction enzyme digests, agarose gel electrophoresis, purification of DNA fragments from agarose gels, electroporation, PCR, ligation of DNA fragments and other routine molecular methods were performed using standard protocols (Sambrook *et al.*, 1989). Isolation of plasmid DNA from *P. syringae* was accomplished by the method of Kado & Liu (1981). Genomic DNA from *P. syringae* was isolated as described by Ausubel *et al.* (1994). Sequencing was carried out commercially (MWG Biotech, Ebersberg, Germany).

Generation of *algD* mutants. *P. syringae* *algD* mutants were generated using the broad-host-range FLP-FRT recombination system (Hoang *et al.*, 1998). Two fragments flanking the *algD* gene were PCR-amplified from PG4180.muc genomic DNA using the oligonucleotide primer pairs *algD_ol2*, *algD_il3* and *algD_ir3*, *algD_or2* (Table 2). PCR products were cloned into vector pGEM-T Easy (Promega), yielding plasmids pGEM_*algD_left#3* (4871 bp) and pGEM_*algD_right#3* (5290 bp). A 2322 bp *XbaI*-*SacI* fragment cut from pGEM_*algD_right#3* was ligated into *XbaI*/*SacI*-digested pGEM_*algD_left#3*, yielding the 7138 bp plasmid pGEM_*algD3_both*. A 1833 bp *SmaI* FRT-cassette fragment cut from pPS858 was ligated into *XbaI*-digested and blunt-ended pGEM_*algD3_both*, yielding the 8975 bp plasmid pGEM_*algD3_GmGFP*. A 5950 bp *BamHI* fragment cut from pGEM_*algD3_GmGFP* was ligated into *BamHI*-digested pEX18Tc yielding the final *algD* gene replacement plasmid pEX18Tc_*algD3_GmGFP* (12.3 kb). This plasmid was mobilized into strains PG4180.muc and PG4180 by triparental matings with the helper plasmid pRK2013 (Figurski & Helinski, 1979). Putative mutants were screened for homologous recombination events by checking their antibiotic resistance and GFP fluorescence. The FRT-cassette carrying *gfp* and the gentamicin determinant was finally excised using the pFLP2 recombinase-encoding plasmid as described by Hoang *et al.* (1998). To confirm the altered genotypes, PCR amplification of *algD* using primers *algD-cl* and *algD-cr* (Table 2) was carried out, yielding a 903 bp fragment for the two mutants, as opposed to the presence of wild-type *algD*, forming a 1258 bp PCR fragment in samples of PG4180 and PG4180.muc.

Isolation and quantification of alginate. Strains were grown on MG agar (three plates per strain) containing the appropriate antibiotics at 28 °C for 96 h. Cells were washed from each plate and resuspended in 0.9 % NaCl. Alginate isolation and quantification were performed as described by May & Chakrabarty (1994), and

alginic acid from seaweed (*Macrocystis pyrifera*; Sigma) was used as a standard. The experiment was repeated twice and mean values were obtained for the quantity of alginate produced per mg protein.

Determination of bacterial growth *in planta*. Growth of *P. syringae* *in planta* was evaluated on soybeans [*Glycine max* (L.) Merr. cv. Maple Arrow]. Soybean seedlings were grown in open shelves equipped with fluorescent lamps at 22–25 °C, 55 % humidity, with a 16 h photoperiod (350 $\mu\text{E m}^{-2} \text{s}^{-1}$). Two days before inoculation, plants were transferred to plant growth chambers (Conviron E/2) at 18 or 28 °C, 55 % humidity, with a 16 h photoperiod (350 $\mu\text{E m}^{-2} \text{s}^{-1}$). PG4180 strains were incubated for 48 h at 28 °C on MG agar. Cells were suspended in distilled water, adjusted to 1×10^7 c.f.u. ml^{-1} and applied to both surfaces of the leaves with an airbrush (~8 p.s.i.) until the leaf surfaces were uniformly wet. Inoculated plants were transferred to shelves equipped with fluorescent lamps, and survival and growth of bacterial strains was monitored by removing random leaf samples at 0–28 days post-inoculation (three replicates per time point). Leaves were weighed separately and macerated in 10 ml sterile PBS (g fresh wt) $^{-1}$ using a mortar and pestle. Bacterial counts [c.f.u. (g fresh wt) $^{-1}$] were determined by plating dilutions of leaf homogenates onto MG agar and counting fluorescent colonies after incubation at 28 °C for 96 h.

***In vitro* and *in planta* gene expression analysis by multiplexed fluorescent Northern hybridization.** *In vitro* and *in planta* gene expression analysis was carried out according to our recently described method (Schenk *et al.*, 2008). In brief, bacterial and plant total RNA was simultaneously isolated from leaf tissue infiltrated with *P. syringae* strains 3 days after inoculation. Total RNA was isolated by an acid-phenol extraction using an extraction buffer containing glycine, SDS, cetyltrimethylammonium bromide, high-molecular-mass polyethylene glycol and β -mercaptoethanol steps followed by LiCl precipitation. Since total RNA samples isolated from infected leaf tissue represent a mixture in which the bacterial RNA portion is unknown, all expression data had to be normalized to a reference, such as the expression of 23S rRNA. Therefore, DIG-labelled gene-specific probes were multiplexed with an Oyster-645 fluorescence-labelled 23S RNA probe in a single Northern hybridization reaction. Hybridization probes of the following sizes internal to the structural genes were generated with respective primer pairs: for *algT*, 536 nt (*algT_fwd*, *algT_revT7*); *algD*, 641 nt (*algD_fwd*, *algD_revT7*); 23S#1, 146 nt (23S_fwd#1, 23S_revT7#1) (Table 2).

Table 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5'–3')*
<i>algD_ol2</i>	CGGATCCTAGAAATCCCCGGACGATCAAAG
<i>algD_il3</i>	TCTAGATTCGGCGAAGTCGGTAGTGC
<i>algD_ir3</i>	TCTAGAACGCACCGATCATCCGCAAG
<i>algD_or2</i>	CGGATCCTTCGGGACGACACGTGTTG
<i>algD_cl</i>	TTGGTTTGGGTTATGTCGG
<i>algD_cr</i>	TTGGTCATGAAGCCACC
<i>algT_fwd</i>	CGGATCCCTTAACGAGGAGTGTTTCATG
<i>algT_revT7</i>	TAATACGACTCACTATAGGGAGGACGGTACCAACAGGACACTG
<i>algD_fwd</i>	GCGTATCAGCATATTTGGTTTGGG
<i>algD_revT7</i>	TAATACGACTCACTATAGGGAGGCGTTGCAGGTGTACTIONGATCA
23SrRNA_fwd#1	ACGTGGACCAGCCCTTAAGTTGTATTG
23SrRNA_revT7#1	TAATACGACTCACTATAGGGAGGCCCATATTCAGACAAG

*Restriction sites incorporated into primers are underlined: GGATCC, *BamHI*; TCTAGA, *XbaI*. T7 RNA polymerase promoter sequences incorporated into primers are in italic type.

RESULTS

Generation and characterization of alginate biosynthesis mutants

The AlgT-producing and alginate-synthesizing *P. syringae* pv. *glycinea* strain, PG4180.muc, was found to be impaired in its *in vitro* growth, but showed increased *in planta* epiphytic fitness and better symptom development compared to the AlgT- and alginate-negative strain, PG4180 (Schenk *et al.*, 2006). To find out whether this phenotype is based on alginate biosynthesis or is mediated by the AlgT sigma factor, *algD* mutants were generated in PG4180.muc and PG4180 by gene deletion (Table 3). The generated *algD* mutants PG4180.muc.alg (mutant of PG4180.muc) and PG4180.alg (mutant of PG4180) were verified by PCR analysis. The *algT* mutation in PG4180 is caused by a single base pair exchange at position 10 of the *algT* ORF (Schenk *et al.*, 2006). Therefore, the *algT* genotype was confirmed for all derivatives by nucleotide sequencing.

Visual examination of the mucoidy of bacterial colonies grown on MG agar plates at 18 and 28 °C showed a clear pattern of alginate production according to the respective genotypes (Table 3). Quantification of alginate production on MG agar plates at 28 °C for 96 h showed that the *algD* and/or *algT* mutants produced only negligible amounts of alginate [less than 2.5 µg alginate (mg protein)⁻¹], approximating the detection limits of the assay (Table 3). In contrast, PG4180.muc produced large amounts of alginate [105.6 µg alginate (mg protein)⁻¹].

In vitro and *in planta* growth studies

The influence of alginate synthesis and AlgT on *in vitro* and *in planta* growth was studied using the four derivatives, PG4180.muc, PG4180.muc.alg, PG4180 and PG4180.alg (Table 3).

For *in vitro* growth studies, bacteria were cultured in HSC medium at 18 and 28 °C, where 28 °C represents the optimal growth temperature and 18 °C represents the temperature at which *P. syringae* pv. *glycinea* is most virulent on its host plant (Ullrich *et al.*, 2000). Optical density of cultures was monitored continuously until the cultures entered the late stationary phase (Fig. 1). PG4180.muc and its *algD* mutant PG4180.muc.alg were both found to be impaired in their *in vitro* growth at 18

and 28 °C compared to PG4180 and its respective *algD* mutant, PG4180.alg. This became most apparent in their extended doubling times (t_d) (1.2- to 1.4-fold at 18 and 28 °C, respectively) for PG4180.muc (t_d 18 °C: 9.3 h; t_d 28 °C: 6.3 h) compared to PG4180 (t_d 18 °C: 7.8 h; t_d 28 °C: 4.3 h). Doubling times of the *algD* mutants showed no significant differences to those of the corresponding parental strains, suggesting that the presence or absence of AlgT were the determining factors for these growth phenotypes.

Virulence, survival and *in planta* growth was evaluated by monitoring development of symptoms and bacterial populations on soybean plants for 28 days (Fig. 2). Plants were spray-inoculated with bacterial suspensions of 1×10^7 c.f.u. ml⁻¹ as determined by living cell counts (data not shown). PG4180.muc and its *algD* mutant PG4180.muc.alg showed 10- to 20-fold better survival 2 h after spraying compared to PG4180 and its respective *algD* mutant, PG4180.alg. To rule out potential biases associated with the spray inoculation technique, the same bacterial suspensions were also sprayed into sterile glass flasks and their c.f.u. ml⁻¹ values were determined before and after spraying. We found that spraying had no effect on the c.f.u. ml⁻¹ values obtained (data not shown). One day post-inoculation, the c.f.u. ml⁻¹ value dropped for all strains by about 50- to 100-fold. Bacterial populations started to increase in numbers from day 2 onwards (Fig. 2). Multiplication rates were about the same for all strains until day 7. From day 7 on, PG4180.muc and PG4180.muc.alg continued to grow to 1×10^8 c.f.u. ml⁻¹, whereas PG4180 and its *algD* mutant remained at a 100-fold lower stationary phase level with 1×10^6 c.f.u. ml⁻¹. Both *algD* mutants, particularly PG4180.muc.alg, showed slightly lower c.f.u. ml⁻¹ values compared to their parental strains, and this decrease in population size was faster at the end of the sampling period. Symptom development was visually monitored 10–12 days post-infection. Although all infected plants showed symptoms characterized by necrotic lesions surrounded by chlorotic haloes, those plants infected with PG4180.muc and PG4180.muc.alg exhibited significantly more abundant symptoms than those treated with PG4180 or its *algD* mutant (data not shown).

PG4180.muc and PG4180.muc.alg, both possessing an intact *algT* gene, had a clear advantage in their ability to survive and to increase population size on soybean leaves compared to PG4180 or its respective *algD* mutant,

Table 3. *P. syringae* pv. *glycinea* genotypes and resulting phenotypes

Strain	Genotype		Phenotype	µg alginate (µg protein) ⁻¹
	<i>algT</i>	<i>algD</i>	Alginate	
PG4180.muc	+	+	+	105.6 ± 11.7
PG4180.muc.alg	+	–	–	1.6 ± 1.5
PG4180	–	+	–	2.1 ± 0.6
PG4180.alg	–	–	–	1.0 ± 0.5

Fig. 1. *In vitro* growth of *P. syringae* PG4180.muc and PG4180.muc.alg (a), and PG4180 and PG4180.alg (b) in HSC medium at 18 and 28 °C as monitored by OD₆₀₀. The data represent the mean values from six independent cultures per strain ($P < 0.001$).

PG4180.alg. The actual *in planta* multiplication rates during exponential growth showed no significant difference between all tested derivatives.

In summary, our results indicated that the presence of a functional AlgT, but not of alginate biosynthesis, hinders the ability to grow *in vitro*. In contrast, AlgT, but not alginate production, strongly promotes *in planta* survival and symptom development of *P. syringae* pv. glycinea.

Analysis of *algT* and *algD* transcript levels *in vitro* and *in planta*

Keith *et al.* (2003) indicated that *algD* transcription in *P. syringae* pv. tomato can be induced by plant-borne signals.

Fig. 2. *In planta* growth of *P. syringae* PG4180.muc and PG4180.muc.alg, and PG4180 and PG4180.alg on soybean leaves. Bacterial strains were spray-inoculated and plants were grown in shelves equipped with fluorescent lamps at 22–25 °C. The data represent the mean values from three independent leaf samples. Error bars represent the SD from the means ($n = 3$, $P < 0.005$).

To explore the situation in *P. syringae* pv. glycinea, *in vitro* and *in planta* transcript levels of *algT* and *algD* at 18 and 28 °C in the alginate-producing strain PG4180.muc and its AlgT-negative counterpart, PG4180, were analysed by multiplexed spot blot Northern hybridization (Schenk *et al.*, 2008) (Fig. 3).

For *in vitro* samples, PG4180.muc and PG4180 were cultured in HSC medium at 18 and 28 °C to an OD₆₀₀ of 1.0. *In planta* samples were obtained by infiltration of soybean leaves with bacterial suspensions of 1×10^7 c.f.u. ml⁻¹ followed by 3 days incubation in growth chambers at 18 or 28 °C, as described previously (Schenk *et al.*, 2006). DIG-labelled *algT* and *algD* probes were each multiplexed with an Oyster-645-labelled 23S#1 probe and used for quantitative spot blot hybridizations with *in vitro* and *in planta* total RNA samples. All probes gave specific signals to samples containing *P. syringae* total RNA, but not to the plant total RNA controls (data not shown). Hybridization signals for *algT* and *algD* were quantified and normalized with their corresponding 23S rRNA signals as described previously (Schenk *et al.*, 2008). Transcript levels for *algT* were 5- to 10-fold higher in all samples of strain PG4180.muc compared to PG4180 (Fig. 3). *In planta* data showed clear temperature-dependent and plant-inducible *algT* expression with highest values at 18 °C, whereas *in vitro* transcription levels were twofold lower and not thermo-responsive. Expression

Fig. 3. Quantitative *in vitro* and *in planta* *algT* and *algD* gene expression. Relative abundance of *P. syringae* pv. *glycinea* *algT* and *algD* mRNA *in vitro* (OD₆₀₀ of 1.0 in HSC medium) and *in planta* (3 days after infiltration of soybean leaves with 1×10^7 c.f.u. ml⁻¹) at 18 and 28 °C. Data was generated by multiplexed spot blot hybridization, quantification of gene-specific signals and normalization to the corresponding 23S rRNA signals. Error bars represent the SD from the means of replica spots ($n=3$, $P<0.001$).

patterns of *algD* resembled that of *algT* with the same temperature-dependent and plant-inducible phenotype. Transcript levels for *algD* were 3- to 10-fold higher for PG4180.muc than for PG4180. Interestingly, the *algT* and *algD* transcript levels in PG4180 were not totally suppressed, suggesting a certain AlgT-independent background expression for both genes.

These data indicated that *algT* as well as *algD* were induced *in planta* in a temperature-dependent manner with higher expression at 18 °C. Furthermore, our findings suggested that alginate biosynthesis in *P. syringae* pv. *glycinea* is transcriptionally activated by AlgT both *in vitro* and *in planta*.

DISCUSSION

In this study, the role of the EPS alginate and the sigma factor AlgT in the soybean plant pathogen *P. syringae* pv. *glycinea* PG4180 was investigated. To identify potential temperature-mediated or plant-borne effects on gene expression of alginate biosynthesis and AlgT, transcription was analysed *in vitro* and *in planta* at two temperatures, 18 and 28 °C.

In a previous study, lack of alginate production in PG4180 was found to be caused by a nonsense mutation in *algT* (Schenk *et al.*, 2006). This mutation reverted in the PG4180.muc derivative into an intact ORF and led to expression of a functional AlgT protein. Interestingly, PG4180.muc was impaired in its *in vitro* growth, but showed increased epiphytic fitness *in planta* and significantly larger numbers of typical disease symptoms compared to the AlgT-deprived strain PG4180. This raised the

question whether the observed phenotype was due to the ability to produce alginate or was based on other regulatory effects of AlgT. In the present study, generation of *algD* knockout mutants in PG4180.muc and PG4180 enabled us to dissect EPS synthesis effects from other potential effects of the alternative sigma factor AlgT.

The characterization of the two *algD* mutants showed that disruption of *algD* led to an alginate-negative phenotype as expected, since *algD* had been shown to be indispensable for alginate biosynthesis in *P. aeruginosa* (Tatnell *et al.*, 1994). We did not observe a significant difference in alginate production between the AlgT-deficient strain PG4180 and its *algD* mutant, PG4180.alg. This adds evidence to the model in which AlgT comprises the main activator and regulatory switch for alginate biosynthesis in *P. syringae* pv. *glycinea* *in vitro*. This had previously been demonstrated for the related phytopathogen *P. syringae* pv. *syringae* FF5 (Fakhr *et al.*, 1999; Keith & Bender, 1999) and the opportunistic human pathogen *P. aeruginosa* (Hershberger *et al.*, 1995; Martin *et al.*, 1994; Ohman *et al.*, 1996).

Comparison of *in vitro* and *in planta* growth and virulence of the PG4180 derivatives analysed (Table 3) demonstrated an impaired *in vitro* growth, an increased *in planta* epiphytic fitness and a significantly larger number of symptoms for PG4180.muc compared to PG4180, which confirmed our previous data (Schenk *et al.*, 2006). The *algD* mutants PG4180.muc.alg and PG4180.alg showed identical *in vitro* and very similar *in planta* phenotypes compared to their respective parental strains. This surprising result led to the conclusion that AlgT is mainly responsible for impaired *in vitro* growth and increased *in planta* epiphytic fitness in strain PG4180.muc. If alginate

biosynthesis had caused this phenotype, the *algD* mutant PG4180.muc.alg should have exhibited a phenotype different from that of its parental strain PG4180.muc, but rather similar to that of PG4180 or PG4180.alg. In contrast to our findings, a different *in planta* scenario had been previously described for an *algL*⁻ alginate mutant in the related phytopathogen *P. syringae* pv. *syringae* FF5 (Yu *et al.*, 1999). A slightly lower *in planta* population size for PG4180.muc.alg in comparison to PG4180.muc could be assigned to lack of alginate biosynthesis and was also found in PG4180.alg, compared to its parental strains. This result demonstrated that *P. syringae* AlgT has a significant impact on *in planta* growth and virulence while alginate production only seems to be of marginal importance. This contradicts results of some other studies where alginate production had been associated with epiphytic fitness, virulence and symptom development (Fett & Dunn, 1989; Gross & Rudolph, 1987b; Osman *et al.*, 1986; Rudolph *et al.*, 1994; Yu *et al.*, 1999). However, in support of our findings, Beattie & Lindow (1994) identified *P. syringae* mutants that were EPS-deficient in culture but were indistinguishable from their parental strain with respect to their epiphytic growth and survival. Likewise, a screen of Tn5 mutants of the closely related phytopathogen *P. syringae* pv. *syringae* B728a revealed several EPS-altered mutants which were only slightly or not altered in growth or survival on bean leaves (Lindow *et al.*, 1993).

It remains unknown which genes, besides those involved in alginate biosynthesis, are controlled by AlgT in *P. syringae*. In *P. aeruginosa* it was shown that AlgT directs transcription of a large set of virulence determinants or toxic factors (Firoved *et al.*, 2002; Firoved & Deretic, 2003) and of the gene encoding the main heat-shock factor RpoH (Schurr & Deretic, 1997). Microarray analysis revealed that AlgT exerts broad-range regulatory effects on approximately 6% of the *P. aeruginosa* genome, with alginate and flagellar genes accounting for only one-fifth of AlgT-regulated genes (Tart *et al.*, 2005). AlgT was found to be essential for the tolerance of a *P. fluorescens* biocontrol strain towards desiccation and osmotic stress (Schnider-Keel *et al.*, 2001). A *P. syringae* pv. *syringae* *algT* mutant displayed increased sensitivity to heat, paraquat and hydrogen peroxide, and *algT* expression could be stimulated by heat shock, osmotic and oxidative stress (Keith & Bender, 1999). Therefore, we propose a role for AlgT as a transcriptional regulator of several yet-to-be determined genetic traits important for *in planta* growth, survival and virulence of *P. syringae*.

Not surprisingly, transcript levels were elevated for *algT* and *algD* in PG4180.muc compared to PG4180. It is known that AlgT auto-induces transcription of the *algT-muc* gene cluster and that of the alginate biosynthetic operon in *P. aeruginosa* (Govan & Deretic, 1996; Schenk *et al.*, 2006). *In vitro*, no temperature-dependent transcription was observed, but *in planta* expression of *algT* and *algD* was clearly induced at the lower temperature of 18 °C, indicating that plant-borne signals combined with low temperature activate *algT* and, in consequence, *algD*

transcription as well. Although at a lower level, *algD* transcription in PG4180 showed a thermoresponsive and plant-inducible pattern comparable to that in PG4180.muc, indicating that additional yet unknown factors influence *algD* transcription besides AlgT.

In conclusion, the sigma factor AlgT contributes to *P. syringae* pv. *glycinea* *in planta* growth, survival and symptom development, whereas *in vitro* bacterial growth is negatively influenced by AlgT. Simultaneously, our observations indicated a negligible role for the EPS alginate for *in planta* growth and virulence. Future studies will focus on the identification of unknown AlgT-dependent genes in *P. syringae* and may thus help to reveal a potential AlgT regulon, which in turn might include novel plant-inducible genes or those important for the plant-microbe interaction.

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