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Proteomic Analysis of a Global Regulator GacS Sensor Kinase in the Rhizobacterium, *Pseudomonas chlororaphis* O6

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The GacS/GacA system in the root colonizer *Pseudomonas chlororaphis* O6 is a key regulator of many traits relevant to the biocontrol function of this bacterium. Proteomic analysis revealed 12 proteins were down-regulated in a *gacS* mutant of *P. chlororaphis* O6. These GacS-regulated proteins functioned in combating oxidative stress, cell signaling, biosynthesis of secondary metabolism, and secretion. The extent of regulation was shown by real-time RT-PCR to vary between the genes. Mutants of *P. chlororaphis* O6 were generated in two GacS-regulated genes, *trpE*, encoding a protein involved in tryptophan synthesis, and *prnA*, required for conversion of tryptophan to the antimicrobial compound, pyrrolitrin. Failure of the *trpE* mutant to induce systemic resistance in tobacco against a foliar pathogen causing soft rot, *Pectobacterium carotovorum* SCCI, correlated with reduced colonization of root surfaces implying an inadequate supply of tryptophan to support growth. Although colonization was not affected by mutation in the *prnA* gene, induction of systemic resistance was reduced, suggesting that pyrrolnitrin was an activator of plant resistance as well as an antifungal agent. Study of mutants in the other GacS-regulated proteins will indicate further the features required for biocontrol-activity in this rhizobacterium.

Keywords **:** induced systemic resistance, proteomic analysis, tryptophan metabolism

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Certain rhizobacteria stimulate plant growth and responses to stress (Bloemberg and Lugtenberg, 2001). Some beneficial bacteria inhibit growth of phytopathogenic fungi by production of antifungal metabolites, and/or exoenzymes. The antifungal agents include hydrogen cyanide (HCN), siderophores, biosurfactants and antibiotics, and enzymes including proteases, lipases, chitinases, and glucanases (Dubis et al., 2007; Haas and Defago, 2005; Raaijmakers et al., 2002). The root colonizer *Pseudomonas chlororaphis* O6 produces several compounds with antifungal activity including a pyoverdine-like siderophore, phenazines, pyrrolnitrin and HCN (Kang et al., 2007; Lee et al., 2011; Park et al., 2011). Effective root colonization by *P. chlororaphis* O6 also protects plants from pathogens through induction of systemic resistance against various plant diseases as well as drought and salinity stress (Cho et al., 2008; 2012; Han et al., 2006).

The two component sensor kinase system involving GacS and GacA is conserved in plant-associated pseudomonads and regulates production of many of the biocontrol active components. The system involves activation of phosphorylation of GacS by an as yet unknown signal followed by phospho-transfer to the GacS regulator. Phosphorylated GacA activates changes expression from genes encoding small regulatory RNAs, such as RsmX, RsmY and RsmZ, which compete with translational repressors, RsmA and RsmB (Brencic et al., 2009). The GacS/GacA regulon is extensive, for example encompassing about 10% of the genes in *P. fluorescens* Pf-5 (Hassan et al., 2010).

The genome of *P. chlororaphis* O6 (Loper et al., 2012) possesses genes potentially encoding GacS, GacA, and the proteins, RsmA and RsmB interacting with rRNAs. However, findings with *P. chlororaphis* O6 and other pseudomonads reveal that the GacS-regulated traits differ between strains. For example, a *gacS* mutant of *P. chlororaphis* O6

has increased swimming and swarming motilities (Kim et al., 2014a), whereas lack of GacS in *P. fluorescens* Pf-5 has no effect on swimming, and decreases swarming activity, due to mainly loss in production of a surfactant (Hassan et al., 2010).

This study employed a proteomic approach for better understanding the genes regulated by GacS in *P. chlororaphis* O6, and we compared proteomes from the wild type and *gacS* mutant strains. Proteins that were down regulated in the *gacS* mutant were identified and their potential functions deduced. The role of two of these proteins, one involved in pyrrolnitrin production and the second in tryptophan biosynthesis, was explored using mutants in the GacS-regulated genes, *prnA* and *trpE*. The abilities of the *prnA* and *trpE* mutants to colonize roots and induce systemic resistance were investigated.

Bacteria were stored at −70°C in 25% glycerol. The *gacS* mutant and the *gacS*-complemented strain of *P. chlororaphis* O6 were constructed previously (Kang et al., 2004) and grown at 28°C with shaking at 200 rpm in King's medium B broth. A *prnA* mutant of *P. chlororaphis* O6 was constructed previously (Park et al., 2011). Cultures of *Escherichia coli* DH5α were grown at 37°C on Luria-Bertani (LB) broth.

Extracts were prepared from the wild type, the *gacS* mutant and the complemented mutant by sonication of cells grown to stationary phase in LB and harvested by centrifugation. Proteins were extracted by washing cells twice in ice-cold phosphate buffered saline before suspension in sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl dimethyammonio-1-propanesulfonate, 1% (w/v) dithiothreitol and 2% (v/v) pharmalyte and 1 mM benzamidine. After sonication for 10 seconds and incubation for one h at room temperature, the mixture was vortexed and centrifuged at 15,000 × g for one h at 15°C to obtain the soluble fraction used in gel analysis. Changes in protein profiles between the wild type, the *gacS* mutant, and the complemented *gacS* mutant were detected by two-dimensional gel electrophoresis using procedures described previously (Oh et al., 2013b). Protein spot intensities of wild type and *gacS* mutant on 2-D PAGE analyses were assessed by Student's *t* test. Three independent two dimensional protein analyses were performed, and significantly up- or down-regulated protein spots in the *gacS* mutant were selected and identified using Q-TOF analysis (Oh et al., 2013b).

Twelve protein spots were observed in the *gacS* mutant at lesser intensities than in the gels from the wild type strain (Fig. 2A). Based on a cut-off value of two-fold change, peptide identification revealed they were a catalase/peroxidase with homology to KatG from *P. fluorescens* Pf-01 (Table 1), a tryptophan halogenase (PrnA), catalyzing the first step in pyrrolnitrin synthesis, a single-strand DNA binding protein (Ssb), a serine protease (PspB), a recombination associated protein (RdgC), and a potential secretin (CpaC), involved in pilus synthesis, and an outer membrane protein (OprF) (Table 1). Proteins showing fold changes between two- and four- fold were: a protein

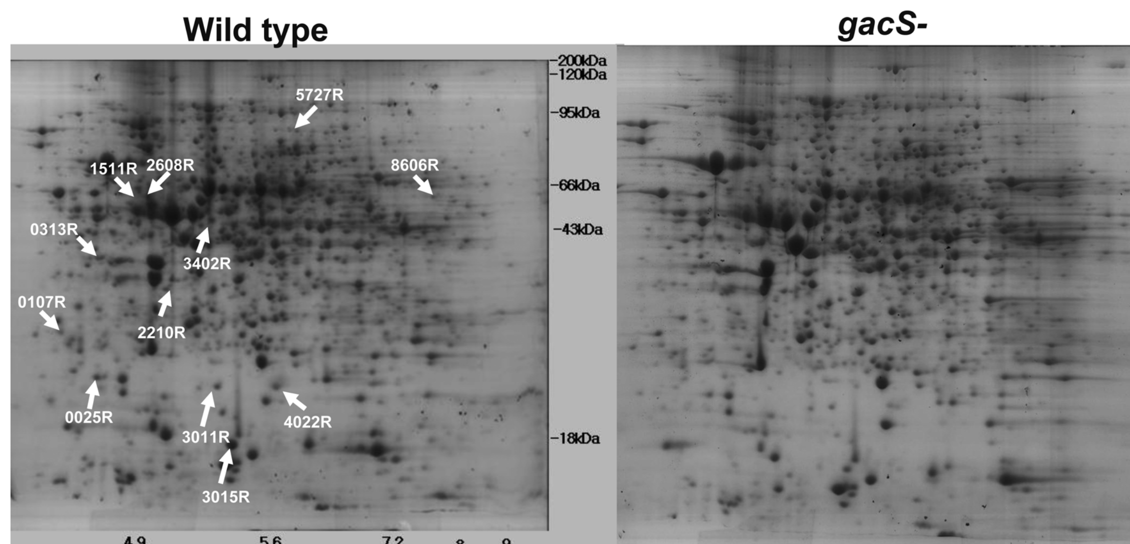

Fig. 1. Representative images of two-dimensional electrophoresis gels showing proteins from the wild type *Pseudomonas chlororaphis* O6 (wild type) and the *gacS* mutant (*gacS*-). Extracts were from cells grown to stationary phase in rich medium. The proteins were stained with silver. Locations of proteins that were present in extracts from the wild type but to a lesser extent in the extracts from the mutant are shown by arrows.

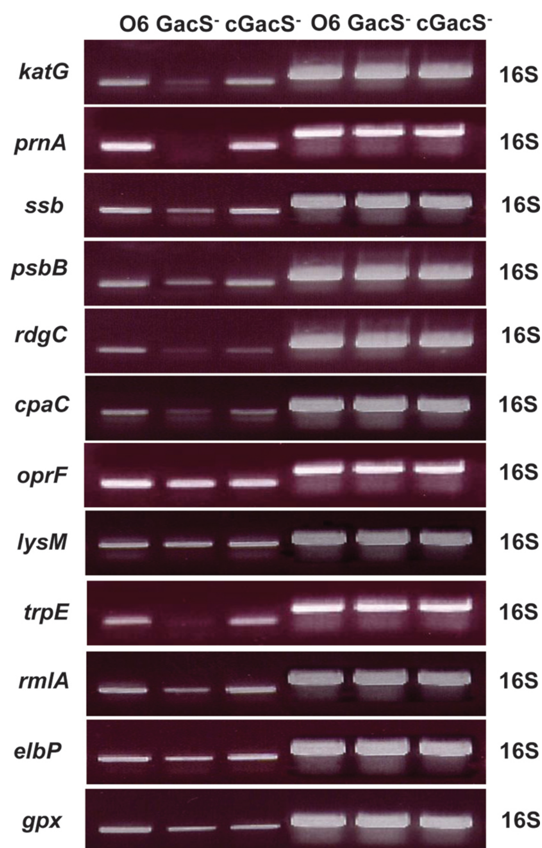

Fig. 2. Transcript accumulations from selected genes in the wild type (O6), the *gacS* mutant (GacS-) and the complemented *gacS* mutant (cGacS-) were assessed by RT-PCR. Data are shown for RNA extracted from stationary phase cells from one of two independent studies showing the same results. PCR bands from RNA transcripts of the 16S rRNA genes are shown to confirm equal loading of the wells.

with a LysM domain associated with binding peptidoglycan (LysM), the anthranilate/para-aminobenzoate synthase component I (TrpE), involved in tryptophan synthesis from chorismate, glucose 1-phosphate thymidilate transferase (RmlA) required for rhamnose synthesis, a protein functioning in isoprenoid biosynthesis (ElbP), and glutathione peroxidase (Gpx) (Table 1).

The study was continued with a transcript analysis to determine the extents to which changes protein level in the *gacS* mutant correlated with altered gene expression. RNA accumulation from the genes predicted to encode the GacS-regulated proteins was evaluated by endpoint RT-PCR. Cells grown in LB broth were harvested in stationary phase ($OD_{600\ nm}$ = 2.4). Total RNA was isolated using the Trizol method following the user's manual (GIBCO BRL, Rockville, MD, USA). RT-PCR was performed using the QuantiTect SYBR Green reverse transcription-PCR kit (Qiagen Inc., Valencia, USA). A reaction mixture of 25 µl was incubated at 50ºC for 30 min for reverse transcription, followed by RT-PCR with each primer set (Supplementary Table 1). A Rotor-Gene 2000 Real Time Cycler machine (Corbett Research Inc., Australia) was used for 35 cycles with denaturation at 94ºC for 15 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 30 seconds. PCR reactions were stop after 20 cycles and PCR products were loaded on 2% agarose gel. The results provided are typical of three independent studies. Findings shown in Fig. 2 are from stationary phase cells and comparable loading between samples was demonstrated from the consistency of the PCR products for the 16S rRNA genes (Fig. 2).

Transcripts in the *gacS* mutant compared to the wild type were most reduced for *katG*, *prnA*, *rdgC*, *cpaC* and *trpE*. For other genes, expression was impaired (*psbB*, *ssb* and *rmlA*) by the *gacS* mutation. However, for *gpx*, *lysM*, and *oprF* transcript levels were near wild type. These findings suggest that other mechanisms other than direct effects on transcription for control of protein levels may be operating.

We have prior evidence that genes, such as *prnA* and *trpE*, showing changes in transcript levels in the *gacS* mutant, were part of the RpoS regulon in *P. chlororaphis* O6 (Oh et al., 2013a; Park et al., 2011). These findings agree with control of pyrronitrin production by quorum sensing as shown in another *P. chlororaphis* isolate PA23 (Selin et al., 2012) and specifically by mutation in *gacA* in *P. chlororaphis* isolate 30–84 (Wang et al., 2013). Indeed, extraction and assay showed pyrrolinitrin formation was eliminated in an *rpoS* mutant (Park et al., 2011), as later confirmed for another isolate by Selin et al. (2012). We believe that the observations with the *gacS* mutant occurred because *rpoS* transcription and RpoS protein abundance are controlled by GacS (Kang et al. 2004: Oh et al., 2013a). Two other proteins also were regulated similarly, the catalase/peroxidase KatG and a potential glutathione peroxidase, Gpx. Both of these proteins have significant roles in cellular protection against oxidative stress. Identical changes in catalase/peroxidase and superoxide dismutase isozyme patterns were observed in *rpoS* and *gacS* mutants (Oh et al., 2013b). A recent report indicated that *P. chlororaphis* 30-84 expression from *rpoS* is under GacA control (Wang et al., 2013).

It is interesting that two of the other GacS-regulated proteins have roles in DNA repair: the single strand DNA binding protein Ssb could be associated with repair of DNA breaks caused by hydrogen peroxide (Ananthaswamy and Eisenstark, 1977) and the protein, RdgC, is proposed to act with RecA to aid in DNA repair when there is damage in replication forks or by double strand breaks (Briggs et

Table 1. Identification of the down-regulated proteins by GacS from *Pseudomonas chlororaphis* O6

Spot number	Observed migration[a]			Identified protein			Fold change		
	Mr (kDa)	pI	Protein[b]	Ion Score or Matching sequence[d]	Mr (kDa)	pI	Mean[c]	SE[c]	p-value[d]
5727R	81	6.12	Catalase/peroxidase I, KatG (*P. fluorescens* Pf0-1 YP347933)	FLANPDQLADAFAR(84) FAPLNSWPDNVSLDK(51) DWWPNQLNLK(19)	83	5.30	>-100		0.001
8606R	62	7.64	Tryptophan halogenase, PrnA (*P. chlororaphis* ACN AAD46360)	IGVGEATIPSLQK(76) TSLPTNYDYLR(39) DQATADFLNLWGLSDNQPLNQIK(55)	61	5.80	>-100		0.001
4022R	21	5.77	Single-strand DNA-binding protein, Ssb (*P. putida* pWWO NP542825)	VAEIAGEYLR(28) VILVGTCGQDPEVR(53)	21	6.13	>-100		0.002
1511R	50	4.99	Serine protease, PspB (*P. brassicaceaum* AF286062)	VNLDYDGLLGSR SFSDVGLTPNQR	107	5.19	>-100		0.018
0313R	37	4.59	Recombination associated protein, RdgC (*P. fluorescens* Pf-5 AAY93657)	LTQDLPFDAEALET	34	4.93	>-100		0.029
3402R	40	5.30	Pilus assembly protein, CpaC (*P. fluorescens* Pf0-1 ABA723932)	LTLTPTLVGNDR	44	8.46	>-100		0.011
0025R	21	4.71	Outer membrane protein, OprF (*P. chlrororaphis* AAD24553)	QVLTSQYGVESSR(59) VQSVGYGESRPVADNATEAGR(36) VAPAPAPVPEPTPEPEAPVAEVVR(48) QYPQTTTVVEGHTDSVGPDAYNQK (36)	34	5.59	-56.6	3.65	0.012
3015R	17	5.44	Peptidoglycan-binding LysM, LysM (*P. fluorescens* PfO-1 ABA72026)	LLDLLTPGNANASEQLK(52)	15	5.25	-4.2	0.60	0.020
2608R	56	5.10	Anthranilate/para-aminobenzoate synthases component I, TrpE (*P. fluorescens* Pf0-1 YP 350846)	LADQPNSYLLESVQGGEK(56) EYILAGDCMQVVPSQR (20)	55	5.02	-2.7	0.61	0.028
2210R	30.	5.11	Glucose 1-phosphate thymidylate trans-ferase, RmlA (*P. stutzeri* CAC44166)	GFAWLDTGTHDSLLEASQYVQTIEHR (80)	28	4.83	-2.6	0.17	0.004
0107R	25	4.41	Isoprenoid biosynthesis protein, GATase1_ES1, ElbP (*P. protegens* Pf-5 YP263046)	LTQDLPFDAEALET(57)	23	5.28	-2.6	0.17	0.011
3011R	21	5.38	Glutathione peroxidase, Gpx (*P. fluorescens* Pf-5 YP258072)	LLAGEGAEFPGDITWNFEK(80)	18	5.35	-2.3	0.3	0.017

[a]The Mr and pI values were estimated from 2-dimensional gels obtained in three independent experiments. Ions score is –10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 49 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Amino acid sequences without ion scores were determined by Q-TOF analysis.
[b]Annotation from NCBI databases using the MASCOT search program (www.matrixscience.com).
[c]The mean and standard error (SE) of fold change of the selected spot was calculated by comparing spot intensities between wild type and *gacS* mutant of three independent gels using quantitative image analysis (PDQest 2-D analysis Software).
[d]Student's t-test.

al., 2010).

Two proteins down-regulated in the absence of GacS, OprF and LysM, were associated with cell wall functions. OprF is a major outer membrane protein with multiple potential roles that includes the formation of outer membrane vesicles (Wessel et al., 2012). Studies with other pseudomonads also linked *oprF* expression with quorum sensing and Gac regulation. Crespo and Valverde (2009) observed mutations in the *oprF* gene sequence in *P. fluorescens* CHA0 that affected activity of the repressor proteins RsmA/E which are involved in GacA regulation. Additionally an *oprF* mutant in *P. aeruginosa* showed reduced production of the quorum-sensing signals, acyl homoserine lactones (Fito-Boncompte et al., 2011). The second GacS-regulated protein has a LysM domain associated with peptidoglycan binding (Bateman and Bycroft, 2000). It is possible that changes in the peptidoglycan binding proteins in the *gacS* mutant were

involved in the elongated growth of these cells observed by atomic force microscopy for cells grown in a biofilm (Anderson et al., 2005) and SEM analysis (Kim et al., 2014b). Additional cell surface changes also are implicated due to the control by GacS in isolate *P. chlororaphis* O6 of the enzyme, RmlA, involved in generating rhamnose (Zuccotti et al., 2001), and a potential secretin, CpaC, involved in pilus formation (Bitter, 2003). We note changes in colony surface morphology and in biofilm formation for the *gacS* mutant compared to the wild type strain (Anderson et al., 2005; Kim et al., 2014b).

To characterize the role of the GacS-regulated genes, *trpE* and *prnA* mutants were constructed. A *trpE* mutant was made by homologous marker exchange mutagenesis. A PCR product from the *trpE* gene was generated using genomic DNA and two specific primers based on the genome sequence of *P. chlororaphis* O6 (Loper et al., 2012); forward (5′-ATG ATC CGC GAA GAA TTC CT-3′) and reverse (5′-TCA GTC CGG GGT TTG CTC GG-3′) in polymerase chain reactions (PCR). The PCR fragment (about 1.5 kb) was cloned into cloning vector pGEM-7Z. The *trpE* sequence in the pGEM-7Z plasmid was disrupted by the insertion of a 0.9 kb *Kpn*I fragment containing the kanamycin resistance gene from the plasmid pRL648 (Elhai and Wolk, 1988). The chromosomal *trpE* gene in *P. chlororaphis* O6 was mutated using the exchange vector pCPP54 (Tc^R) and pRK2073 helper plasmid strain as previously described (Miller et al., 1997). The mutants were selected on LB agar containing 5% sucrose based on their sensitivity to tetracycline and resistance to kanamycin. A *prnA* mutant of *P. chlororaphis* O6 was constructed previously (Park et al., 2011).

The effects of mutations in *prnA* and *trpE* mutants on the requirement for tryptophan for growth was studied (Fig. 3) using cells pre-grown on LB agar plates for 2 days at room temperature. The cells were washed several times by re-suspension in sterile distilled water, and the suspension adjusted to $OD_{600nm} = 0.1$ before being applied to M9 minimal medium agar plates with or without a supplement of 5 mM tryptophan (Sigma Co., MO, USA) to assess growth. As anticipated, the *trpE* mutation rendered the mutant unable to grow on minimal medium without the addition of 5 mM tryptophan (Fig. 3). The *prnA* mutant grew well on the minimal medium with or without the tryptophan (Fig. 3).

Induction of systemic resistance by colonization of roots with the mutants was measured in tobacco *cv.* Xanthi seedlings using a challenge of the soft rot bacterium, *Pectobacterium carotovorum* SCCI (Han et al., 2006). Briefly, sterilized seeds were placed on 1 ml of 0.5% (w/v) MS agar supplemented with 3% sucrose contained in the wells of a 12-well microtiter plate (SPL Inc., Korea). After growth for three weeks, the seedlings were inoculated with suspensions of cells of the wild type, the *gacS* mutant, the complemented *gacS* mutant, the *trpE* mutant and the *prnA* mutant. The cells for the inocula were grown to an $OD_{600nm} = 2.0$ in LB broth, pelleted by centrifugation and suspended in sterile 50 mM potassium phosphate buffer (pH 7.5) to an $OD_{600nm} = 0.2$. The inocula, 10 μl, of bacterial suspensions containing 1×10^8 colony forming units (cfu)/ml, were applied to the seedling roots. Sterile 50 mM potassium phosphate buffer was applied to control plants. One week after bacterial treatments, tobacco plants were

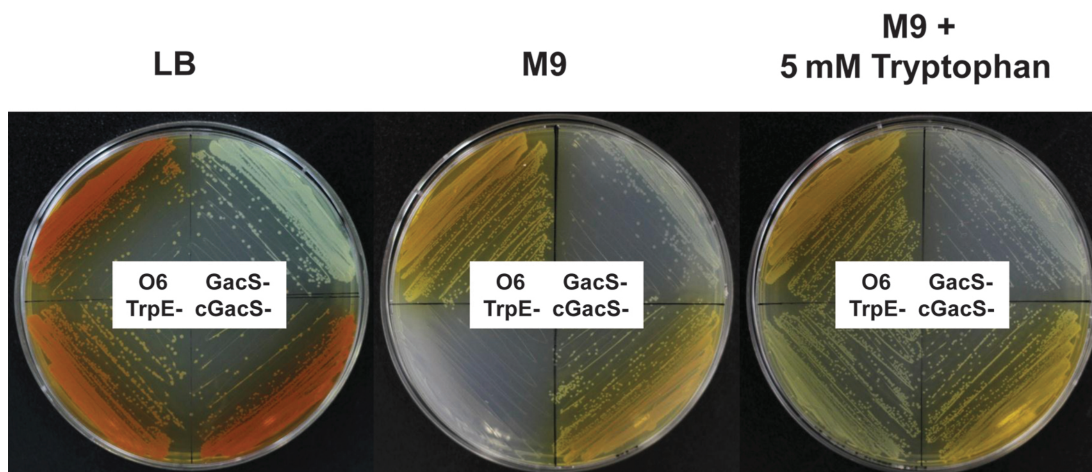

Fig. 3. Auxotrophic phenotype of *Pseudomonas chlororaphis* O6 *trpE* mutant under minimal growth conditions. Each bacterial strain O6 (wild type), the *gacS* mutant (GacS-), the complemented *gacS* mutant (cGacS-), and the *trpE* mutant (TrpE-) was applied to Luria Bertani agar (LB), M9 minimal agar (M9), or M9 minimal agar with 5 mM tryptophan. The growth images were photographed two days after inoculation on the plates. The images are representative of three independent experiments.

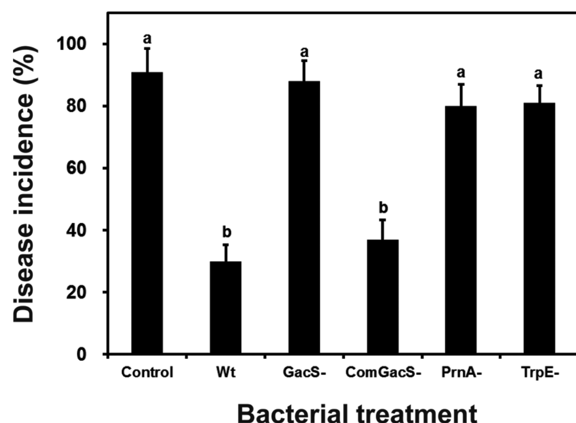

Fig. 4. Effect of mutations in *prnA* and *trpE* of *Pseudomonas chlororaphis* O6 on induced systemic resistance activity in tobacco against *Pectobacterium carotovorum* SCCI. Roots of three week-old tobacco grown in microtiter wells were inoculated with wild type (Wt), the *gacS* mutant (GacS-), the complemented *gacS* mutant (ComGacS-), the *trp* mutant (TrpE) and the *prnA* mutant (PrnA) or were treated with water as a negative control prior to pathogen challenge. After one week, leaves were challenged with *P. carotovorum* subsp. *carotovorum* SCCI and soft rot symptomic leaves were scored after two days. Different letters indicate significant differences between treatments according to Duncan's multiple range test ($p < 0.05$). Two independent experiments were performed with at least 21 plants/treatment.

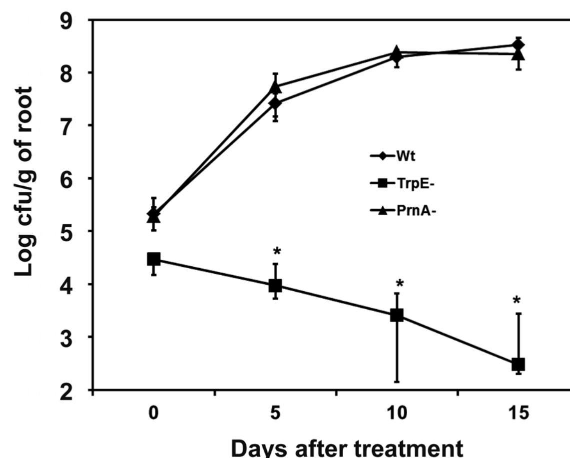

Fig. 5. Effect of mutations in *prnA* and *trpE* in *Pseudomonas chlororaphis* O6 on colonization of roots of tobacco under sterile, non-competitive growth conditions. Three week-old tobacco roots in microtiter plates were inoculated with the wild type strain (Wt), the *prnA* mutant (PrnA-) and *trpE* mutant (TrpE-) of *P. chlororaphis* O6. At the defined days after inoculation, root colonization of each bacterial strain was measured based on culturable cells obtained from excised roots. Data are the means of three independent studies with three plants per treatment in each study. Vertical bars represent standard errors. * indicates differences between bacterial strains by Duncan's multiple range test at $p < 0.05$.

challenged with *P. carotovorum* subsp. *carotovora* SCCI by pipetting 2 µl of pathogen inoculum onto a leaf (1×10^8 cfu/ml) as described by Han et al. (2006). One to two days after pathogen challenge, the extent of soft-rot was rated visually and the disease severity assessed. The *trpE* and *prnA* mutants displayed less ability than the wild type or the complemented *gacS* mutant to protect the plants against the pathogen (Fig. 4). The extent of protection from the *prnA* and *trpE* mutants was low and similar to that displayed by the *gacS* mutant.

The efficacy of root colonization of the wild type, the *trpE* mutant and *prnA* mutant was compared on the tobacco cv. *Xanthi* seedlings using methods described previously with three week old seedlings raised in the well plates (Han et al., 2006). Roots were excised at defined times after inoculation, and the fresh weight was measured before transferred into 10 ml of sterile distilled water. After vigorous vortexing for 1 min, serial dilutions of these washings were plated onto LB-agar plates containing antibiotics appropriate to the strain. Colonies were scored after incubation at 28°C for two days. Studies were repeated two times with three plants/each treatment. Root colonization in terms of cfu/g fresh weight roots was calculated for each time point. Data were statistically analyzed by ANOVA with the IBM SPSS Statistics version 21 (IBM Corp., Somers, New York, USA). The lack of induced resistance for the *trpE* mutant correlated with loss of root colonization which declined with time over a 15-d trial (Fig. 5). In contrast both the wild type and the *prnA* mutant colonized the root surfaces with increases in cfu/root during the first 10 d of the 15 d assessment period (Fig. 5).

The failure of the *trpE* mutant to colonize the plant roots suggested that the supply of tryptophan in the tobacco root exudates was inadequate to maintain wild type level of growth at the root surface. The ability of the *prnA* mutant to colonize but not to induce systemic resistance suggested that pyrronitrin was active as an effector. This suggestion indicated that pyrrolnitrin had a dual role for the biocontrol-active pseudomonad, both as a direct anti-microbial compound as well as an effector of induced resistance (Park et al., 2011). A similar role was determined for the phenazines produced by this bacterial isolate (Kang et al., 2007). However, our findings of impaired colonization with the *trpE* mutant emphasized that the formation of pyrrolnitrin in the rhizosphere would be influenced by the supply of tryptophan in the plant root exudates.

In summary, the proteomics analysis of control by GacS confirmed its role as a key regulator of proteins with antici-

pated roles not only in the formation of antimicrobials but also in oxidative stress, cell signaling, secretion and cell surface properties. Production of KatG and Gpx, involved in protection against oxidative stress, overlapped between the *rpoS* mutant and the *gacS* mutant. The newly identified GacS-regulated proteins in this paper, Ssb, PspB, RdgC, LysM, RmlA, and ElbP, indicate further the diverse role of this regulatory system in a rhizosphere-competent pseudomonad with biocontrol potential.

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