

Short communication

Polyamine is a critical determinant of *Pseudomonas chlororaphis* O6 for GacS-dependent bacterial cell growth and biocontrol capacity

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SUMMARY

The Gac/Rsm network regulates, at the transcriptional level, many beneficial traits in biocontrol-active pseudomonads. In this study, we used Phenotype MicroArrays, followed by specific growth studies and mutational analysis, to understand how catabolism is regulated by this sensor kinase system in the biocontrol isolate *Pseudomonas chlororaphis* O6. The growth of a *gacS* mutant was decreased significantly relative to that of the wild-type on ornithine and arginine, and on the precursor of these amino acids, *N*-acetyl-L-glutamic acid. The *gacS* mutant also showed reduced production of polyamines. Expression of the genes encoding arginine decarboxylase (*speA*) and ornithine decarboxylases (*speC*) was controlled at the transcriptional level by the GacS sensor of *P. chlororaphis* O6. Polyamine production was reduced in the *speC* mutant, and was eliminated in the *speAspeC* mutant. The addition of exogenous polyamines to the *speAspeC* mutant restored the *in vitro* growth inhibition of two fungal pathogens, as well as the secretion of three biological control-related factors: pyrrolnitrin, protease and siderophore. These results extend our knowledge of the regulation by the Gac/Rsm network in a biocontrol pseudomonad to include polyamine synthesis. Collectively, our studies demonstrate that bacterial polyamines act as important regulators of bacterial cell growth and biocontrol potential.

Keywords: arginine, Gac/Rsm network, GacS sensor kinase, metabolic phenotypes, ornithine, polyamines, putrescine.

Pseudomonas chlororaphis O6, isolated from the wheat rhizosphere, promotes plant growth and induces systemic resistance against various environmental and biotic stresses (Spencer *et al.*, 2003). Strain O6 produces a variety of secondary metabolites with

direct antimicrobial activities, such as phenazines (Kang *et al.*, 2004), pyrrolnitrin (Park *et al.*, 2011) and hydrogen cyanide (HCN) (Lee *et al.*, 2011). Production of the volatile (2*R*,3*R*)-butanediol promotes plant growth and induces systemic resistance to biotic and abiotic stresses (Han *et al.*, 2006). The Gac/Rsm network controls many of the traits involved in the biocontrol of strain O6 (Han *et al.*, 2006; Kang *et al.*, 2004) and other pseudomonads, including *P. protegens* CHA0 (Humair *et al.*, 2009; Kay *et al.*, 2005) and *P. brassicacearum* (Lalaouna *et al.*, 2012). The environmental signal that activates the GacS sensor kinase to initiate a signal cascade through GacA, resulting in a changed expression of genes associated with biocontrol, is unknown.

Other studies have shown that, in addition to the GacS/A system, nutrient availability influences the production of antifungal metabolites. For *P. chlororaphis* O6, the ratio of antifungal phenazines to pyrrolnitrin changes with the level of glucose (Park *et al.*, 2011). Similarly, in a *P. fluorescens* isolate, glucose increases the production of the antifungal agent 2,4-diacetylphloroglucinol, but reduces the synthesis of another, pyoluteorin (Kraus and Loper, 1995; Nowak-Thompson *et al.*, 1994). In addition, phenazines are not synthesized by isolate O6 in a defined minimal medium, irrespective of the cell number (Housley *et al.*, 2009). The relationship between nutrition and biological control is important in considering how to culture beneficial biocontrol-active microbes for use in formulations for the effective suppression of diseases in the field.

Little is known about the importance of nitrogen metabolism in biocontrol-active pseudomonads. Therefore, we analysed the catabolic differences between the wild-type strain O6 and its *gacS* mutant using the Phenotype MicroArray™ system developed by Biolog (Hayward, CA, USA). This system, with Phenotype MicroArray (PM) plates 1–10, allows the growth of a wild-type and mutant strain to be compared by measurement of the rates of respiration on nearly 1000 substrates (Bochner *et al.*, 2008).

Differential growth of the mutant and wild-type strains indicated that nitrogen metabolism associated with arginine and ornithine was affected by the *gacS* mutation. The annotation of

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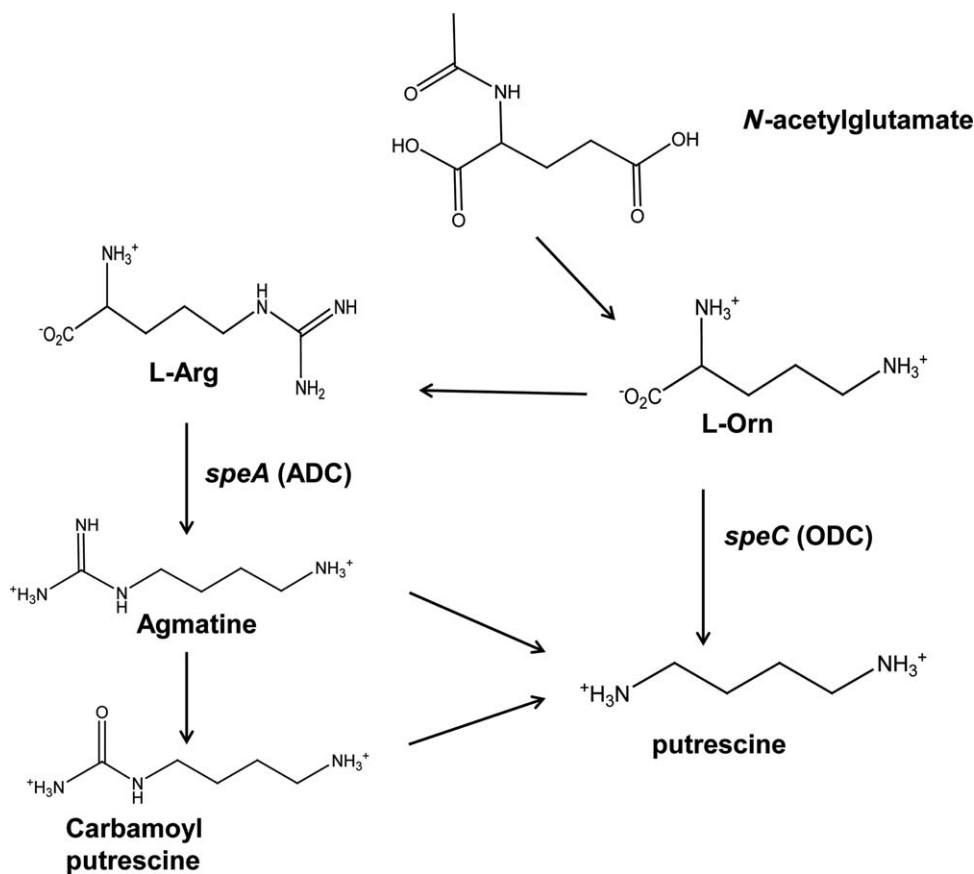


Fig. 1 Predictive pathways for polyamine synthesis in *Pseudomonas chlororaphis* O6 based on MetaCyc analysis (Caspi *et al.*, 2016). *speA* encodes for ADC and *speC* encodes for ODC. ADC, arginine decarboxylase; ODC, ornithine decarboxylase; L-Arg, L-arginine; L-Orn, L-ornithine.

the *P. chlororaphis* O6 genome showed genes encoding enzymes that produce ornithine and arginine from *N*-acetylglutamate, and the use of these amino acids as precursors for putrescine. *In silico* analysis by Metacyc.org predicted three potential pathways for putrescine synthesis in *P. chlororaphis* O6: one pathway involves the transformation of ornithine by ornithine decarboxylase encoded by *speC* (PchlO6_0864), and two pathways originate from arginine, both of which use arginine decarboxylase *speA* (PchlO6_0666) to produce agmatine as an intermediate (Fig. 1). Consequently, we examined whether the *gacS* mutation in *P. chlororaphis* O6 affected the production of polyamines, such as putrescine, spermine and spermidine. We made a single mutant lacking *speA* and a double mutant *speA**speC* to probe the roles of their gene products on putrescine production. To connect the metabolic changes with the biological control potential, we examined the impact of polyamine production on fungal growth inhibition by the wild-type and mutant strains.

EFFECT OF THE *GACS* MUTATION ON THE METABOLISM OF *P. CHLORORAPHIS* O6

The bacterial strains and plasmids used in the experiments (Table 1) were stored at -70°C in 20% glycerol. King's B (KB)

medium (King *et al.*, 1954), Luria–Bertani (LB) medium, modified M9 minimal medium (14 mM Na_2HPO_4 , 7 mM KH_2PO_4 , 18 mM NH_4Cl , 8 mM NaCl, 2 mM MgSO_4 , 0.1 mM CaCl_2 and 0.2% glucose per litre) (Sambrook, 2001) and *Pseudomonas* minimal medium (PMM) (Housley *et al.*, 2009) were used for the growth of *P. chlororaphis* O6 at 28°C . *Escherichia coli* strains were cultured in LB medium at 37°C . The antibiotics ampicillin (50 $\mu\text{g}/\text{mL}$), tetracycline (25 $\mu\text{g}/\text{mL}$), kanamycin (50 $\mu\text{g}/\text{mL}$) and streptomycin (50 $\mu\text{g}/\text{mL}$) were used when appropriate for the different mutants (Table 1).

Growth of the *P. chlororaphis* O6 wild-type and *gacS* mutant was compared using Biolog PM Technology, which employs 10 PMs (PM 1–10) to assess the utilization of defined carbon or nitrogen sources. This method relies on the development of colour from a tetrazolium dye based on active respiration. Cells of the wild-type and *gacS* mutant strains were cultured at 28°C for 2 days on LB agar plates; medium was supplemented with kanamycin (25 $\mu\text{g}/\text{mL}$) for the *gacS* mutant. Cells from a single colony were suspended in the inoculation medium provided by Biolog, and the suspension was adjusted to 85% using a Biolog turbidity meter. Aliquots of 100 μL of bacterial suspensions were dispensed into PM 1–10 to allow for growth, indicated by chromogenic changes in the tetrazolium dye in each well. The PM plates were incubated for up to 48 h at 28°C , and the growth of bacteria was

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or description*	Source or reference
Bacterial strains		
<i>Pseudomonas chlororaphis</i> O6		
Wild-type	Sm ^r , Am ^r	Radtke <i>et al.</i> (1998)
<i>gacS</i> mutant	<i>gacS::nptII</i> , Sm ^r , Am ^r , Km ^r	Kang <i>et al.</i> (2012)
Com <i>gacS</i> mutant	Complemented <i>GacS</i> mutant	Kang <i>et al.</i> (2012)
	Sm ^r , Am ^r , Km ^r , Tc ^r	
<i>odc</i> mutant	<i>speC::nptII</i> , Sm ^r , Am ^r , Km ^r	This study
<i>adcOdc</i> mutant	<i>speC::nptII</i> and <i>speA::aacC</i> , Sm ^r , Am ^r , Km ^r , Gm ^r	This study
<i>Escherichia coli</i> DH5 α	Host strain	BRL Inc., Grand Island, NY, USA
Plasmid		
pRK2073	Helper plasmid, Mob ⁺ , Tra ⁺ , Sp ^r	D. R. Helinski (University of California at San Diego)
pSB1A3	Cloning vector, Am ^r	Ginkgo Bioworks, Boston, MA, USA
pCRII	Cloning vector, Am ^r , Km ^r	Invitrogen, San Diego, CA, USA
pCPP54	Marker exchange eviction plasmid <i>sacB-sacR</i> ⁺ , Tc ⁺	D. Bauer (Cornell University)
pRL648	pRL vector containing 1-kb Km ^r cassette	C. P. Wolk (Michigan State University)
pML122	pML vector containing Gm ^r cassette	M. Labes (McMaster University)

*Am^r, ampicillin resistance; Gm^r, gentamycin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance.

Table 2 Changes in the use of substrates related to polyamine metabolism affected by the *gacS* mutation.

Plate number	Substrate	Relative use of a substrate by the <i>gacS</i> mutant/wild-type strain*
PM 2	<i>N</i> -Acetylglutamic acid	-112
PM 3	L-Arginine	-180
PM 3	L-Ornithine	-59
PM 6	Met-Arg	-175
PM 6	Arg-Arg	-91
PM 7	Arg-Met	-109
PM 2	Putrescine	77
PM 3	Agmatine	136

*Relative use by the wild-type and *GacS* mutant is the mean of two independent experiments using Phenotype MicroArrays (PM 1–10) with Biolog PM Technology.

recorded using an OmniLog reader (Biolog Inc., Hayward, CA, USA) which measured the dye intensity at 15-min intervals. The study was repeated twice.

The monitoring of dye formation in PM plates, with each well containing different substrates for catabolism, indicated differences in growth between the wild-type and *gacS* mutant. Growth of the *gacS* mutant was enhanced by over 165 substrates in the PM plates, whereas the *gacS* mutant showed less respiration than the wild-type with only nine specific substrates (Fig. S1 and Table S1, see Supporting Information). The increased utilization of many nitrogen and carbon sources by the *gacS* mutant may explain the observation that *gacS/gacA* mutants arise spontaneously in nutrient-rich medium (van den

Broek *et al.*, 2005; Duffy and Defago, 2000). These mutants lacking *Gac/Rsm* regulation thus appear to have increased fitness compared with the wild-type. Such changes in growth potential may be important in mutualism between the wild-type and *gac* mutants, as proposed by Pierson's group (Chancey *et al.*, 2002; Driscoll *et al.*, 2011).

Data from PMs 3, 6 and 7 revealed that the mutation in *gacS* decreased growth on L-acetyl-L-glutamic acid, L-ornithine and L-arginine relative to that of the wild-type strain (Fig. S1 and Table 2). Growth of the *gacS* mutant was impaired on dipeptides containing arginine (Arg-Arg, Arg-Met and Met-Arg) as sole nitrogen sources. However, the *gacS* mutant retained the wild-type ability to degrade agmatine and putrescine. Annotation of the isolate O6 genome in MetaCyc predicted the presence of genes involved in three pathways for putrescine degradation to γ -aminobutyric acid (GABA, data not shown). These changes in substrate use by the *gacS* mutant suggest that the pathway for putrescine production is blocked prior to agmatine formation.

Modified M9 minimal medium was used to confirm the results of the PM assay. L-Acetyl-L-glutamic acid or L-glutamic acid was added at a concentration of 0.2% to replace glucose as the defined carbon source. Nitrogen sources, L-arginine or L-ornithine, were used at 2–5 μ M to replace NH₄Cl. At defined times, the colony-forming units of cultures grown at 28°C were determined by the plating of serial dilutions on LB medium; kanamycin was added to assess the growth of *gacS* cells. Two independent replicates of the assay were performed. Impaired growth of the *gacS* mutant relative to that of the wild-type was confirmed with arginine and ornithine as the substrates (Fig. 2). With arginine amendment, the cell numbers of the *gacS* mutant decreased as

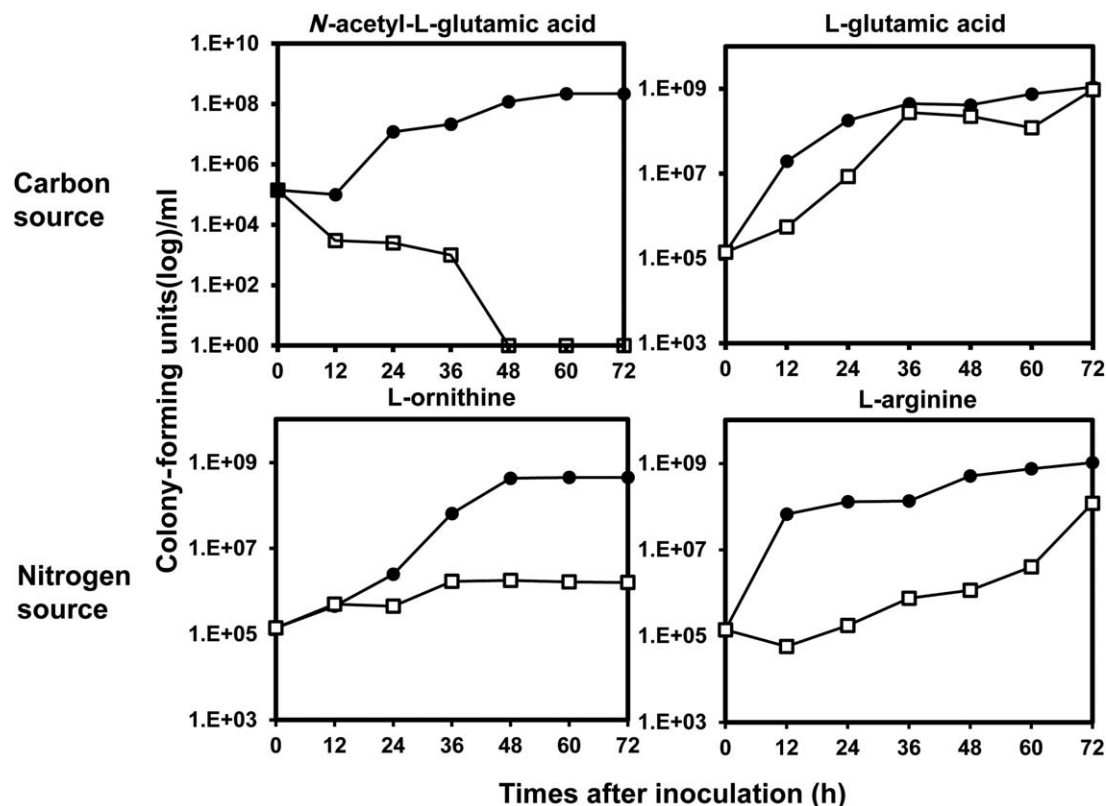


Fig. 2 Growth of the wild-type *Pseudomonas chlororaphis* O6 (circles) and the *gacS* mutant of *P. chlororaphis* O6 (rectangles) in modified M9 minimal medium containing sole carbon or nitrogen sources. At the defined times, samples were removed and the colony-forming units were determined. Data shown are from one of two independent experiments with the same results.

the wild-type strain increased in cell density. With ornithine, cell numbers increased for the mutant about 10-fold, and then plateaued with no further increases in growth. With *N*-acetylglutamate, growth of the *gacS* mutant was delayed up to 24 h, after which time adaptation occurred, allowing the wild-type cell density to be reached 3 days after inoculation. These findings were again consistent with the *gacS* mutation causing a blockage in metabolism after ornithine and arginine formation, but before agmatine synthesis.

POLYAMINE PRODUCTION IN THE WILD-TYPE AND *GACS* MUTANT OF *P. CHLORORAPHIS* O6

The production of polyamines was compared between wild-type and *gacS* mutant cells grown for 72 h with agitation in PMM containing 0.2% acetylglutamic acid as a single carbon source. Cells from 50-ml cultures were harvested by centrifugation and homogenized in 1 mL of 5% perchloric acid at 4°C. The pellet, obtained by centrifugation at 10 000 *g* for 20 min, was suspended in 1 mL of 1 M NaOH, and 200 µL of 30% HCl was added to each 100-µL suspension. After incubation at 110°C for 15 h, the mixtures were filtered through a 0.45-µm membrane (Millipore, Billerica, MA, USA) and freeze-dried (Seiler and Wiechmann, 1967). The freeze-

dried sample was treated with 5% perchloric acid and 400 µL of dansyl chloride (30 mg/mL in acetone), followed by the addition of 20 mg Na₂CO₃. After incubation at 65°C for 1 h in the dark, 100 µL of proline (100 mg/mL) was added. Dansylated compounds were extracted using 500 µL of toluene and loaded onto LK60 Whatman silica gel thin layer chromatography (TLC) plates (GE Healthcare, Cleveland, OH, USA). Compounds were separated using chloroform–triethylamine (4 : 1, v/v). The dansylated derivatives were detected by visible fluorescence with excitation at 365 nm. Putrescine dihydrochloride, spermine and spermidine trihydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dansylated as described and used as standards. Studies were performed in triplicate.

In silico analysis from strain O6 genomic mining predicted that genes for putrescine, but not spermidine, production were present, because a gene for spermidine synthase was lacking. However, uncharacterized alternative routes for spermidine and spermine synthesis may exist (Lee *et al.*, 2009). Intermediates in this synthesis process include carboxynorspermidine, produced from putrescine by carboxynorspermidine decarboxylase (Lee *et al.*, 2009). The production of putrescine was verified from the wild-type strain (Fig. 3) grown in M9 medium with *N*-

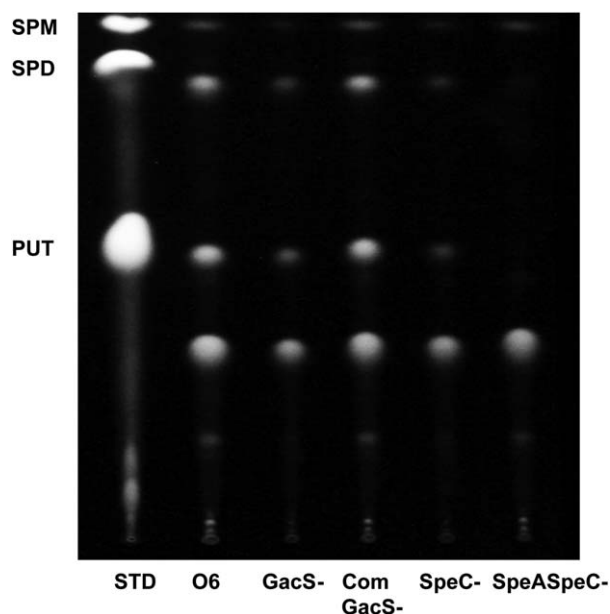


Fig. 3 Polyamine production in wild-type *Pseudomonas chlororaphis* O6 (O6), the *gacS* (*gacS*-), complemented *gacS* (*ComgacS*-), *speC* (*speC*-) and *speASpeC* (*speASpeC*-) mutants. Cells were grown in *Pseudomonas* minimal medium with 0.2% acetylglutamic acid as the sole carbon source. Each strain was grown for 72 h before total polyamines, extracted from the bacterial cells, were resolved on silica gel-coated thin layer chromatography (TLC) plates using a mobile phase consisting of chloroform–triethylamine (4 : 1, v/v). The dansylated polyamines were visualized after UV irradiation. Dansylated putrescine dihydrochloride (PUT), spermidine trihydrochloride (SPD) and spermine (SPM) were used as standards. The image is from one of three independent experiments with the same results.

acetylglutamate, the precursor of both arginine and ornithine. Production levels were reduced by the *gacS* mutation and restored by its complementation. Another fluorescent spot was observed with retention factor (Rf) values similar, but not identical, to those of the dansylated derivatives of authentic spermidine in extracts from wild-type cells (Fig. 3). The intensity of this spot was changed in the same way as for putrescine, showing reduced levels in the *gacS* mutant. These products are yet to be identified. These data confirm that the production of putrescine in *P. chlororaphis* O6 requires a functional GacS protein.

The mechanism of control through the Gac/Rsm network remains unknown. The *gacS* mutant lacks the production of the cell signalling compounds, the acyl homoserine lactones (AHLs) (Kim *et al.*, 2014). However, in *P. aeruginosa*, a mutant lacking AHL synthesis showed increased levels of polyamines (Davenport *et al.*, 2015). Thus, in strain O6, another regulatory parameter controlled by GacS, in addition to the lack of AHLs, is in play. Similarly, metabolite profile analysis of *P. fluorescens* CHA0 indicated that arginine and *N*-acetylglutamic acid concentrations were higher, and putrescine and ornithine concentrations were lower, in the *gacA* mutant relative to the wild-type (Takeuchi *et al.*,

2012). The cellular changes in the levels of polyamines in the Gac mutant cells could contribute to higher reactive oxygen species (ROS) stress. Polyamines have been documented as free radical scavengers (Das and Misra, 2004). Previously, a higher level of ROS in *gacS* cells correlated with impaired production of key enzymes, such as superoxide dismutase and catalases (Gade *et al.*, 2016; Kang *et al.*, 2004).

ANALYSIS OF POLYAMINE BIOSYNTHETIC GENE TRANSCRIPTION

Analysis of transcripts from genes *speA* and *speC* involved in polyamine biosynthesis was performed for the wild-type strain, the *gacS* mutant and the complemented *gacS* mutant. The cells were grown in sucrose-free PMM containing 0.2% acetylglutamic acid as the sole carbon source and incubated at 28°C with shaking at 200 rpm. After growth to stationary phase, cells were harvested by centrifugation and total RNA was prepared using TRIzol™ (Gibco BRL, Gaithersburg, MD, USA). The following reverse transcription primer sets were designed based on the *P. chlororaphis* O6 genome sequence (Loper *et al.*, 2012): *speA* (PchlO6_forward primer, 5'-ACTACCAGCACGGCTTCAA-3'; reverse primer, 5'-AGTTGATCGAACTGGCGTTG-3'); *speC* (PchlO6_forward primer, 5'-GACTTACGCGCTACCAGCAC-3'; reverse primer, 5'-ACTACATCACCCGCACCAA-3'). A QuantiTect SYBR Green RT-PCR Kit (Qiagen Cat. No. 204243, Hilden, Germany) and Stratagene Mx3000P qPCR System (Agilent Technology, Santa Clara, CA, USA) were used. The initial reverse transcription reaction was performed at 50°C for 30 min, and real-time polymerase chain reaction (PCR) was carried out using the conditions described previously (Kim *et al.*, 2014). Expression levels of the target gene were analysed using MxPro software after normalization with *P. chlororaphis* O6 16S ribosomal RNA levels. Relative expression levels between the wild-type and *gacS* mutant were determined by the delta-delta CT method, as described previously (Kim *et al.*, 2014). Three independent studies were performed to extract and analyse transcript levels for the three strains.

In the minimal defined medium, the wild-type produced transcript levels from *speA* and *speC* (Fig. 4). This finding shows independence of the production of these transcripts by AHLs, because the signalling compounds were not produced on this medium (Housley *et al.*, 2009). Transcript levels for both genes were reduced in the *gacS* mutant, but were detected at levels higher than for the wild-type strain with gene complementation (Fig. 4). These changes in transcription in the *gacS* mutant correlated with reduced levels of polyamine production for the mutant versus wild-type cells (Fig. 3).

The pattern of gene regulation in strain O6 differs from that observed in *P. protegens* strain Pf-5 (Hassan *et al.*, 2010; Kidarsa *et al.*, 2013). In Pf-5, expression from *speA* was enhanced by a mutation in the gene, *gacA*, encoding the sensor for GacS. In addition,

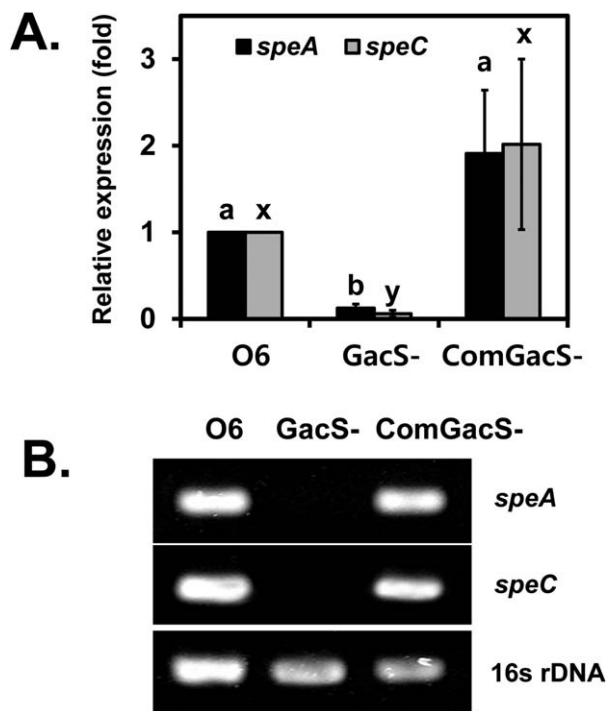


Fig. 4 Gene transcript analysis of *speA* and *speC* in the wild-type, *gacS* mutant and complemented *gacS* mutant of *Pseudomonas chlororaphis* O6. Total RNA was isolated from bacterial cells grown in M9 minimal medium broth with 0.2% *N*-acetylglutamic acid. Quantitative real-time polymerase chain reaction (PCR) was performed for 20 cycles (A), and the products were visualized on 2% agarose gels to determine their intensities (B). The relative expression levels of the *speA* and *speC* genes represent the means of three independent experiments, and different letters indicate significant differences in transcript expression amongst *P. chlororaphis* O6 strains according to Duncan's analysis of variance test ($P < 0.05$).

the expression of genes encoding arginine deiminase and an arginine/ornithine antiporter was lower when compared with expression in the Pf-5 wild-type (Hassan *et al.*, 2010). The apparent differences between strains O6 and Pf-5 in these expression patterns could be a result of the different experimental conditions or variability between the Gac regulons of the two isolates. No genes related to polyamines have been reported to show changes in expression by modifications in the Gac regulon for *P. fluorescens* SBW25 (Cheng *et al.*, 2013). These findings support differences in the Gac-regulated genes between pseudomonads.

BACTERIAL CELL GROWTH AND POLYAMINES

To further understand the roles of SpeC and SpeA in polyamine synthesis in strain *P. chlororaphis* O6, mutants were constructed by the insertion of antibiotic resistance genes. The *speC* mutant eliminated synthesis from ornithine, and a double mutant *speAspeC* was engineered to block synthesis from arginine (Fig. 1). The *speC* and *speA* genes in isolate O6 were modified by the

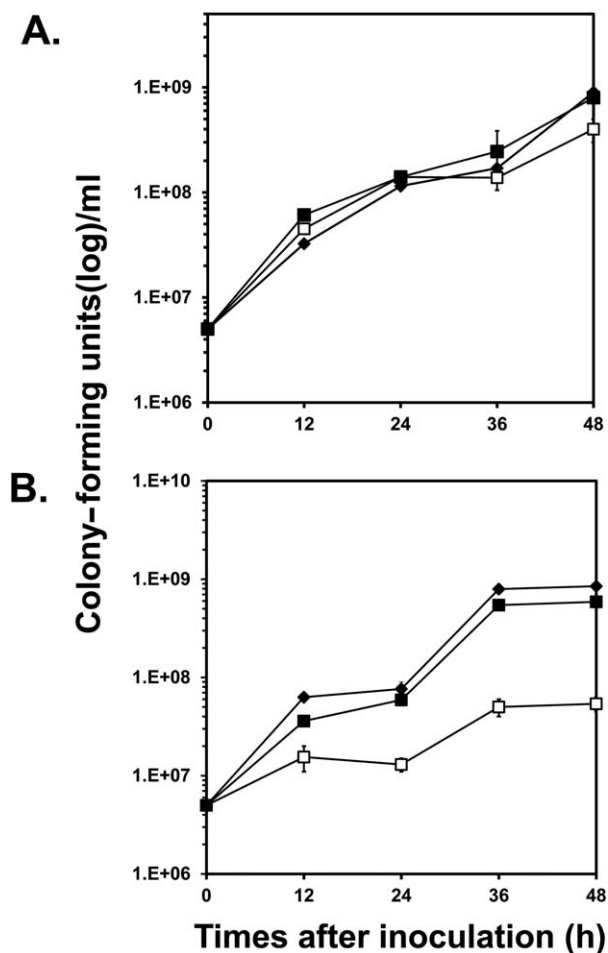


Fig. 5 Effects of polyamine supplementation on the growth of *Pseudomonas chlororaphis* O6 and the *speAspeC* mutant on Luria-Bertani (LB) broth (A) or M9 minimal broth (B) with glucose as the carbon source, with or without the addition of 50 μM spermidine. Symbols: wild-type (filled diamonds) and *speAspeC* mutant without 50 μM spermidine (open squares) or with spermidine (filled squares). Data shown are the means of two independent experiments.

attachment of sequences specific for restriction enzymes (Table S2 and Fig. S2, see Supporting Information) through the use of the pSB1A3 BioBrick Assembly Kit, as described previously (Shetty *et al.*, 2008). PCR products from the *nptII* gene, encoding kanamycin resistance from pRL648 (Wolk *et al.*, 1991), and the *aacC* gene, encoding gentamycin resistance from pML122 (Labes *et al.*, 1990), were amplified using primers attached with *Xba*I and *Spe*I restriction sites at the ends (Table S2). These fragments were inserted into open reading frames of the *speC* and *speA* genes to disrupt the target genes. Each mutated gene sequence was digested with *Eco*RI and *Not*I, cloned into the pCRII vector (Invitrogen, San Diego, CA, USA) and then digested with *Nsi*I before transfer to the marker exchange vector pCPP54. Exchange of the mutated genes and selection of the mutants were performed as

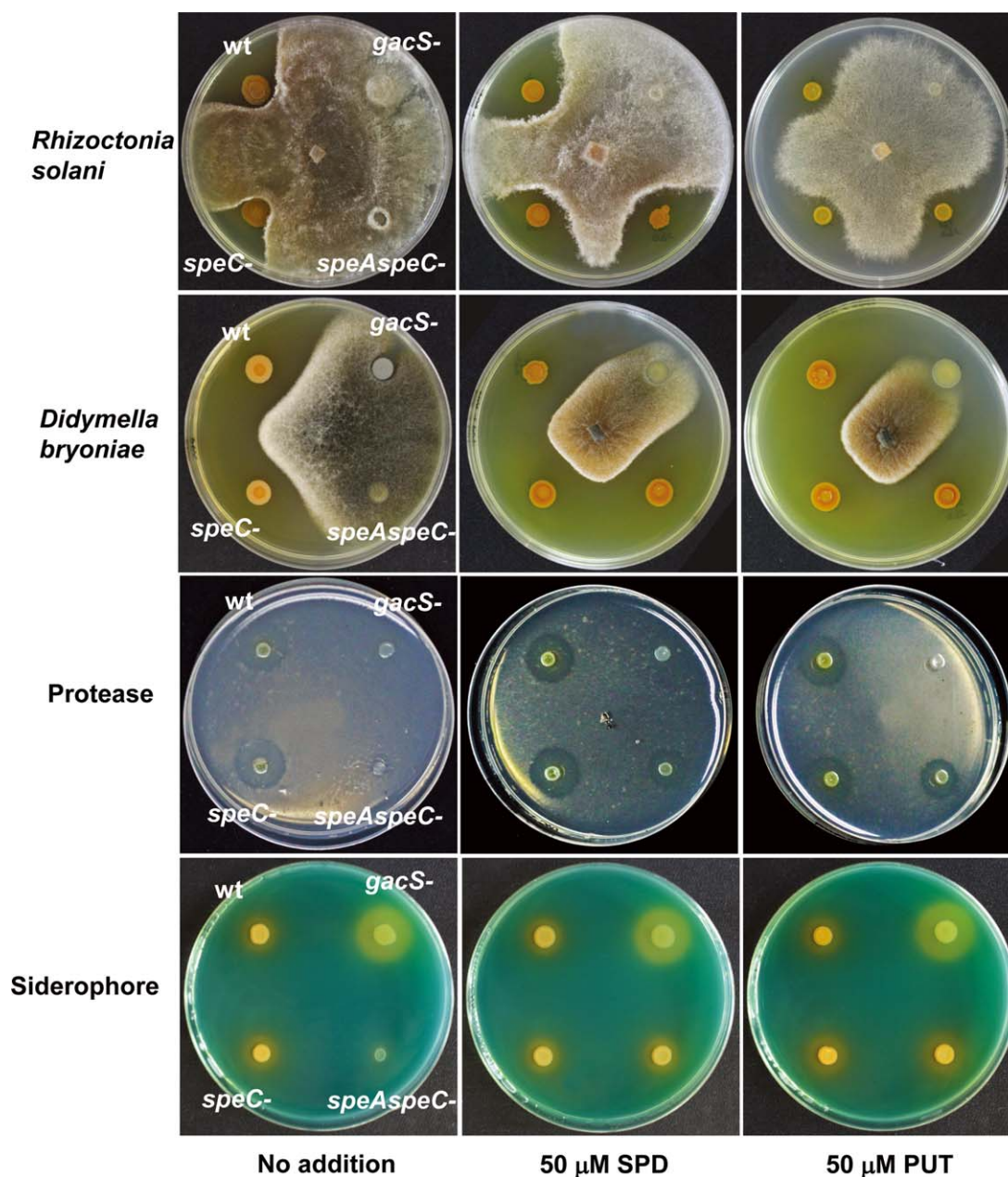


Fig. 6 Analysis of biocontrol traits in wild-type (wt) and *gacS* (*gacS*-), *speC* (*speC*-) and *speAspeC* (*speAspeC*-) mutants of *Pseudomonas chlororaphis* O6. Antifungal activities against phytopathogenic fungi, *Rhizoctonia solani* and *Didymella bryoniae*, were tested on potato dextrose agar (PDA) with or without the addition of 50 μM spermidine (SPD) or putrescine (PUT). The production of extracellular proteases and siderophores by the bacterial cells was assessed on specialized media described in the text with or without the addition of SPD or PUT. The data shown are from one of two separate experiments with similar results.

outlined elsewhere (Miller *et al.*, 1997). A single *speC* mutant and *speAspeC* double mutant were selected on 5% (w/v) sucrose and LB agar containing kanamycin and gentamycin. The presence of the antibiotic resistance genes in the mutants was confirmed by detection of the PCR products corresponding to *aacC* and *nptII* in the appropriate mutant (Fig. S2). This analysis showed that regions of the *speA* gene (from the 22nd V to 598th E) and the

speC gene (from the 105th S to 380th P) were replaced with the *aacC* and *nptII* genes in the mutants, respectively (Fig. S2).

The production of putrescine decreased in the *speC* mutant to levels seen with the *gacS* mutant and was absent in the *speAspeC* double mutant (Fig. 3). Although the *speAspeC* mutant grew at wild-type levels on rich LB medium, an initially slower growth rate was observed in glucose M9 minimal medium. However, the

addition of either spermidine or putrescine was required for the double mutant to attain growth equivalent to that of the wild-type strain (Fig. 5). Important roles of polyamines have been proposed for the determination of the growth rate and in connection with virulence in *Salmonella enterica* serovar Typhimurium (Jelsbak *et al.*, 2012).

BIOCONTROL TRAITS OF THE POLYAMINE-DEFECTIVE MUTANTS

The Gac/Rsm network controls antimicrobial properties and thus affects the biocontrol potential of pseudomonads, including isolate O6 (Kang *et al.*, 2007; Park *et al.*, 2011). Antagonism of the growth of fungal pathogens is frequently used to illustrate antimicrobial production. Consequently, the potential role of polyamines in fungal growth antagonism was assessed. Inhibition of the growth of two fungal pathogens, *Rhizoctonia solani* (KACC 40111), which causes root rot and damping off in a variety of hosts, and *Didymella bryoniae* (KACC40937), the causal agent of black rot in cucumber and squash, was determined on potato dextrose agar (PDA) plates (Fig. 6). Growth inhibition, apparent on PDA with the wild-type strain, was not observed for either pathogen with the *gacS* mutant. The *speC* mutant showed inhibition of fungal growth similar to the wild-type, but, when polyamine synthesis was eliminated in the *speAspeC* mutant, there was no *in vitro* antifungal activity. Antagonism was restored with the *speAspeC* mutant by the addition of spermidine or putrescine. However, these polyamines did not restore antagonism to the *gacS* mutant, suggesting that the polyamines act as regulators for traits that require a functional Gac/Rsm network.

Based on these findings with antagonism, we examined the effects of polyamines on metabolites/enzymes that could be involved in the inhibition of fungal growth. Protease secretion, detected by a zone of clearing around the colonies on agar plates containing skimmed milk, was not affected in the *speC* mutant, was reduced in the *speAspeC* mutant and was eliminated in the *gacS* mutant (Fig. 6). Siderophore secretion, assessed by a change in colour on LB plates containing chrome azure as a result of iron chelation (Schwyn and Neilands, 1987), was enhanced above wild-type levels by the *gacS* mutation (Fig. 6). Mutation in *speC* had no effect on siderophore production, but this was reduced in the *speAspeC* mutant (Fig. 6). Both protease and siderophore production were restored by the incorporation of putrescine or spermidine into the medium for the *speAspeC* mutant (Fig. 6). However, as with antagonism activity, the addition of polyamines did not affect protease or siderophore production in the *gacS* mutant (Fig. 6), indicating that GacS affected the synthesis of protease and siderophore by polyamine-independent mechanisms. The inclusion of putrescine or spermidine into the medium did not affect colony morphology, suggesting that these findings were not associated with changes in cell growth.

Table 3 Production of phenazines and pyrrolnitrin from cell-free supernatants of wild-type and mutants of *Pseudomonas chlororaphis* O6.

Strain	Phenazine (absorbance at 367 nm)*	Pyrrolnitrin (μM) [†]
Wild-type	1.17 \pm 0.05 a	210.9 \pm 60.3 a
<i>gacS</i> mutant	0.16 \pm 0.05 d	0.5 \pm 0.2 b
<i>gacS</i> mutant supplemented with putrescine	0.17 \pm 0.04 d	0.6 \pm 0.2 b
<i>speAspeC</i> mutant	0.52 \pm 0.05 c	9.1 \pm 13.4 b
<i>speAspeC</i> mutant supplemented with putrescine	0.65 \pm 0.06 b	399.6 \pm 163.7 a

Data are expressed as the means of two independent experiments with three replicates, and the different letters indicate significant differences between bacterial strains according to one-way analysis of variance (ANOVA) at $P < 0.05$.

*Bacteria were grown in nutrient broth with or without 50 μM putrescine to stationary phase. After centrifugation to remove the cells as a pellet, the supernatants were acidified with concentrated HCl. Phenazines were extracted for 1 h on a rotary shaker with an equal volume of benzene. The benzene fraction was evaporated and the residue containing phenazines was resuspended in 0.1 M NaOH. Serial dilutions were quantified via absorbance at 367 nm.

[†]Pyrrolnitrin in the ethyl acetate extracts was quantified by high-performance liquid chromatography (HPLC).

Other secondary metabolites produced by isolate O6 that have direct antimicrobial activity are phenazines and pyrrolnitrin (Park *et al.*, 2011). The production of these secreted metabolites was determined after growth of O6 cells in suspension to stationary phase, as described previously (Kang *et al.*, 2007; Park *et al.*, 2011). The level of phenazines was reduced significantly in the *gacS* mutant and partially by the mutations in *speAspeC*; the addition of polyamines partially restored phenazine production (Table 3). Pyrrolnitrin production was severely reduced in both the *gacS* and *speAspeC* mutants; putrescine fully restored pyrrolnitrin levels to those of the wild-type for the double mutant (Table 3). Thus, these studies reveal that the production of metabolites in the *gacS* regulon is sensitive to polyamine levels in such a way that the antimicrobial effects of the cell are affected.

In conclusion, global regulation by Gac/Rsm was required for polyamine production in the biological control strain *P. chlororaphis* O6. Our results suggest that polyamines are important regulators at the level of gene expression for optimal biocontrol potential of this pseudomonad. Such regulation by polyamines parallels their control of expression of ribosomal genes in *E. coli* (Huang *et al.*, 1990) and genes associated with pathogenicity in *S. enterica* serovar Typhimurium (Jelsbak *et al.*, 2012). Multiple roles of putrescine in stress resistance and virulence of *S. enterica* serovar Typhimurium have been investigated recently (Espinel *et al.*, 2016).

The observation that polyamines regulate the biocontrol potential is relevant to the complex processes in the rhizosphere. Dependent on the level at the root surface, the polyamines could reduce the colonization potential of biocontrol bacteria (Kuiper *et al.*, 2001). Polyamines are produced by rhizosphere bacteria and are important in the biocontrol process. The polyamines produced by *Bacillus* strains are implicated in plant growth promotion (Xie *et al.*, 2014) and induced in drought tolerance in plants (Zhou *et al.*, 2016). Plants also produce polyamines in the symplast and apoplast, and they are secreted in root exudates (Moschou *et al.*, 2012; Warren, 2015). Polyamine transformation by surface copper oxidases in plants generates the ROS hydrogen peroxide, which regulates plant development (Moschou *et al.*, 2012) and could cause stress in root-associated microbes. However, the polyamines also scavenge hydroxyl radicals (Das and Misra, 2004; Potosin *et al.*, 2014). The utilization of polyamines as sources of C and N by rhizosphere bacteria will affect these processes.

This work with the *P. chlororaphis* O6 isolate suggests an additional pivotal role of bacterial polyamines in biological control activity in the rhizosphere through the control of the production of antimicrobial metabolites. In addition, polyamine production could act as a key signal to induce systemic tolerance, as reported in *Bacillus amyloliquefaciens* (Chen *et al.*, 2017). A more complete understanding of the multiple roles of polyamines in the rhizosphere and its associated microbiome requires further investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Raw data indicating the relative use of substrates by *Pseudomonas chlororaphis* O6 wild-type and *GacS* mutant cells on three Phenotype MicroArray plates with different substrates.

Fig. S2 Overall scheme showing the construction of the *speC* and *speAspeC* mutants of *Pseudomonas chlororaphis* O6. The gene *speA* was interrupted with another gene, *aacC*, that encodes for gentamycin resistance (A), and *speC* was interrupted with a gene, *nptII*, that encodes for kanamycin resistance (B). Red coloration indicates the position of the genes upstream and downstream of the gene into which the insertion was made. Green coloration shows the portions of the sequences for the *nptII* or *aacC* genes that were inserted into the *spe* genes shown with yellow coloration. PCR was performed with primers that corresponded to sequences at the ends of the *aacC* and *nptII* genes to confirm their insertion into the constructed mutants. The construction of the mutants involved three steps. First, the upstream region of each gene was amplified with *EcoRI* and *XbaI* tagged primers, and cloned into the pSB1A3 vector. Second, the *aacC* and *nptII* genes were amplified with the *XbaI* and *SpeI* tagged specific primers, and cloned into the recombinant vector containing the upstream region of the each gene. Third, the downstream regions of each gene were amplified with the *SpeI* and *NotI* tagged primers, and cloned into the recombinant vector containing the upstream region of the gene with the appropriate antibiotic resistance gene. The mutated genes were transferred to the plasmid pCPP54 and the mutants were selected on 5% sucrose and Luria–Bertani (LB) agar containing kanamycin and/or gentamycin depending on the mutant. The sequences of the specific primers are shown in Table S2 (Supporting Information).

Table S1 Gained and lost phenotypes of the *Pseudomonas chlororaphis* O6 *gacS* mutant versus wild-type using Biolog Phenotype MicroArrays.

Table S2 Specific polymerase chain reaction (PCR) primers used to construct *Pseudomonas chlororaphis* O6 mutants.