Disruption of MiaA provides insights into the regulation of phenazine biosynthesis under suboptimal growth conditions in *Pseudomonas chlororaphis* 30-84

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**Abstract**

Many products of secondary metabolism are activated by quorum sensing (QS), yet even at cell densities sufficient for QS, their production may be repressed under suboptimal growth conditions via mechanisms that still require elucidation. For many beneficial plant-associated bacteria, secondary metabolites such as phenazines are important for their competitive survival and plant-protective activities. Previous work established that phenazine biosynthesis in *Pseudomonas chlororaphis* 30-84 is regulated by the PhzR/PhzI QS system, which in turn is regulated by transcriptional regulator Pip, two-component system RpeA/RpeB and stationary phase/stress sigma factor RpoS. Disruption of MiaA, a tRNA modification enzyme, altered primary metabolism and growth leading to widespread effects on secondary metabolism, including reduced phenazine production and oxidative stress tolerance. Thus, the miaA mutant provided the opportunity to examine the regulation of phenazine production in response to altered metabolism and growth or stress tolerance. Despite the importance of MiaA for translation efficiency, the most significant effect of miaA disruption on phenazine production was the reduction in the transcription of *phzR, phzI* and *pip*, whereas neither the transcription nor translation of RpeB, a transcriptional regulator of *pip*, was affected. Constitutive expression of *rpeB* or *pip* in the miaA mutant completely restored phenazine production, but it resulted in further growth impairment. Constitutive expression of RpoS alleviated sensitivity to oxidative stress resulting from RpoS translation inefficiency in the miaA mutant, but it did not restore phenazine production. Our results support the model that cells curtail phenazine biosynthesis under suboptimal growth conditions via RpeB/Pip-mediated regulation of QS.

**INTRODUCTION**

Phenazines are heterocyclic, nitrogen-containing compounds that are produced by a number of bacterial genera. They have been studied as antimicrobial compounds that are determinants of the biological control abilities of certain rhizosphere-associated *Pseudomonas* species, including *Pseudomonas chlororaphis* and *Pseudomonas fluorescens*, as well as virulence factors important in *Pseudomonas aeruginosa* infections. The antimicrobial activity and virulence traits associated with phenazine production are related to their ability to generate reactive oxygen species. Phenazines also contribute significantly to the lifestyle of the producing bacteria, such as by facilitating biofilm production and growth under anoxic conditions [1–5]. *P. chlororaphis* 30-84 is a biological control strain isolated from the wheat rhizosphere for the control of take-all disease of wheat caused by *Gaeumannomyces graminis var. tritici* [6]. In addition to pathogen inhibition, phenazine production by *P. chlororaphis* 30-84 is essential for rhizosphere persistence [7], biofilm formation [8] and extracellular DNA release [9]. Phenazine production also alters the expression of many genes, including those involved in oxidative stress response, cell autolysis, production of other secondary metabolites, iron transport and efflux mechanisms [9, 10].

Because phenazine production has such an extensive influence on bacterial fitness and microbe–host interactions, the regulation of phenazine production has received much attention [2, 11, 12]. As in other *Pseudomonas* species, phenazine biosynthesis in *P. chlororaphis* 30-84 is regulated by the PhzR/PhzI quorum sensing (QS) system, which is located directly upstream of the phenazine biosynthetic operon (annotated as *phzXYFABCD* in *P. chlororaphis* 30-84) [2, 12, 13]. PhzI, a LuxI homologue, encodes an AHL synthase that produces N-acyl-homoserine lactone (AHL)
signals [14], PhzR, a LuxR-type transcriptional regulator, activates phz gene expression in response to accumulation of AHL signals [15].

Despite sufficient population density for QS, phenazine synthesis may be limited under suboptimal growth conditions, although the mechanisms by which phenazine production is fine-tuned under these conditions remain to be determined. Previous studies with P. chlororaphis 30-84 established that a hierarchical network of genes regulates the PhzR/PhzI QS system that controls the phenazine biosynthetic operon [16, 17]. This includes regulation by the transcriptional regulator Pip, the two-component signal transduction system RpeA/RpeB, the stationary sigma factor RpoS and the Gac/Rsm system [16–20]. Pip is an important positive regulator of QS in P. chlororaphis 30-84 and PCL1391 [16, 18]. In P. chlororaphis 30-84, the RpeA/RpeB two-component system is required for pip gene expression. RpeA is a putative sensor kinase shown previously to impact phenazine operon expression in response to medium conditions, suggesting that it may function in detecting the metabolic state or growth rate of cells [21]. RpeB is a response regulator that positively regulates the transcription of pip; however, the mechanism by which RpeB influences pip expression is unknown [16]. Additionally, it was proposed that RpoS may regulate Pip in response to nutrient conditions, since constitutive expression or mutation of rpoS can alter pip expression and phenazine production under different medium conditions [16, 18, 22]. Moreover, RpoS regulation of phenazine production is plausible since phenazine production is correlated with the expression of RpoS-regulated genes important for oxidative stress response [9, 23]. Pip has been shown to be involved in the curtailment of phenazine production in response to other abiotic stresses (fusaric acid, kanamycin or NaCl) hypothetically to conserve resources for stress management, although RpoS did not affect Pip levels under these stress conditions [23]. Another component of the phenazine regulatory network is the sensor/regulator GacS/GacA, which controls phenazine production via the expression of small RNAs [17]. A central question is how the regulators upstream of Pip are integrated to control phenazine biosynthesis in response to different nutritional, metabolic or stress conditions that affect growth.

Investigation of this question was enabled by the identification of a Tn5 plasposon-generated mutant of P. chlororaphis 30-84 that was significantly impaired in phenazine production. DNA sequence analysis revealed that the insertion occurred in the bacterial chromosome within the open reading frame of miaA, which is a gene that encodes the tRNA modification enzyme, tRNA prenyltransferase. In Escherichia coli, this tRNA prenyltransferase enzyme catalyses the addition of a Δ2-isopentenyl group from dimethylallyl diphosphate to the N9-nitrogen of adenosine in position 37 of specific tRNA species (i6A-37) [24, 25]. After i6A-37 is formed by MiaA, the tRNA is further methylthiolated by the action of the MiaB (thiolation) and MiaC (methyl transfer) enzymes to form 2-methylthio-N9-(Δ2-isopentenyl) adenosine (ms2 i6A-37) [26, 27]. The tRNAs requiring MiaA-mediated modification recognize UXX codons, which includes all codons encoding Trp, Cys, Tyr and Phe, four of six tRNAs encoding Ser and two of six encoding Leu [25, 28–30].

Although the mechanisms by which tRNA modification enzymes modulate translational efficiency, specificity and fidelity have been well described, their potential role in mediating bacterial metabolism and physiology is generating new interest [31]. Studies of tRNA modification mutants demonstrate extensive phenotypic consequences due to the disruption of specific tRNA modification enzymes. In miaA mutants, A-37 remains unmodified, leading to deficiencies in tRNAs with ms2 i6A-37 (E. coli) or ms2 i6A-37 (Salmonella typhimurium) and resulting in multiple defects in translation efficiency, codon context sensitivity and fidelity [32]. Several studies have shown that mutation of miaA caused decreased growth rates and yield, and it altered sensitivity to amino acid analogues [33], altered aromatic amino acid biosynthesis and transport [34], altered utilization of primary carbon sources [35] and reduced leu operon expression [36]. It was hypothesized that mutation of MiaA influences the expression of genes important for primary metabolism in a context-specific manner, leading to widespread effects on secondary metabolism and growth. Recently, it was suggested that tRNA modification may regulate bacterial physiology via effects on global transcriptional regulators such as RpoS, based on observations in E. coli that MiaA was required for robust expression of RpoS [29]. The effect may be due to the high proportion of MiaA-dependent codons in the RpoS coding sequence, resulting in reduced translation and levels of that sigma factor [29, 37].

Although miaA mutants probably do not occur widely in nature, the P. chlororaphis 30-84 miaA mutant, having altered metabolism and growth as well as reduced tolerance to oxidative stress, provided the opportunity to examine which of these conditions is more important in limiting phenazine production, and the potential role of the Pip regulators RpeA/RpeB and RpoS in modulating phenazine production in response to each condition. MiaA-mediated effects on biological control capabilities, including soil persistence, are discussed.

**METHODS**

**Bacterial strains and growth conditions**

Bacterial strains and plasmids are listed in Table 1, and oligonucleotides and primer sets are listed in Table S1 (available in the online Supplementary Material). A spontaneous rifampicin-resistant derivative of P. chlororaphis 30-84 was used in all experiments [6]. P. chlororaphis 30-84 and its derivatives were grown at 28 °C in various types of media including LB medium containing 5 g of NaCl per litre, M9 medium, PPMD (pigment production medium D), AB medium and AB medium supplemented with 2% casamino acids (AB-C) (Difco, Franklin Lakes, NJ) [14]. E. coli strains...
were grown at 37 °C in LB with appropriate antibiotics. Potato dextrose agar was used for fungal inhibition assays, and skim milk agar (Difco) was used for exoprotease assays. The following antibiotics were added to the media when necessary: ampicillin (Ap) 100 µg ml⁻¹, gentamicin (Gm) 50 µg ml⁻¹, kanamycin (Km) 50 µg ml⁻¹, rifampicin (Rif) 100 µg ml⁻¹ and tetracycline (Tc) 50 µg ml⁻¹.

DNA manipulation and sequence analysis

Plasmid DNA isolation, cloning, restriction enzyme digestion and T4 DNA ligation were performed using standard procedures [38]. PCR was carried out using Fidelity Taq DNA polymerase (Affymetrix, Santa Clara, CA) at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 s, 55 °C (or at the recommended Tm for the primers) for 30 s and 72 °C for 90 s, as well as a final elongation step of 72 °C for 10 min. DNA sequencing was performed by the Laboratory for Genome Technology within Institute for Plant Genomics and Biotechnology, Texas A&M University, using an ABI 3130xl Genetic Analyzer. Nucleotide and amino acid homology searches were conducted using the BLAST programmes at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

**Triparental conjugation and electroporation**

Triparental conjugation was performed as described previously [6]. Electrocompetent cells were prepared and electroporation was performed as described previously [16]. Transconjugants and transformants were selected on LB agar supplemented with appropriate antibiotics.

**Isolation of the miaA mutant**

*P. chlororaphis* 30-84 was mutagenized using the pRL27 :: Tn5KmR cassette inserted into the unique Scal site within *bla*. Isolation of the *miaA* mutant 30-84 was mutagenized using the pRL27 :: Tn5KmR cassette inserted into the unique Scal site within *bla*.

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Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
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<td><strong>P. chlororaphis</strong></td>
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<tr>
<td>30-84WT</td>
<td>Phz⁺ Rif⁺ wild-type</td>
<td>Pierson and Thomashow [6]</td>
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<td>30-84ZN</td>
<td>Phz⁺ Rif⁺ phzB::lacZ genomic fusion</td>
<td>Wood et al. [14]</td>
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<td>30-84MiaA</td>
<td>Phz⁺ Rif⁺ miaA::Tn5KmR (ID: 30-84 :19-30)</td>
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<td>30-84I/Z</td>
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<td>Whitler and Pierson [21]</td>
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<td>30-84RpoS</td>
<td>Phz⁺ Rif⁺ rpoS::uidA</td>
<td>Wang et al. [16]</td>
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<td><strong>E. coli</strong></td>
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<td>DH5α</td>
<td>F− recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argF-lacZYA)169 φ80lacZ ΔM15A−</td>
<td>Gibco BRL</td>
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<td>HB101</td>
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<td><strong>Plasmid</strong></td>
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<td>pUCP20G</td>
<td>GmR, pUCP20 derivative containing constitutive promoter pLac with SmaI-flanked GmR cassette inserted into the unique Scal site within <em>bla</em></td>
<td>Chiang and Burrows [40]</td>
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<td>pGT2pTacAcZ</td>
<td>GmR, pGT2lacZ containing constitutive promoter pTac</td>
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<td>GmR lacZ carried on promoter trap vector pPROBE-GT⁺</td>
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*KmR, GmR, RifR and TcR = kanamycin, gentamicin, rifampin and tetracycline resistance, respectively.*
28 °C for 24 h. Putative transconjugants were selected on LB plates supplemented with Km and Rif. The mutant (ID: 30-84::19-30) was selected based on its light orange appearance consistent with reduced phenazine production.

The plasposon and adjacent chromosomal regions of 30-84::19-30 were isolated as a plasmid as described previously [39]. Using the outwardly directed sequencing primers (tpnRL17 and tpnRL13, Table S1), the sequence adjacent to the insertion was determined. Sequence analysis revealed that the Tn5 was inserted into the coding sequence of miaA, so the mutant is hereafter referred to as 30-84MiaA. To verify the location of insertion, the primer pair miaAseqF-miaAseqR was used to amplify the entire coding sequence of miaA with the Tn5 insertion. Sequence analyses of the PCR product verified that the Tn5 was inserted 785 bp downstream of the start codon and 188 bp upstream of the stop codon in miaA.

Cloning and in trans expression of the miaA, htf, pip, rpoS, phzR and rpeB genes

The coding sequences for miaA, htf, pip, rpeB and rpoS were PCR amplified using primer pairs with appropriate restriction enzyme sites for cloning (Table S1). The PCR fragments miaA (1.1 kb), htf (299 bp), pip (784 bp), rpeB (1.2 kb) and rpoS (1.4 kb) were purified and cloned into the appropriate restriction enzyme sites within the multiple cloning site of pUCP20G (Table 1) for constitutive expression of the promoters and flanking sequences of miaA with the Tn5 insertion. Sequence analyses of the PCR product verified that the Tn5 was inserted 785 bp downstream of the start codon and 188 bp upstream of the stop codon in miaA.

Growth curves

Growth curves of the wild-type, 30-84MiaA and 30-84MiaA(miaA) were complemented by extra copies of constitutively expressed miaA, henceforth 30-84MiaA(miaA), were determined in different types of media (PPMD, LB, AB-C, AB and M9 minimal media). Three replicates of each strain started from single colonies were inoculated into 3 ml of broth and grown overnight at 28 °C with agitation (200 r.p.m.). Cell densities were normalized to an OD620 of 0.8, and cells were re-inoculated at a 1:100 dilution into fresh medium. The cultures were grown at 28 °C with agitation until an OD620 of 2.4 was reached. Phenazines were extracted and quantified as previously described [6]. The assays were repeated twice.

AHL extraction and quantification using AHL-specific reporter 30-84I/Z

To extract total AHL, single colonies of the wild-type, 30-84MiaA and 30-84MiaA(miaA) were inoculated into AB, LB or PPMD broth and grown at 28 °C with agitation. When cell density reached an OD620 of 2.0, total AHLs were extracted as described previously [21]. AHL production was quantified by inoculating cell-free supernatants that contained the AHLs and the AHL-specific reporter 30-84I/Z (phzI, phzB::lacZ), as described previously [16]. Briefly, the cell density of the overnight culture of 30-84I/Z was normalized to an OD620 of 0.8 and then cells were added at a 1:100 dilution to each medium type containing AHL. The β-galactosidase activity of 30-84I/Z was determined after 24 h according to Miller [41]. The assays were repeated three times.

Construction of the phzI, phzR and phzX transcriptional reporters

Transcriptional reporters of phzI, phzR and phzX were constructed to compare phzI, phzR and phzX expression in the wild-type and 30-84MiaA. PCR was used to generate PCR fragments containing the promoters and flanking sequences of phzI, phzR and phzX (Table S1), and these were ligated into the TOPO vector (Invitrogen) by TA cloning. EcoRI–BamHI fragments containing each construct were ligated into the promoter trap vector pGT2lacZ to make the phzI, phzR and phzX transcriptional fusion reporter strains pGT2phzI-lacZ, pGT2phzR-lacZ and pGT2phzX-lacZ, respectively (Table 1). These reporters were introduced separately into the wild-type and 30-84MiaA by triparental mating. The expression from each promoter was quantified as β-galactosidase activity.

RNA preparation and quantitative PCR analysis

Single colonies of the wild-type and 30-84MiaA from three separate AB-C plates were chosen and grown with rapid agitation at 28 °C in 3 ml of AB-C to an OD620 of 1.2. Prior to RNA extraction, the RNA was stabilized by Qiagen RNA Protect reagent (Qiagen, Hilden, Germany). Cells were harvested by centrifugation and total RNA was extracted using a Qiagen RNeasy Protect Bacteria Mini Kit according to the manufacturer’s protocol (Qiagen). Genomic DNA was removed using Qiagen RNase-Free DNase Kit (Qiagen) on-column digestion for 30 min. RNA concentration and purity were determined with a GE NanoVue spectrophotometer (GE Healthcare). Five micrograms of total RNA were reverse-transcribed using random primers (Invitrogen Life Technologies, Carlsbad, CA) and Superscript III (Invitrogen) at 50 °C for 1 h and inactivated at 75 °C for 15 min. For the
negative control, the same reaction was performed using sterilized water instead of reverse transcriptase.

Quantitative PCR (qPCR) assays were performed to measure the expression levels of the target genes as previously described [16]. Synthesized cDNA (2 ng per reaction) or a negative control was used for qPCRs with Fast SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers (500 nM final concentration). qPCR amplification was used to detect the expression of gacA, gacS, rpeB, rpeA, pip, phzR, phzI, hfg, rpoS, 16S rDNA and rpoD genes in the wild-type and 30-84MiaA (see Table S1 for primers). qPCR amplifications were carried out at 95°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min and a final dissociation curve analysis step from 65 to 95°C. Three technical replicate experiments were performed for each triplicate biological samples. Amplification specificity for each reaction was confirmed by dissociation curve analysis. The relative expression of the target gene was determined based on the mean cycle threshold (Ct) values of triplicate samples, and ΔΔCt analysis was performed to normalize the results using 16S rDNA as the reference genes.

The transcript abundances of genes involved in tryptophan utilization or exoprotease production in the wild-type and 30-84MiaA, including aroF, trpE, trpG, trpD, antA, iaaM, aprA and rpoD, were measured following the same protocol (see Table S1 for primers). The relative expression of target genes was determined based on the mean cycle threshold (Ct) values of triplicate samples.

**Construction of the RpoS, RpeB and GacA translational reporters**

Translational reporters were constructed to compare the translational efficiency of RpoS, RpeB and GacA in the wild-type and 30-84MiaA. The putative promoter, the 5’ untranslated region and the partial coding sequences of RpoS (155th codon from the first codon), RpeB (110th codon from the first codon) and GacA (156th codon from the first codon) were amplified using the primer sets shown in Table S1. The PCR fragments were purified, digested with EcoRI and BamHI and inserted in-frame with the E. coli lacZ gene in the translational fusion vector pME6015 [42, 43]. The RpoS, RpeB and GacA translational fusion vectors of pME6015-RpoSTL, pME6015-RpeBTL and pME6015-GacATL (Table 1), respectively, were introduced separately into the wild-type and 30-84MiaA by electroporation, and translational efficiency was measured by β-galactosidase assay.

**Stress tolerance, pathogen inhibition and exoprotease activity**

Sensitivity to oxidative stress was determined by exposing bacteria to hydrogen peroxide. Bacterial cultures were grown in 3 ml AB minimal broth at 28°C with agitation. Overnight cultures were washed with PBS, and cell densities were adjusted to an OD₆₂₀ of 0.8 with PBS. A solution containing 30% hydrogen peroxide was added to bacterial cultures at a final concentration of 0.45% hydrogen peroxide, and cells were incubated at 28°C for 30 min. For the control, the same volume of PBS was added. Serial dilutions of bacterial cultures were spotted on AB minimal agar and incubated at 28°C for 48 h. The population densities were calculated by determining c.f.u., and survival rate for each strain was expressed as a percentage, e.g. the c.f.u. on the hydrogen peroxide plates relative to the control plates.

To measure the ability of strains to inhibit the pathogen G. graminis var. graminis, bacterial cultures of the wild-type, 30-84MiaA and 30-84MiaA (miaA) were used to carry out in vitro mycelium inhibition assays as described previously [44] on potato dextrose agar (Difco). Zones of inhibition were measured after 5 days by calculating the distance between the edge of the bacterial colony and the fungal mycelium.

To measure the ability of strains to produce exoprotease, cultures of the wild-type, 30-84MiaA and 30-84MiaA (miaA) were spotted onto skim milk agar plates as described previously [8]. The proteolytic activity was determined after 48 h by measuring diameter of the clearing zone.

**Soil persistence assay**

A bacterial soil persistence assay was performed as described previously [7] with a few modifications. The soil used in this study was a clay loam collected from the upper 20 cm of the soil profile from the Texas A&M University Horticultural Research Farm, College Station, Texas. The soil was passed through a 0.5 cm mesh screen and pasteurized by treating moist soil with a steam–air mixture at 95°C for 90 min before use. Cultures of the wild-type, 30-84MiaA and 30-84ZN (deficient in phenazine production, Table 1) were grown in LB broth to an OD₆₂₀ of 1.2. Bacterial cells were collected by centrifugation and washed twice with PBS and then normalized to an OD₆₂₀ of 0.1. Ten grams of pasteurized natural soil were mixed with 5 ml bacterial suspension and then transferred into 25×200 mm sterile plastic tubes. The tubes were arranged in a random design and incubated at room temperature. Samples were collected at 0, 2, 4 and 8 weeks, and total bacterial population was determined by serial dilution plating on LB plates supplemented with rifampicin. The assay was conducted twice.

**Statistical analysis**

All data presented are mean±SEM. Data were analysed using ANOVA and Fisher’s protected LSD test (P<0.05). Data were processed with GraphPad Prism (GraphPad Software, San Diego, CA).

**RESULTS**

**Isolation of a phenazine-deficient mutant**

During the screening of a Tn5 plasposon library of P. chlororaphis 30-84 created using pRL27::TnsKm, a colony (ID: 30-84:19-30, Table 1) was isolated that produced less orange pigment than the wild-type, indicative of reduced phenazine production (Fig. 1a). Sequence analysis of genomic DNA isolated from the mutant revealed that the transposon
was inserted into a gene annotated as miaA (973 bp) at a position 785 bp downstream from the ATG start codon (Fig. 1b). The miaA gene encodes a tRNA Δ^2-isopentenyl pyrophosphate transferase [45].

The predicted protein encoded by miaA is 323 amino acids in length and showed 94% amino acid identity to the MiaA protein in Pseudomonas protegens Pf-5 (GenBank accession: AAY95971). In addition, protein alignment revealed that the MiaA homologues were highly conserved between Pseudomonas species and other Gram-negative bacteria (Fig. S1).

### Decreased phenazine biosynthesis in the miaA mutant

Consistent with the reduced orange pigmentation, phenazine production by the miaA mutant was significantly lower than the wild-type in all medium types, especially LB and AB-C media where almost no phenazine was detected spectrophotometrically at OD₆₇₇ mm (Fig. 1a). Complementation of the mutant via constitutive expression of miaA (on plasmid pUCMiaA, Table 1) restored wild-type levels of orange pigmentation and phenazine production (Fig. 1a, c), confirming that the mutation in miaA is responsible for the decreased phenazine production. The results demonstrate that a functional MiaA is required for robust phenazine production in P. chlororaphis 30-84.

### MiaA affects growth of P. chlororaphis 30-84

A major phenotype of the miaA mutant is poor growth compared to the wild-type. Growth curves confirmed that the P. chlororaphis miaA mutant exhibited growth impairment compared to the wild-type in all media tested (PPMD, LB, AB-C and minimal AB and M9 media). For example, in AB-C medium, the population densities at stationary phase (cellular yields) of the miaA mutant (OD₆₂₀ = 8.55±0.08) were significantly lower than observed for the wild-type (OD₆₂₀ = 9.84±0.17) (Fig. 2a). The largest growth defect was observed in the phenazine-stimulating PPMD medium, where the cellular yields of the wild-type and the miaA mutant measured at OD₆₂₀ were 11.25±0.44 and 6.57±0.65, respectively (Fig. 2b). Complementation of the miaA mutant with the plasmid pUCMiaA fully restored growth of 30-84MiaA(miaA) to wild-type levels in all media tested, including AB-C and PPMD where cellular yields measured at OD₆₂₀ were 9.70±0.11 and 10.98±0.51, respectively (Fig. 2a, b). These results demonstrate that a functional MiaA is required for vigorous bacterial growth and that the miaA mutant’s growth defect is more pronounced in a medium supporting high phenazine production and cellular yield.

### Reduced phenazine production due to MiaA, not Hfq

In P. chlororaphis 30-84, miaA is the last of five genes in an operon (containing Pchl3084_0549, Pchl3084_0551, Pchl3084_0550, Pchl3084_0551, mutL and miaA) and is located directly upstream of hfg (Fig. 1b). Hfg is an RNA chaperone protein that acts as a global post-transcriptional regulatory protein by interacting with target mRNAs or facilitating the complementary base pairing between small non-coding RNAs and mRNAs [46]. Hfg was shown previously to positively regulate the production of bacterial secondary metabolites, including the antibiotic 2,4-diacyetylphloroglucinol and phenazines in Pseudomonas species [47–50]. In E. coli K-12, the hfg gene

![Fig. 1. Genomic organization of the chromosomal region of P. chlororaphis 30-84 surrounding miaA and phenazine production by the wild-type, the miaA mutant and the complemented miaA mutant. (a) Pigment production by the wild-type (containing the empty vector pUCP20G), the miaA mutant (containing pUCP20G) and the miaA mutant complemented by plasmid pUCMiaA in 2 ml PPMD at 28 °C for 24 h. (b) The position of miaA and flanking genes in the chromosome of P. chlororaphis 30–84. Each open reading frame is represented by an arrow (indicating the transcriptional direction) and the sizes of the coding sequences are shown in parentheses. An arrowhead indicates the position of the Tn5::Km₆ insertion located 785 bp from the start codon of miaA. (c) Phenazines were extracted from the wild-type (containing the empty vector pUCP20G), the miaA mutant (containing pUCP20G) and the miaA mutant complemented by plasmid pUCMiaA after growth in different media (PPMD, LB and AB-C) at 28 °C with agitation until OD₆₂₀=2.4 was reached. Phenazines were quantified by UV-visible light spectroscopy at OD₆₇₇. The different letters indicate significant differences by Fisher’s protected LSD test (P<0.05).](image-url)
contains three functional promoters, two of which are located inside the open reading frame of miaA [29, 30, 35].

In our study, disruption of miaA resulted in a decrease in the transcript abundance of hfq (Fig. 3a). Therefore, it seemed possible that the reduction in phenazine production was due to a polar effect of the Tn5 insertion on hfq expression. To test this, multiple copies of hfq (behind a constitutive promoter in pUCHfq) were introduced into the wild-type and the miaA mutant, and the amount of phenazine production was quantified in PPMD and AB-C. In the wild-type, additional copies of hfq significantly increased phenazine production compared to the parental strain (with the empty vector), demonstrating both the functionality of the construct and the positive role of Hfq in phenazine production (Table 2). However, phenazine production was not restored in the miaA mutants carrying either multiple copies of hfq or the empty vector (pUCP20G) (Table 2). These results suggest that even though Hfq has a positive role in phenazine production, the reduced phenazine production by the miaA mutant is not due to a polar effect on hfq expression.

**Disruption of miaA reduces expression of the phenazine biosynthetic and phzR/phzI QS genes**

The transcript abundance of phzX, the first gene in the phenazine biosynthetic operon, was significantly lower in the miaA mutant than the wild-type as determined by qPCR using the 16S rDNA gene as the reference gene (Fig. 3a). This difference in the level of expression of the phenazine biosynthetic operon was confirmed using the transcriptional reporter pGT2phzX-lacZ, having the phzX promoter fused to lacZ (P_{phzX}::lacZ). The β-galactosidase activity in the wild-type was 218.44±30.36 MU compared to 67.94±25.21 MU in the miaA mutant. These results demonstrate that diminished phenazine production in the miaA mutant compared to the wild-type is a result of reduced expression of the phenazine biosynthetic operon.

The transcript abundances of phzI and phzR determined by qPCR were significantly reduced in the miaA mutant compared to the wild-type (Fig. 3a). The qPCR results were confirmed using the transcriptional reporters pGT2phzI-lacZ (P_{phzI}::lacZ) and pGT2phzR-lacZ (P_{phzR}::lacZ). The expressions of P_{phzI}::lacZ and P_{phzR}::lacZ were significantly lower in the miaA mutant compared to that of the wild-type (Fig. 3b). By comparison, no differences in the expression of lacZ in the control plasmid (constitutively expressing the lacZ gene, pGT2pTac-lacZ, Table 1) in the miaA mutant or the wild-type were observed (1059.89±35.14 vs 1012.41±49.28 MU, respectively). These results confirm that, in *P. chlororaphis* 30-84, a functional MiaA is required for wild-type expression levels of both phzI and phzR.

To verify the amount of AHL produced by the wild-type, the miaA mutant and the complemented miaA mutant 30-84MiaA(miaA), *P. chlororaphis* 30-84I/Z (phzI, phzB::lacZ) was used as a reporter of AHL production. The amount of AHL (measured as Miller units) produced by the miaA mutant was significantly lower than that produced by
the wild-type in all media tested. Complementation of miaA restored AHL production to the wild-type level (Fig. 3c), which demonstrates that the reduction in phenazine expression in the miaA mutant was due in part to the decreased AHL accumulation.

To verify that the reduced phenazine production in the miaA mutant was due to reduced amounts of the QS regulator PhzR, multiple copies of phzR behind a constitutive promoter (pGT2PhzR, Table 1) were introduced into the wild-type and the miaA mutant, and phenazine production was quantified. As expected, constitutive expression of phzR fully restored phenazine production in the miaA mutant (Fig. 4a).

**The effect of miaA mutation on the regulatory genes pip, rpeB and rpoS**

Disruption of miaA also resulted in decreased pip transcripts as compared to the wild-type (Fig. 3a), indicating that MiaA may influence the regulation of phenazine biosynthesis and QS via effects on the expression of pip. Previous work demonstrated that Pip regulates both QS genes as well as the phenazine biosynthetic operon [16]. As shown in Fig. 4(b), constitutive expression of pip (via pUCPip) in
both the wild-type and the miaA mutant significantly increased phenazine production compared to the same strains carrying an empty vector. These results indicate that reduced phenazine production by the miaA mutant was due to the reduced expression of pip, which led to the reduction in phzI/phzR QS-mediated activities.

In contrast to phzX, phzI, phzR and pip, the transcript abundance of rpeA, rpeB and rpoS did not differ between the mutant and the wild-type (Fig. 3a). Previous work demonstrated that pip expression is controlled by RpeA/RpeB (two-component signal transduction system) [16]. It was proposed that RpoS (stationary sigma factor) may also regulate Pip since constitutive expression or mutation of rpoS can alter pip expression and phenazine production [16, 18]. To further characterize the role of the Pip regulatory genes in phenazine biosynthesis, rpeB (pUCRpeB) or rpoS (pUCRpoS) were constitutively expressed independently in the wild-type and the miaA mutant. Constitutive expression of rpeB in the miaA mutant completely restored phenazine production and significantly enhanced phenazine production in the wild-type compared to the same strains carrying an empty vector (Fig. 4c). These results indicate that phenazine production in the miaA mutant was mediated primarily by RpeA/RpeB regulation.

Because the transcript abundances of rpeB in the miaA mutant and the wild-type were not significantly different (Fig. 3a), we hypothesized that miaA might affect phenazine production via RpeB at the translational level. To test this hypothesis, a translational fusion of rpeB::lacZ was constructed in pME6015 and introduced into the wild-type and the miaA mutant, and the translational efficiency was quantified by β-galactosidase activity. As a control, a gacA::lacZ translational fusion was similarly constructed in pME6015 to test whether the effect of the miaA mutation on translation efficiency is a general phenomenon rather than context specific. The transcriptional regulator GacA was selected because its transcription abundance was not changed in the miaA mutant compared to the wild-type (Fig. 3a), and the GacA coding sequence contained fewer UXX codons than RpoS (see Discussion). The GacA translation efficiency in the miaA mutant was not significantly different from the wild-type, indicating that translation of neither gacA nor lacZ is significantly impaired by miaA mutation (Fig. 5a).

The translational efficiency of RpeB in the miaA mutant also was unchanged compared to the wild-type (Fig. 5b).

In contrast to rpeB, constitutive expression of rpoS (pUCRpoS) in the miaA mutant did not restore phenazine production, but the wild-type having extra copies of rpoS exhibited a slight increase in phenazine production in AB-C medium (Table 3), consistent with previous observations related to rpoS overexpression [22]. To test whether miaA might affect rpoS at the translational level, a translational fusion, rpoS::lacZ, was introduced into the wild-type and the miaA mutant. The translation of RpoS was markedly lower in the

Table 2. Effect of extra copies of hfq on phenazine production in different media

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Phenazine absorbance†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PPMD</td>
</tr>
<tr>
<td>WT pUCP20G</td>
<td>2.39±0.08 b</td>
</tr>
<tr>
<td>WT pUCHfq</td>
<td>3.17±0.28 a</td>
</tr>
<tr>
<td>30-84MiaApUCP20G</td>
<td>1.18±0.35 c</td>
</tr>
<tr>
<td>30-84MiaApUCHfq</td>
<td>1.15±0.35 c</td>
</tr>
</tbody>
</table>

*Plasmid pUCP20G is the empty vector (control) and plasmid pUCHfq is the plasmid carrying hfq.
†Absorbance at 367 nm of phenazine extracted from culture supernatant at OD<sub>630</sub>=2.4 and resuspended in 0.1 N NaOH. The values are means ± standard errors based on eight replicates per treatment. Means followed by the same letter are not significantly different as determined by Fisher’s protected LSD test (P>0.05).

![Fig. 4](https://example.com/fig4.png)

Phenazine production by the wild-type and the miaA mutant with additional copies of phzR, pip and rpeB. (a) Quantification of phenazines produced by the wild-type and the miaA mutant containing GT2 (pGT2pTaclacZ) or pGT2PhzR, (b) pUCP20G or pUCPip or (c) pUCP20G or pUCRpeB in AB-C liquid medium. For all experiments, the bacterial cultures were incubated at 28 °C with agitation until an OD<sub>630</sub>=2.4 was reached. Phenazines were extracted with acidified benzene and quantified at OD<sub>367</sub>. Data are the means and standard errors of eight replicates. Different letters indicate significant differences as determined by Fisher’s protected LSD test (P<0.05).
miaA mutant compared to the wild-type (430.8±3.2 vs 626.2±63.9 MU, respectively, Fig. 5c). These results indicate that, in *P. chlororaphis* 30-84, MiaA is required for efficient RpoS translation.

To confirm that RpoS levels are lower in the MiaA mutant than the wild-type, susceptibility to hydrogen peroxide was tested, since RpoS is required for hydrogen peroxide tolerance. Strains evaluated included the following: the wild-type, the miaA mutant and the *rpoS* mutant 30-84*RpoS* (*rpoS::uidA*, Table 1) containing the empty vector (pUCP20G); the miaA mutant complemented with constitutively expressed *miaA* (pUCMiaA) or *rpoS* (pUCRpoS); and the *rpoS* mutant complemented with *rpoS*. Hydrogen peroxide tolerance of 30-84MiaA was much lower than that of the wild-type and similar that of 30-84RpoS (survival rates in 0.45 % hydrogen peroxide were 45, 24.1 and 25.8 %, for the wild-type, 30-84MiaA and 30-84RpoS, respectively) (Fig. 6a). Constitutive expression of *rpoS* in the *miaA* mutant fully restored tolerance of hydrogen peroxide to the wild-type level (49.3 % survival, Fig. 6a), but it did not significantly improve cellular yield [OD$_{620}$ of the wild-type (pUCP20G), 30-84MiaA (pUCP20G) and 30-84MiaA (pUCRpoS) in PPMD was 10.32±0.28, 7.61±0.37 and 8.12±0.42, respectively]. These data indicate that the reduction in the translational efficiency in RpoS in the *miaA* mutant significantly reduced oxidative stress tolerance but that it is not responsible for the reduced phenazine production or growth of the mutant.

Constitutive expression of *pip* or *rpeB* in either the wild-type or the *miaA* mutant had no significant effect on hydrogen peroxide tolerance. However, constitutive expression of these genes reduced growth of the wild-type and the *miaA* mutant in all medium types tested (PPMD, LB, AB-C and AB minimal media), including AB minimal medium where differences between the wild-type and the *miaA* mutant in growth are the smallest (Fig. 6b). These results indicate that overriding regulatory control by coercing cells to produce phenazines via constitutive expression of RpeB or Pip has a negative impact on growth.

**Table 3.** Effect of extra copies of *rpoS* on phenazine production in different media

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Phenazine absorbance†</th>
<th>PPMD</th>
<th>AB-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT pUCP20G</td>
<td>2.95±0.06 a</td>
<td>2.14±0.05 b</td>
<td></td>
</tr>
<tr>
<td>WT pUCRpoS</td>
<td>2.79±0.09 b</td>
<td>2.37±0.05 a</td>
<td></td>
</tr>
<tr>
<td>30-84MiaA pUCP20G</td>
<td>1.23±0.07 c</td>
<td>0.69±0.01 c</td>
<td></td>
</tr>
<tr>
<td>30-84MiaA pUCRpoS</td>
<td>1.17±0.03 c</td>
<td>0.71±0.02 c</td>
<td></td>
</tr>
</tbody>
</table>

*Plasmid pUCP20G is the empty vector (control) and plasmid pUCRpoS is the plasmid carrying *rpoS*.
†Absorbance at 367 nm of phenazine extracted from culture supernatant at OD$_{620}$=2.4 and resuspended in 0.1 N NaOH. The values are means ± standard errors based on eight replicates per treatment. Means followed by the same letter are not significantly different as determined with Fisher’s protected LSD test (*P*<0.05).

Loss of MiaA decreased pathogen inhibition and exoprotease production

Consistent with the role of phenazines in pathogen inhibition, the *miaA* mutant was significantly reduced in its ability to inhibit *G. graminis* compared to the wild-type (Fig. 6c). In contrast, fungal inhibition ability was fully restored in the complemented *miaA* mutant (e.g. mean zone of inhibition was 7.6 mm) (Fig. 6c). These observations indicate that, by
controlling phenazine production, MiaA influences *P. chlororaphis* 30-84’s capacity to inhibit *G. graminis*.

The transcript abundance of *aprA*, encoding a metalloprotease involved in biological control activity, also was reduced in the *miaA* mutant compared to the wild-type (Table 4). The *miaA* mutant also produced significantly smaller clearing zones compared to the wild-type (1.74 ±0.04 vs 2.175±0.02, respectively; N=6), confirming the reduced protease production. Exoprotease activity of the mutant was improved by complementation with *miaA* (2.04 ±0.04), although the reason it did not fully restore production is unknown. The results suggest that MiaA also influenced the production of exoprotease, which is important for biological control activity [51–53].

**MiaA is required for persistence in the soil**

Phenazines are important for the persistence of *P. chlororaphis* 30-84 in soil [7]. Given the growth deficiency observed with the disruption of *miaA* in liquid medium, we hypothesized that the persistence of the *miaA* mutant in soil might be impaired even when compared to the phenazine-deficient mutant 30-84ZN (*phzB::lacZ*). After inoculation into soil, the wild-type populations remained high for up to 8 weeks (Fig. 6d). Consistent with previous studies, the bacterial populations of 30-84ZN were similar to the wild-type for the first 4 weeks but were significantly smaller at 8 weeks. However, bacterial populations of the *miaA* mutant were significantly smaller than either the wild-type or 30-84ZN by 2 weeks and, at 8 weeks, were 1000-fold less. The
Table 4. Relative expression of genes in the miaA mutant compared to the wild-type

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein description</th>
<th>Relative expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>aroF</td>
<td>3-Deoxy-7-phosphoheptulonate synthase</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>trpE</td>
<td>Anthranilate synthase, component I</td>
<td>6.5±1.0</td>
</tr>
<tr>
<td>trpG</td>
<td>Anthranilate synthase, component II</td>
<td>8.4±1.6</td>
</tr>
<tr>
<td>trpD</td>
<td>Anthranilate phosphoribosyltransferase</td>
<td>5.1±1.0</td>
</tr>
<tr>
<td>antA</td>
<td>Anthranilate 1, 2-dioxigenase, large subunit</td>
<td>2.4±0.6</td>
</tr>
<tr>
<td>iaaM</td>
<td>Tryptophan 2-monooxygenase</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>aprA</td>
<td>Extracellular alkaline metalloprotease</td>
<td>−1.9±0.3</td>
</tr>
<tr>
<td>rpoD</td>
<td>RNA polymerase sigma factor</td>
<td>1.2±0.2</td>
</tr>
</tbody>
</table>

*The relative expression determined by qPCR of the selected genes in the miaA mutant compared to the wild-type. Values are the means of three replicates ± standard error. The16S rRNA was used as the reference gene.

results indicate that a functional MiaA is important for soil persistence and that, in addition to phenazine biosynthesis, it regulates other traits important for survival in soil.

DISCUSSION

Although miaA mutants probably do not occur widely in nature, the effects of the MiaA defect on primary metabolism, growth and oxidative stress tolerance provided insight into how the RpeA/RpeB and RpoS regulatory pathways modulate phenazine production in response to changes in the metabolic state or stress response of the cells. Characteristically, mutations in miaA cause broad pleiotropic phenotypes, including decreased growth rate and yield, broad changes in the biosynthesis and utilization of certain amino acids, defects in translation efficiency and fidelity (especially of specific regulatory genes), increased susceptibility to antibiotics and reduced virulence [32, 54]. For example, cellular yields of miaA mutants in S. enterica sv. Typhimurium LT2 were 20–50 % lower than the wild-type, but the yield reductions were more pronounced in media that supported shorter doubling times [33]. Our study also showed reductions in the cellular yields of 30-84MiaA compared to the wild-type in all medium types tested, with the largest differences in yield observed in phenazine-promoting PPMD medium, supporting the highest phenazine production and cellular yield by the wild-type. One hypothesis for the impaired growth is that disruption of MiaA altered the biosynthesis and utilization of primary metabolites such as amino acids. For example, the absence of a functional MiaA in P. putida resulted in 30-fold higher expression of the tryptophan biosynthetic genes trpE and trpGCD regardless of the presence or absence of exogenous tryptophan [55]. In P. chlororaphis 30-84, the transcripts of genes involved in the biosynthesis and metabolism of anthranilate and tryptophan (aroF, antA, trpE, trpGD and iaaM) were significantly more abundant (two to eightfold) in 30-84MiaA than in the wild-type (Table 4). These results suggest that, in P. chlororaphis 30-84, MiaA has regulatory roles in the expression of genes involved in the biosynthesis or utilization of certain amino acids and that its influence is strongly context dependent. In P. chlororaphis 30-84MiaA, these changes resulted in reduced cellular yield and secondary metabolite production, including phenazines and exoprotease important for biological control.

It was hypothesized that tRNA modification is a mechanism for regulating bacterial physiology via effects on global transcriptional regulators such as the stationary phase/general stress response sigma factor RpoS [29]. A recent study in E. coli suggested context-dependent effects of loss of MiaA on efficient translation of rpoS mRNA [29, 37]. Steady-state levels of RpoD and RpoS were compared by Western blot analysis and showed a two to threefold decrease in the steady-state levels of RpoS, but not RpoD, in the E. coli miaA mutant [29]. Given the apparent difference in the translational efficiency of RpoS and RpoD in the E. coli miaA mutant, codon usage was compared. Interestingly, usage of the Leu UXX codons that require MiaA modification in the E. coli RpoS sequence was 28 % compared to 10 % in RpoD [29]. In the current study, we did not find a bias in the usage of Leu codons, but the usage of Ser UXX codons in the P. chlororaphis 30-84 RpoS sequence was 75 % compared to 65 % in RpoD and 50–60 % in other phenazine regulatory genes such as GacA (60 %). Consistent with this bias, the expression of an rpoS::lacZ but not a gacA::lacZ translational fusion was significantly reduced in 30-84MiaA compared to the wild-type. These data support the hypothesis that tRNA modifications affected the translation efficiency of RpoS, which may have contributed to the pleotropic metabolic and growth deficiencies we observed.

Relationship between MiaA and the genes in the phenazine regulatory network

Despite the importance of MiaA for translation efficiency, the most significant effect of mutation of miaA was on the transcription of the phenazine biosynthetic operon and genes regulating this operon, including the phzR/phzI QS genes and pip. Constitutive expression of phzR or pip in the miaA mutant fully restored phenazine production, but not cellular yield. Whether mutation of miaA also affected translation of pip and/or phzR is unclear; however, under our experimental conditions, constitutive expression of pip and phzR was sufficient to overcome potential translational deficiencies, consistent with transcriptional control of pip being the limiting factor. These results demonstrate that the miaA mutant was metabolically capable of producing phenazines, and are consistent with Pip having a role in the regulation of phenazine production in the miaA mutant under suboptimal growth conditions.

Consistent with the role of RpeB in controlling the transcription of pip and phzR, constitutive expression of rpeB in the miaA mutant also completely restored phenazine production, but it did not improve cellular yield. In fact, constitutive expression of rpeB or pip in the wild-type or 30-84MiaA reduced cellular yield or further amplified the mutant growth defect, respectively. A translational fusion of
rpeB was not significantly affected by miaA disruption, indicating that the reduction in phenazine production in the miaA mutant was not due to a reduction in translation efficiency of RpeB. Taken together, these results support the model that reduced phenazine production in the miaA mutant is due to RpeA/RpeB-mediated regulation of pip in response to poor growth conditions.

Given the effect of the miaA mutation on RpoS translation, we hypothesized that reduced RpoS-mediated oxidative stress response, potentially important for managing oxidative stress associated with phenazine production [9], may contribute to the reduced phenazine production by 30-84MiaA. Analysis of the wild-type (empty vector, pUCP20G), 30-84MiaA (pUCP20G) and 30-84RpoS (pUCP20G) susceptibility to hydrogen peroxide demonstrated that the miaA mutant was as susceptible to hydrogen peroxide as an rpoS mutant, confirming the RpoS deficiency in the miaA mutant. Complementation of the RpoS defect in 30-84MiaA or 30-84RpoS restored wild-type levels of hydrogen peroxide tolerance, but it did not restore phenazine production or cellular yield in the miaA mutant. These results indicate that diminished RpoS translation in the miaA mutant was not responsible for the reduced phenazine production and that reduced oxidative stress tolerance was not limiting phenazine production.

**Insights into the regulation of phenazine biosynthesis gained from study of 30-84MiaA**

One of the unique aspects of our study was the demonstration that phenazine biosynthesis in 30-84MiaA was affected more by the cell’s metabolic state and ability to reach its yield potential (e.g. the wild-type population level) than by reduced oxidative stress tolerance associated with lower RpoS levels. Constitutive expression of rpeB in the miaA mutant restored phenazine production, apparently by-passing the influence of cell metabolism and growth on phenazine production, but it did not improve oxidative stress tolerance. Reductions in the cellular yields of the wild-type and 30-84MiaA that occurred when the RpeA/RpeB regulation was bypassed via constitutive expression of pip or rpeB indicated that RpeB regulation of Pip is important for limiting phenazine production at the expense of optimal growth, as well as in curtailing phenazine biosynthesis under suboptimal growth conditions. Previous work using *P. chlororaphis* PCL1391 demonstrated that constitutive expression of pip restored phenazine production under stressful abiotic conditions that were repressive to phenazine production, but sufficient for QS, such as sub-inhibitory concentrations of fusaric acid or NaCl; however, similar to our study, this resulted in further reductions in cellular yields [23]. Their observations support the model that phenazine biosynthesis is constrained under poor growth conditions and that Pip regulation is important for this control; however, the regulators of Pip were not identified [23].

Our findings lend further support to the model that RpeA/RpeB is the primary sensor/regulator system responsible for modulating Pip and phenazine production in response to the metabolic state of the cell [16, 21]. According to this model, RpeA acts as a metabolic state sensor to determine whether production of phenazines is appropriate and controls phenazine biosynthesis by modifying the level of active, phosphorylated RpeB and, in turn, pip expression. This observation is supported by RpeA/RpeB mutation and constitutive expression studies demonstrating loss of Pip regulation of QS and phenazine production in different medium types [16, 21]. RpeB shows a high degree of similarity (≥90% amino acid similarity) with several functionally uncharacterized response regulators in other environmental *Pseudomonas* species, including those that also have genes annotated as Pip and do not produce phenazines [16]. These findings introduce the possibility that RpeB may play additional, as yet uncharacterized, roles in modulating Pip regulation of secondary metabolism in other pseudomonads.

**Importance of MiaA for biological control**

In *P. chlororaphis* 30-84, phenazine production is important for pathogen inhibition and soil persistence and is associated with broad transcriptomic changes in gene expression [9]. As expected, decreased phenazine production reduced the ability of the miaA mutant to inhibit *G. graminis*. Exoprotease, another product of secondary metabolism, was also reduced in the mutant. The transcription of exoprotease was linked previously to phenazine production [9], which may explain the effect of MiaA on exoprotease production, although the mechanism by which phenazine production influences exoprotease production is unknown. As expected from observations of the miaA mutant in liquid culture, persistence of 30-84MiaA in soil was less than that of the wild-type or 30-84ZN (lacking phenazine production). Taken together, these data suggest that a functional MiaA affects many traits important for the biological control capabilities of *P. chlororaphis* 30-84.

As Jackman and Alfonzo [31] suggest, tRNA modifications expand the genetic information encoded by tRNA genes and are potentially important determinants of eukaryotic and prokaryotic physiology. Under normal cell conditions, it is likely to be difficult to predict the effects of selection for modified amino acids in specific coding sequences, or the fluctuations in the amounts of tRNA modification enzymes [31]. However, studies such as this utilizing MiaA-deficient mutants reveal the far-reaching effects of tRNA modification enzymes on cell physiology and secondary metabolite production important for survival in favoured bacterial niches and biological control applications.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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