Small RNA-dependent Expression of Secondary Metabolism Is Controlled by Krebs Cycle Function in Pseudomonas fluorescens

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Kasumi Takeuchi, Patrick Kiefer, Cornelia Reimmann, Christoph Keel, Christophe Dubuis, Joëlle Rolli, Julia A. Vorholt, and Dieter Haas

From the 4 Département de Microbiologie Fondamentale, Bâtiment Biophore, Université de Lausanne, CH-1015 Lausanne, Switzerland, the 5 National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan, and the 4 Institute of Microbiology, Eidgenössische Technische Hochschule Zürich, CH-8093 Zürich, Switzerland

Pseudomonas fluorescens CHA0, an antagonist of phytopathogenic fungi in the rhizosphere of crop plants, elaborates and secretes several secondary metabolites with antibiotic properties. Their synthesis depends on three small RNAs (RsmX, RsmY, and RsmZ), whose expression is positively controlled by the GacS-GacA two-component system at high cell population densities. To find regulatory links between primary and secondary metabolism in P. fluorescens and in the related species Pseudomonas aeruginosa, we searched for null mutations that affected central carbon metabolism as well as the expression of rsmY-gfp and rsmZ-gfp reporter constructs but without slowing down the growth rate in rich media. Mutation in the pycAB genes (for pyruvate carboxylase) led to down-regulation of rsmXYZ and secondary metabolism, whereas mutation in fumA (for a fumarase isoenzyme) resulted in up-regulation of the three small RNAs and secondary metabolism in the absence of detectable nutrient limitations. These effects required the GacS sensor kinase but not the accessory sensors RetS and LadS. An analysis of intracellular metabolites in P. fluorescens revealed a strong positive correlation between small RNA expression and the pools of 2-oxoglutarate, succinate, and fumarate. We conclude that Krebs cycle intermediates (already known to control GacA-dependent virulence factors in P. aeruginosa) exert a critical trigger function in secondary metabolism via the expression of GacA-dependent small RNAs.

Secondary metabolism occurs in certain bacteria and fungi as part of developmental processes, which are often accompanied by morphological changes (1, 2). In natural environments, secondary metabolites are believed to confer a selective advantage to the producers when these organisms cannot rely on their full growth potential to compete with other organisms (3). Such a role is most plausible for secondary metabolites having antibiotic activities (4). In pure cultures, secondary metabolites are non-essential for the producers and are typically formed when cell population densities are high and growth is restricted. This distinct production phase, sometimes called idiophase, usually follows the phase of optimal growth, also termed trophophase (5). A fundamental question is what triggers the onset of the idiophase. Both extracellular and intracellular signal molecules are known to be involved. For instance, excreted quorum-sensing signal molecules, such as N-acyl-homoserine lactones of Pseudomonas species or γ-butyrolactones of Streptomyces species, positively regulate the expression of antibiotic compounds, and the intracellular alarmone ppGpp is required for antibiotic production in Streptomyces coelicolor under conditions of nitrogen starvation (1, 6). However, these findings do not provide a generally valid picture of how secondary metabolism is initiated in microorganisms. For instance, some Pseudomonas species produce secondary metabolites without N-acyl-homoserine lactones and phosphate-limited S. coelicolor does not rely on ppGpp for antibiotic production (1, 6).

In fluorescent pseudomonads, the Gac/Rsm signal transduction pathway is instrumental for the expression of secondary metabolites. These metabolites contribute effectively to virulence in animal- and plant-pathogenic Pseudomonas species as well as to plant protection in root-colonizing Pseudomonas species having biocontrol activity. Thus, depending on the species, mutants defective in the Gac/Rsm pathway have lost part or all of their virulence or have a reduced ability to suppress plant diseases (7–9). The Gac/Rsm cascade is initiated by the GacS/GacA two-component system (8, 10). At high cell population densities, Pseudomonas species excrete chemically uncharacterized signal molecules that can act as inducers of the Gac/Rsm pathway by favoring phosphorylation of the GacS sensor kinase and hence of the cognate GacA response regulator (11–14). Activated GacA promotes the transcription of two or three non-coding small RNAs (sRNAs), depending on the species. In the closely related biocontrol strains Pseudomonas fluorescens CHA0 and Pf-5 (15), GacA controls the expression of three sRNAs, termed RsmX, RsmY, and RsmZ. In the opportunistic
human pathogen *Pseudomonas aeruginosa*, there are only two functionally equivalent, GacA-controlled sRNAs, RsmY and RsmZ. These sRNAs have a high affinity for the RNA-binding protein RsmA and additionally for the RsmA parologue RsmE in strain CHA0 (11, 12, 16, 17). The RsmA and RsmE proteins repress the translation of genes involved in secondary metabolism during the trophophase. When the RsmX/Y/Z sRNAs are induced in the idiophase, they relieve translational repression of target genes by sequestering the RsmA and RsmE proteins, thereby allowing the synthesis of secondary metabolites (12, 18–20).

We have undertaken the present genetic and metabolic study to find intracellular factors that favor or hinder the expression of the *rsmX/Y/Z* genes in *P. fluorescens* CHA0 and to reveal regulatory links that may exist between primary and secondary metabolism. To this end, we have screened mutant libraries of *P. fluorescens* carrying an *rsmZ-gfp* fusion (21) and of *P. aeruginosa* carrying an *rsmY-gfp* fusion for diminished or enhanced GFP expression. We find that mutational loss of pyruvate carboxylase or fumarase A strongly affects *rsmX/Y/Z* expression without major changes of growth properties.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The bacterial strains and plasmids used are listed in Table 1. Strains of *Escherichia coli* and *P. fluorescens* were routinely grown in nutrient yeast broth (NYB; 2.5% (w/v) nutrient broth, 0.5% (w/v) yeast extract) with shaking or on nutrient agar plates (4% (w/v) blood agar base, 0.5% (w/v) yeast extract) amended with the following antibiotics when required: ampicillin, 100 μg/ml; gentamicin, 50 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 1 mg/ml; or tetracycline, 25 μg/ml (125 μg/ml for selection of *P. fluorescens*). Transposon mutants of *P. aeruginosa* were screened on a medium described by Carmi et al. (22). For antibiotic activity assays, bacteria were grown on glycerol-casamino acids medium (GCM) (23). For metabolic analyses, bacteria were grown in diluted and modified GCM to minimize the effects of metabolism during the trophophase. When the RsmX/Y/Z sRNAs are expressed, the translation of genes involved in secondary metabolism is repressed by biparental matings between *E. coli* S17-1pir/pLM1 and the pyoverdin-negative *P. aeruginosa* strain PAO6382.

Transposon Tn5Gm Mutagenesis of PAO6382 and Mutant Screening—About 10,000 random Tn5Gm insertions were generated by biparental matings between *E. coli* S17-1pir/pLM1 and the pyoverdin-negative *P. aeruginosa* strain PAO6382. Transposon insertion mutants were selected on nutrient agar containing 50 μg/ml gentamicin and 10 μg/ml chloramphenicol and stored at −80 °C in 96-well microtiter plates in NYB containing 15% (v/v) glycerol and 50 μg/ml gentamicin. The Tn5Gm mutant library was then screened for mutants with altered *rsmY* expression. As a reporter construct, we chose pME7415 because preliminary tests had shown that detection of GacA-dependent regulation was easier on plates with an *rsmY* expression fusion than with an *rsmY* fusion (data not shown). Plasmid pME7415 was constructed by inserting the *rsmY* promoter region on a 0.31-kb BamHI-PstI fragment (recruited from pME6916) into pPROBE-TT. Plasmid pME7415 was introduced into the mutant library by conjugation as follows. Mutants were grown overnight in 96-well microtiter plates at 43 °C in NYB and subsequently transferred with a 48-needle replicator onto nutrient agar plates where 100 μl of an NYB-grown overnight culture of *E. coli* S17-1pir/pME7415 had been spread. Donor and recipients were incubated together on nutrient agar at 37 °C for 3–4 h and subsequently transferred, with the replicator, onto plates containing Carmi (22) medium and 50 μg/ml gentamicin plus 100 μg/ml tetracycline. To avoid fluorescence due to siderophore production, FeCl3 was added to the selective medium at a final concentration of 100 μM. Plates were incubated at 37 °C, and GFP production was scored regularly by visual inspection in daylight and under UV light. The transposon insertion sites of mutants with altered GFP production were determined by sequence analysis as described previously (27) except that cloning out of the transposon was done with BamHI.

**Complementation of the pycB-negative Mutant CHA1201**—To restore pycB function in the mutant CHA1201, a 3.8-kb fragment carrying *pycA* and *pycB* was amplified with primers PycAB1 and PycAB2, high fidelity DNA polymerase PrimeSTAR™ (Takara), and genomic DNA of *P. fluorescens* CHA0 as a template. The amplified fragment was digested with BamHI and HindIII and cloned into BamHI- and HindIII-digested pBluescript II KS (Stratagene). The insert obtained was confirmed by sequencing and digested with HindIII and BamHI; the BamHI sticky end was filled in with T4 polymerase. This fragment was subcloned into the mini-Tn7 vector pME6182 cleaved at the HindIII and SmaI sites. The resultant plasmid, pME9951, was introduced together with pUX-BF13 into CHA1201 by electroporation. Transposition of the mini-Tn7 carrying the *pycAB* genes into the chromosome was confirmed by PCR with primers Gm and Glms (28). The resultant strain was named CHA1201C. The *pycAB* genes were also subcloned into pME6031 (at the BamHI and HindIII sites), and the resulting plasmid, pME9952, was introduced into CHA1201 for complementation. The resultant strain was named CHA1201Cp and used for assaying the chromosomal *rsmX-lacZ* and *rsmY-lacZ* fusions carried by mini-Tn7, which occupied the Tn7 attachment site in these strains.
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TABLE 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| B. subtilis M168  | Wild type   | C. Keel             |
| E. coli DH5α, HB101 | Laboratory strains | Ref. 37 |
| S17-1-1::Δpir     | pro the hsdr recA chromosome::RP4-2 (Tc::Mu Km::Tn7) Δpir; Tp' Sm' | Ref. 38 |
| P. aeruginosa PA01 | Wild type   | ATCC15692           |
| PAO6382           | ΔpdfF       | C. Reimmann         |
| P. fluorescens    |             |                     |
| CHA0              | Wild type   | Ref. 39             |
| CHA19 gacAΔlacI    |            | Ref. 40             |
| CHA89             | gacA-Km'    | Ref. 41             |
| CHA1201 fumAΔΔlacI | pycB-Tn5, insertion 1.6 kb from 5'-end | This study |
| CHA1201 Cp        | pycB-Tn5 strain with pycAB' region in mini-Tn7 | This study |
| CHA1201 Cp        | pycB-Tn5 strain harboring pME9952 | This study |
| CHA1202 ΔretS     |            | Ref. 30             |
| CHA1204 ΔladS     |            | Ref. 30             |
| CHA1313 ΔgacAΔlacI | pycA::ΔSp/Sm | This study |
| CHA1314 gacA-Km'pycA::ΔSp/Sm | This study |
| CHA1315 ΔretS pycA::ΔSp/Sm | This study |
| CHA1316 ΔladSΔlacI | pycA::ΔSp/Sm | This study |
| CHA1317 ΔgacAΔfumA |            | This study |
| CHA1318 gacA-Km'ΔfumA |            | This study |
| CHA1319 ΔretSΔfumA |            | This study |
| CHA1320 ΔladSΔlacI | pycA::ΔSp/Sm | This study |
| CHA1321 pycA::ΔSp/Sm |            | This study |
| CHA1322 ΔfumA |            | This study |
| CHA1322C ΔfumA strain with fumA' region in mini-Tn7 | This study |
| CHA1322 Cp ΔfumA strain harboring pME9956 | This study |

Plasmids

| Plasmid | Description | Source or reference |
|---------|-------------|---------------------|
| pBluescript II KS Cloning vector, ColE1 replicon; Ap' | Stratagene |
| pHP451 | Source of the ΔSp/Smr cassette, ColE1 vector; Ap' | Ref. 29 |
| pLM1 | Tn5Gm delivery vector, Gm' derivative of plpL27 | Ref. 27 |
| pME497 | Mobilizing plasmid, IncP-1, Tra, RepA(A); Ap' | Ref. 42 |
| pME3087 | Suicide vector, ColE1 replicon, Mobi, Tc' | Ref. 43 |
| pME3266 | Translational gacAΔlacI fusion; Tc' | F. Carruthers |
| pME5031 | pACYC177-pVS1 shuttle vector; Tc' | Ref. 44 |
| pME6060 | Translational aprAΔ-lacZ fusion; Tc' | Ref. 45 |
| pME6091 | Transcriptional rsmZ-lacZ fusion; Tc' | Ref. 11 |
| pME6182 | Mini-Tn7 gene delivery vector based on pME280a, HindIII-Smal-KpnI-Nool-Sphl cloning site, ColE1 replicon, Gm' Ap' | Ref. 30 |
| pME6702 | Translational phIA'Δ-lacZ fusion under ptcA; Tc' | Ref. 17 |
| pME6827 | Translational gacA'Δ-lacZ fusion; Tc' | C. Reimmann |
| pME6916 | Transcriptional rsmY-lacZ fusion; Tc' | Ref. 16 |
| pME7402 | Transcriptional rsmZ-gfp fusion; Tc' | Ref. 21 |
| pME7415 | Transcriptional rsmX-gfp fusion; Tc' | This study |
| pME7698 | pME6182 containing a transcriptional rsmX-lacZ fusion in mini-Tn7 | B. Humair |
| pME7699 | pME6182 containing a transcriptional rsmY-lacZ fusion in mini-Tn7 | B. Humair |
| pME9951 | pME6182 containing the pycAB genes in mini-Tn7 | This study |
| pME9952 | pME6031 containing the pycAB genes | This study |
| pME9953 | pME3087 containing pycAB region with a 0.5-kb EcoRV deletion in pycA replaced by the 2-kb ΔSp/Sm fragment | This study |
| pME9954 | pME3087 containing a 1.4-kb fumA region with a 0.9-kb deletion in the fumA gene | This study |
| pME9955 | pME6182 containing the fumA gene in mini-Tn7 | This study |
| pME9956 | pME6031 containing the fumA gene | This study |
| pPROBE-TT' | gfp promoter probe vector; Tc' | Ref. 46 |
| pUX-BF13 | Helper plasmid encoding Tn7 transposition fusions; Ap' | Ref. 47 |

Generation of the pycA-negative Mutant CHA1321—To inactivate the chromosomal pycA gene of *P. fluorescens* CHA0, we utilized a derivative of the suicide plasmid pME3087. pBluescript II KS carrying the 3.8-kb pycAB fragment mentioned above was digested with EcoRV to replace a 0.5-kb internal region of pycA with the 2-kb ΔSp/Sm cassette from pHP451 (29). The resulting plasmid was digested with XbaI and KpnI to excise the mutated pycA gene, which contained 1248 bp upstream and 889 bp downstream of ΔSp/Sm. This fragment was subcloned into pME3087 at the KpnI and XbaI sites to give pME9953. This plasmid was mobilized from *E. coli* DH5α to *P. fluorescens* by triparental mating using *E. coli* HB101/pME497. A pycA::ΔSp/Sm mutant, which was resistant to spectinomycin but sensitive to tetracycline, was obtained after an enrichment for tetracycline-sensitive cells (30), and the ΔSp/Sm insertion was confirmed by PCR in the resulting strain CHA1321.

Generation of the fumA-negative Mutant CHA1322 and Its Complementation—An in-frame deletion in the chromosomal fumA gene of *P. fluorescens* CHA0 was created as follows. Fragments of ~700 bp located on each side of the fumA gene were amplified by PCR with primer pairs FumAUF/FumAUR and FumADF/FumADR, annealed, and amplified as a 1.4-kb fragment using primers FumAUF/FumADR. After sequencing, this
1.4-kb fragment was cloned into pME3087 cut with BamHI and HindIII to give pME9954. This plasmid was mobilized from E. coli DH5α by triparental mating with E. coli HB101/pME497. Excision of the vector via a second crossing over was obtained after enrichment for tetracycline-sensitive cells (30), generating the fumA mutant CHA1322. This strain was complemented with a 2.2-kb fragment carrying fumA, which had been amplified by PCR with primers FumAUF and FumADR and sub-cloned into pME6182 (resulting in plasmid pME9955) to give strain CHA1322C based on the mini-Tn7 method described above. The fumA region was also cloned into pME6031 (cut with BamHI and HindIII) and the resulting plasmid, pME9956, was introduced into CHA1322, thereafter named CHA1322C.

RNA Extraction and Northern Blot Analysis—RNAs used for Northern blot analysis were isolated using a hot acid phenol extraction protocol (31). RNAs (10 μg/lane) were separated on a denaturing urea-polyacrylamide gel and analyzed by Northern blotting using digoxigenin-labeled probes as described previously (32).

Enzyme Assays—For β-galactosidase assays, P. fluorescens strains were grown at 30 °C in 50-ml flasks containing 20 ml of NYB amended with 0.05% Triton X-100 with shaking at 180 rpm. Specific activities were determined by the Miller method (33). For fumarase assays, exponential phase cultures of P. fluorescens in GCM were harvested at 4 °C by centrifugation at 6,000 × g for 10 min. Cells were washed twice, resuspended in 1 ml of chilled 50 mM sodium phosphate buffer, pH 7.0, containing 1 μg/ml Pefabloc SC (Roche Applied Science), and broken with a VibraCell sonicator (Danbury, CT) in an ice bath for 1 min. Cell debris was removed by centrifugation at 10,000 × g for 10 min. Fumarase activity was measured in the extracts by following the increase in absorbance at 240 nm in the presence of 6 mM fumarate, 7 mM malate, succinate, and amino acids) were determined after overnight incubation at 30 °C, cells were killed by UV irradiation on a transilluminator for 5 min. An overlay of B. subtilis revealed antibiotic production by growth inhibition zones.

Plant Disease Suppression and Root Colonization Assays—Ten flasks containing natural sandy loam soil were planted with three cucumber seedlings each and treated with Pythium ultimum and/or P. fluorescens. After 7 days of incubation, the biocontrol activity of each strain was estimated as described previously (16).

LC-HRMS Analysis of Central Metabolites—P. fluorescens strains harboring pME7402 were grown in 20 ml of modified GCM in 50-ml Erlenmeyer flasks with shaking at 180 rpm and 30 °C. For each strain, three cultures were incubated in parallel. Samples were taken from each culture during the exponential trophophase (at A400 nm = 0.5) and the idiophase (at A400 nm = 1.7) when maximal GFP formation was observed. Cells were sampled by fast filtration using RC membrane filters (diameter 47 mm, pore size 0.2 μm; Sartorius Göttingen, Germany) without washing. Subsequently, filters were directly transferred into 8 ml of boiling water for 8 min. An internal standard solution (i.e. a uniformly 13C-labeled metabolite extract (35) from Methylobacterium extorquens AM1 (36)) was added simultaneously. The volumes of the sample and the internal standard were measured by weight. The extracts were cooled on ice, filtered through an RC Sartorius Minisart filter (pore size 0.2 μm), chilled with liquid nitrogen, and freeze-dried. The dried samples were redissolved in 80 μl of aqueous acetonitrile (70% (v/v)) for LC-HRMS analysis as described (36). For each metabolite, the peak intensity of the non-labeled mass peak M₀ and that of the uniformly 13C-labeled mass peak M₁₃ were determined, and the intracellular concentrations (μmol/g) were calculated as follows,

\[
[M_{\text{metabolite}}] = \frac{I_{M_0} \cdot V_S \cdot c_{MUL} \cdot \frac{M_{UL}}{\Delta M}}{I_{MUL} \cdot V_S \cdot c_{MUL} \cdot \frac{M_{UL}}{\Delta M}} \quad (Eq. 1)
\]

where I_M₀ represents intensity of the metabolite mass peak of the cell extract in sample, I_M₁₃ is intensity of the metabolite mass peak of the 13C-labeled internal standard in sample, V_S is culture volume of sample, V_S is volume of internal standard added to sample, c_MUL is concentration of the 13C-labeled internal standard, and X_S is biomass concentration (in g cell dry weight) of sample. Average values and S.D. were determined from three cultures.

Metabolites present in the culture supernatant (citrate, malate, succinate, and amino acids) were determined after removing cells with an RC Sartorius Minisart filter (pore size 0.2 μm). For LC-HRMS analysis, the filtrate was diluted 1:100 using 1 μM sucrose in aqueous acetonitrile (85% (v/v)) as an internal standard. LC-HRMS analysis was performed as described (36). This method was not suited for the analysis of arginine, lysine, cysteine, and tryptophan. The consumption of amino acids was determined by following the decrease of chromatogram peak intensities (relative to the initial peak intensities) for each amino acid.

RESULTS

Transposon Insertion Mutations Affecting the Expression of GacA-dependent sRNAs in Fluorescent Pseudomonads—We used two collections of Tn5 insertion mutants. The first library consists of about 20,000 P. fluorescens CHA0 mutants carrying an rsmZ_CHA0·gfp fusion on plasmid pME7401 and has previously been used in a search for biocontrol-negative mutants (21). The second library was constructed in this study and consists of about 10,000 P. aeruginosa PAO mutants carrying an rsmY_CHA0·gfp fusion on pME7415. These mutants were generated with a gentamicin resistance variant of Tn5 in a pyoverdin-negative background (PAO6382; see “Experimental Procedures”). The rationale for using two different Pseudomonas species and two different sRNA gene reporters is to improve the chances to find regulatory mechanisms that are common to fluorescent pseudomonads. Both collections were screened on rich media for dim and superbright colonies, thus differing with respect to GFP expression from the normally bright fluorescent phenotype seen in the parent strains. In both screens, we used gacA and gacS mutants (dim) as a control. Candidate clones
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(about 70 from each library) were reexamined for GFP expression in rich liquid media, and, when clear cut phenotypes could be confirmed, the Tn5 insertions were mapped by sequencing.

Mutants having insertions in genes with unknown functions were not analyzed further at this stage.

If conditions of suboptimal growth per se were conducive to high expression of secondary metabolism, via the expression of rsmX/Y/Z, we might expect to find a number of small colony variants with a superbright phenotype. However, this did not appear to be the case, although in the P. aeruginosa screen, two independent isolates of this kind were found; both were mutated in the retS gene. The product of this gene has recently been identified as an antagonist of GacS and hence as a negative regulator of rsmZ expression (14), confirming the validity of our approach. Both P. fluorescens and P. aeruginosa screens yielded biotin- and threonine-auxotrophic mutants, which had a slight growth handicap under the conditions used. Both types of mutants were dim rather than superbright. We decided not to examine these further but to concentrate on normally growing mutants.

Two independently generated dim mutants of P. fluorescens CHA0 had a Tn5 insertion in the PFL_6157 gene, which appears to form a transcription unit with the upstream PFL_6158 gene (Fig. 1). These genes were originally annotated as oadA (for oxaloacetate decarboxylase subunit) and accC (for a biotin carboxylase), respectively (15). However, it has been shown recently that the PA5435 and PA5436 homologues of P. aeruginosa are in fact the structural genes (pycB and pycA) for pyruvate carboxylase (48), an enzyme that catalyzes the biotin- and ATP-dependent carboxylation of pyruvate, yielding oxaloacetate (49). We cured the pycB::Tn5 mutant CHA1201 of the rsmZ-gfp reporter construct and instead introduced the transcriptional fusions rsmX-lacZ (chromosomal), rsmY-lacZ (chromosomal), or rsmZ-lacZ (on a plasmid). The expression of all three fusions was significantly reduced in the pycB mutant, compared with that in the wild type and in the complemented pycB mutant (Fig. 2, A–C), when these strains were growing in rich medium (NYB). It was important to use chromosomal rsmX-lacZ and rsmY-lacZ reporter fusions; when they were carried by plasmids, their expression of β-galactosidase was too high to be tolerated by the cells. We also constructed a pycA (PFL_6158) ΔSp/Sm insertion mutant, CHA1321. In this strain, the expression of the three GacA-dependent sRNAs was similarly down-regulated (supplemental Fig. S1).

Besides the retS mutants, one more superbright, normally growing P. aeruginosa mutant attracted...
our attention. It was found to contain Tn5Gm in the PA4333 open reading frame, tentatively annotated as a fumarase (fumA) gene (Pseudomonas Genome Database web site). We constructed a fumA (PFL_4801) deletion mutant in P. fluorescens CHA0, termed CHA1322 (Fig. 1). The rsmX-, rsmY-, and rsmZ::lacZ fusions all showed a strongly elevated expression in this background, compared with the wild type and the complemented fumA mutant (Fig. 2, D–F), indicating that the influence of fumA is similar in both Pseudomonas species. In the rich medium used (NYB), the pycB and fumA mutants grew almost at wild type rates (Fig. 2, G and H).

Phenotypic Characterization of the pycAB and fumA Mutants—In agreement with the growth properties of P. aeruginosa pyruvate carboxylase-negative mutants (48, 49), the P. fluorescens pycA and pycB mutants grew very poorly in minimal media containing glycerol, pyruvate, or glucose as the only carbon source but grew normally in succinate or fumarate media (supplemental Fig. S2) (data not shown). These characteristics confirm that pyruvate carboxylase is an important anaplerotic enzyme also in P. fluorescens. The P. fluorescens fumA mutant, by contrast, was strongly handicapped in minimal media containing fumarate or succinate as the only carbon source, but grew well in glycerol medium (supplemental Fig. S2). Because the PFL_4801 and PA4333 genes had only been tentatively annotated, we measured the fumarase specific activity in P. fluorescens after growth in rich medium. The fumA mutant CHA1322 gave 0.4 ± 0.1 units/mg, whereas the wild type CHA0 and the complemented mutant each had 1.5 ± 0.1 units/mg. The residual fumarase activity in the fumA mutant was probably due to the presence of two additional fumarase isoenzymes, FumC1 (PFL_0907) and FumC2 (PFL_4328). In conclusion, our mutant screen identified two enzymes involved in the function of the Krebs (tricarboxylic acid) cycle (i.e. fumarase and pyruvate carboxylase) as key factors in the regulation of the Gac/Rsm signal transduction pathway, in that both fumA and pycAB mutations had a profound influence on rsmX/Y/Z expression.

Altered RNA Levels in pycB and fumA Mutants—The results obtained with the rsmX-, rsmY-, and rsmZ::lacZ fusions (Fig. 2) indicate that Krebs cycle intermediates influence the promoter activities of the three GacA-dependent sRNA genes. We validated this finding by determining the levels of RsmZ sRNA by Northern blotting. The RsmZ level was lower in the pycB mutant and higher in the fumA mutant by comparison with the wild type and the complemented mutant (Fig. 3).

Antibiotic and Protease Expression in the pycB and fumA Mutants—An important GacA-regulated secondary metabolite and biocontrol factor of strain CHA0 is 2,4-diacetylphloroglucinol (20, 41, 43). The translational expression of the 2,4-diacetylphloroglucinol biosynthetic gene phlA was reduced in the pycB mutant CHA1201 and increased in the fumA mutant CHA1322 by comparison with the wild type (Fig. 4), as we expected from the sRNA expression data (Figs. 2 and 3). Moreover, a parallel pattern of antibiotic production was seen in a biotest using B. subtilis as a 2,4-diacetylphloroglucinol-sensitive indicator (Fig. 5). The major GacA-controlled exoprotease of strain CHA0 is the product of the aprA gene (45, 50). We also followed the expression of a translational aprA’::lacZ fusion in the pycB and fumA mutants; again, the effects of both mutations were consistent with the rsmX/Y/Z expression data (Fig. 6).

Consequences of pycB and fumA Mutations on Biocontrol Efficacy—Root exudates in the rhizosphere, the natural habitat of P. fluorescens CHA0, are rich in Krebs cycle intermediates...
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and amino acids; sugars are less abundant (51). We wondered how the pycB and fumA mutations would influence the biocontrol efficacy of \( P. \) \textit{fluorescens} in a microcosm under natural soil conditions. In a cucumber/P. \textit{ultimum} microcosm, the wild type CHA0 afforded excellent protection of the plant from the oomycete, in terms of root weight as well as shoot weight (Table 2). Both the pycB and the fumA mutant were somewhat less effective as biocontrol agents, with \( \approx 95\% \) confidence (Table 2). All bacterial strains reached similar levels of root colonization (Table 2). These results indicate that in vitro levels of secondary metabolites do not translate directly into \textit{in vivo} biocontrol efficacy. The data also confirm previous work on strain CHA0 showing that any impairment of the Gac/Rsm system generally results in reduced biocontrol efficacy (4, 6) but that constitutive up-regulation of the Gac/Rsm system also negatively affects biocontrol efficacy (40).

**Metabolite Analysis of the \textit{pycB} and \textit{fumA} Mutants—** We wished to examine the consequences of the \textit{pycB} and \textit{fumA} mutations on intracellular metabolite pools, especially with respect to Krebs cycle intermediates and key metabolites of carbohydrate utilization. This proved to be technically difficult because, on the one hand, simple minimal media could not be used with the mutants and, on the other hand, our commonly used rich media interfered strongly with cell sample preparation. As a compromise, we used a diluted and modified glycerol-casamino acids medium (see “Experimental Procedures”) in which the wild type and the \textit{fumA} and \textit{pycB} mutants grew well and showed the same pattern of differential \textit{rsmZ}-\textit{gfp} expression as observed in full-strength medium (supplemental Fig. S3). Nevertheless, growth yields were about 2-fold lower, compared with those in full-strength medium (data not shown). Samples were taken during the exponential trophophase (at \( A_{600} \approx 0.5 \)) and during the idiophase (at \( A_{600} \approx 1.7 \)). From an analysis of amino acids in the culture supernatant, it became evident that upon entry into the idiophase, all three strains had similarly used up their most preferred substrates, such as glutamate, aspartate, proline, histidine, and phenylalanine, whereas they had not exhausted some of the less favorable nutrients, such as leucine, isoleucine, valine, threonine, and methionine (data not shown). Thus, carbon, nitrogen, and sulfur sources were still available to the bacteria, and the oxygen supply never fell below 70% of saturation (data not shown).

The wild type as well as the \textit{pycB} and \textit{fumA} mutants had lower intracellular pools of Krebs cycle intermediates, phosphoenolpyruvate, pyruvate, and sugars in the idiophase than in the trophophase (Table 3). However, there were important differential responses in the mutants. In the \textit{pycB} mutant, the pools of succinate, malate, and 2-oxoglutarate suffered a much stronger decline than in the wild type and in the \textit{fumA} mutant. In the \textit{pycB} mutant, the pyruvate pool was only transiently elevated during the trophophase, but not in the idiophase. In the \textit{fumA} mutant, the fumarate pool was dramatically increased, whereas the pools of succinate, malate, and 2-oxoglutarate were mildly elevated by comparison with the wild type and the \textit{pycB} mutant (Table 3). Fumarate, succinate, and malate were detectable in the culture supernatant of the \textit{fumA} mutant but not in the wild type and the \textit{pycB} mutant (data not shown).

In summary, a direct comparison between the \textit{fumA} and the \textit{pycB} mutant in the idiophase reveals the following salient features. A ratio of 15:1 was seen for \textit{rsmZ}-\textit{gfp} expres-

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**TABLE 2**

Contribution of \textit{fumA} and \textit{pycB} to the suppression of \textit{Pythium} damping off and root rot of cucumber by \textit{P. \textit{fluorescens}} CHA0 in natural soil

Data represent the means from two individual repetitions of the same experimental setup, with 10 replicates (flasks containing three cucumber plants) per treatment in each experiment. ND, not detected.

| Bacterial strain added\(^a\) | \textit{Pythium} added\(^a\) | Surviving plants/flask | Shoot fresh weight/flask | Root fresh weight/flask | Colonization by \textit{P. \textit{fluorescens}}\(^b\) |
|-----------------------------|---------------------------|------------------------|-------------------------|------------------------|-----------------------------|
| None                        | –                         | 100\(^c\)              | 1.17\(^d\)              | 0.26\(^e\)             | \( \log_{10} \) colony-forming units/g root |
| CHA0 (wild type)            | –                         | 100\(^c\)              | 1.31\(^d\)              | 0.26\(^e\)             | 7.10 ± 0.20                 |
| CHA1322 (\textit{fumA})     | –                         | 100\(^c\)              | 1.23\(^d\)              | 0.29\(^e\)             | 7.27 ± 0.17                 |
| CHA1201 (\textit{pycB}:Tn5) | –                         | 100\(^c\)              | 1.23\(^d\)              | 0.27\(^e\)             | 7.25 ± 0.15                 |
| None                        | +                         | 19\(^d\)               | 0.21\(^d\)              | 0.05\(^d\)             | ND                          |
| CHA0 (wild type)            | +                         | 96\(^e\)               | 1.18\(^d\)              | 0.28\(^e\)             | 7.87 ± 0.58                 |
| CHA1322 (\textit{fumA})     | +                         | 65\(^f\)               | 0.90\(^e\)              | 0.18\(^h\)             | 8.37 ± 0.66                 |
| CHA1201 (\textit{pycB}:Tn5) | +                         | 78\(^g\)               | 0.94\(^h\)              | 0.18\(^h\)             | 7.91 ± 0.59                 |

\(\textit{a}\) \textit{P. \textit{fluorescens}} strains were added at \( \approx 10^7 \) colony-forming units/g of natural soil contained within 200-ml flasks (60 g of soil/flask), after planting three 92-h-old, sterile-grown cucumber seedlings per flask. \textit{P. \textit{ultimum}} was added as a millet seed inoculum at 2.5 g/kg of soil before planting. Plants were harvested after 7 days.

\(\textit{b}\) The rhizosphere-stable plasmid pME6031 containing a tetracycline resistance determinant (44) was introduced as a selective marker into the bacterial strains to determine their root colonization capacity in natural soil.

\(\textit{c}\) Means within the same column followed by different letters are significantly different (\( p \leq 0.05 \)) according to Fisher’s protected least squares difference test. Prior to separation of means by the least squares difference test, data of the two individual experiments could be pooled following an analysis of variance of trial-by-treatment interactions.
sion (supplemental Fig. S3), and this was mirrored by ratios of more than 100:1 for fumarate, ~10:1 for succinate, and ~5:1 for 2-oxoglutarate and malate (Table 3). It thus appears that the relative pool sizes of fumarate and, to a lesser extent, succinate, 2-oxoglutarate, and malate correlate with RsmZ expression. Whether the pool sizes of these metabolites are a direct cause of differential RsmX/Y/Z expression or simply effects of a metabolic imbalance in the mutants cannot be deduced from this experiment.

**Role of the Sensor Kinases RetS, LadS, and GacS**—In *P. fluorescens* and *P. aeruginosa*, the accessory sensors RetS and LadS have negative and positive effects, respectively, on the GacS sensor (14, 30, 52). Krebs cycle intermediates might conceivably interact with RetS and LadS. However, in both retS and ladS mutant backgrounds, a *fumA* mutation still positively influenced *rsmZ-lacZ* expression, and a *pycA* mutation still negatively affected *rsmZ-lacZ* expression (supplemental Fig. S4). It is therefore unlikely that RetS and LadS sense Krebs cycle intermediates. In *Salmonella enterica*, metabolic loss of the BarA (GacS) sensor can be compensated by high concentrations of acetate in the growth medium, and it has been hypothesized that acetylphosphate derived from acetate might directly phosphorylate the SirA (GacA) response regulator (53). However, in a *P. fluorescens* gacS mutant, introduction of a *fumA* mutation did not elevate the low level of *rsmZ-lacZ* expression (data not shown), suggesting that there is no compensating bypass activation of GacA by tri-carboxylic acid compounds. Moreover, we checked that the *fumA* and *pycB* mutations had no effects on the expression of the gacS and gacA genes, as measured with translational ′lacZ′ fusions (data not shown).

**Regulation of sRNA Gene Expression by Krebs Cycle Intermediates**—*P. fluorescens* CHA0, like other fluorescent pseudomonads, grows on most Krebs cycle intermediates as the only carbon source. (An exception is oxaloacetate, which was a poor growth substrate.) We reasoned that Krebs cycle interme-

| Metabolite          | Phase | Concentration (µmol/g) |
|---------------------|-------|------------------------|
|                      |       | Wild type | *fumA*  | *pycB* |
| Hexose-P            | T     | 5.4 ± 1.0 | 4.6 ± 0.3 | 4.2 ± 0.3 |
|                     | I     | 2.0 ± 0.1 | 2.5 ± 0.5 | 1.7 ± 0.1 |
| 3-Phosphoglycerate  | T     | 1.5 ± 0.3 | 1.1 ± 0.1 | 1.1 ± 0.1 |
|                     | I     | 0.6 ± 0.1 | 0.7 ± 0.2 | 0.9 ± 0.5 |
| Phosphoenolpyruvate | T     | 1.5 ± 0.2 | 0.8 ± 0.1 | 1.2 ± 0.1 |
|                     | I     | 0.4 ± 0.1 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| Pyruvate            | T     | 9.2 ± 4.9 | 5.7 ± 0.4 | 26 ± 1  |
|                     | I     | 2.1 ± 0.2 | 4.5 ± 0.5 | 1.8 ± 0.4 |
| Citrate + isocitrate| T     | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.1 ± 0.1 |
|                     | I     | 1.6 ± 0.5 | 1.9 ± 0.3 | 0.8 ± 0.2 |
| Succinate           | T     | 7.1 ± 4.4 | 29 ± 5  | 6.4 ± 3.0 |
|                     | I     | 4.1 ± 3.3 | 12 ± 3  | 0.9 ± 0.4 |
| Fumarate            | T     | 0.4 ± 0.05| 140 ± 7 | 0.5 ± 0.05|
|                     | I     | 0.2 ± 0.05| 62 ± 16 | 0.2 ± 0.05|
| Malate              | T     | 0.1 ± 0.05| 0.3 ± 0.05| 0.3 ± 0.05|
|                     | I     | <0.1 ± 0.05| 0.2 ± 0.05| <0.05 ± 0.05|
| 2-Oxoglutarate      | T     | 2.3 ± 0.1 | 10 ± 0.6 | 1.8 ± 0.05|
|                     | I     | 0.4 ± 0.05| 0.5 ± 0.1 | 0.1 ± 0.05|

* Values outside linear range.

DISCUSSION

Our present study shows that an imbalance in the Krebs cycle can strongly change the expression of GacA-dependent sRNAs in *P. fluorescens* and hence an ecologically relevant development (i.e. the production of secondary metabolites and biocontrol factors). There is an interesting precedent for this finding. In *B. subtilis*, a major developmental process, sporulation, depends on the function of the Krebs cycle as well. In this case Krebs cycle intermediates and/or enzymes provide a signal for the phosphorylation of the transcription factor SpoOA, which is the master regulator of sporulation. Strong evidence comes from a triple mutant blocked in citrate synthase, aconitase, and isocitrate dehydrogenase; this mutant sporulates very poorly and lacks SpoOA′-P-mediated functions (54). Furthermore, there is evidence that citrate synthase function may be involved, indirectly, in morphological differentiation and antibiotic biosynthesis of *S. coelicolor*, although the mechanism by which this effect occurs is not clear (55) and that Krebs cycle function regulates adhesion and virulence factor expression in *Staphylococcus aureus* (56). Our previous finding that thiamine limita-

![FIGURE 7. Effect of Krebs cycle intermediates and other carbon sources on rsmZ-gfp expression.](image-url)

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| Metabolite | Citrate | Gluconate | Isocitrate | 2-Oxoglutarate | Succinate | Fumarate | Malate | Glycerol | Glucose | Acetate |
|------------|---------|-----------|------------|----------------|-----------|----------|--------|----------|---------|---------|
| Wild type  | 0.0 ± 0.0| 0.0 ± 0.0 | 0.0 ± 0.0  | 0.0 ± 0.0      | 0.0 ± 0.0 | 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0|
| CHA0       | 50.0 ± 5.0| 50.0 ± 5.0 | 50.0 ± 5.0 | 50.0 ± 5.0     | 50.0 ± 5.0| 50.0 ± 5.0| 50.0 ± 5.0| 50.0 ± 5.0| 50.0 ± 5.0| 50.0 ± 5.0|
| CHA0/pME7402| 0.0 ± 0.0| 0.0 ± 0.0 | 0.0 ± 0.0  | 0.0 ± 0.0      | 0.0 ± 0.0 | 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0|

* T, trophophase; I, idiophase.
tion leads to down-regulation of the Gac/Rsm pathway in P. fluorescens CHA0 may also be due to an underlying metabolic deficiency in the Krebs cycle (21). Thiamine is necessary for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities. Conceivably, the pools of Krebs cycle intermediates, such as citrate, succinate, or fumarate might be negatively affected when thiamine is limiting.

In P. aeruginosa, mutations in Krebs cycle enzymes or in enzymes replenishing the Krebs cycle can strongly affect the expression of the type III secretion system (TTSS). Pyruvate dehydrogenase (aceAB) mutants have lost TTSS function and are non-virulent in a rat model of acute pneumonia (57). A mutation that massively up-regulates histidine utilization enzymes, resulting presumably in elevated glutamate and 2-oxoglutarate pools, also shuts down the TTSS (58). By contrast, P. aeruginosa cells overexpress the TTSS when two citrate synthase isoenzymes (gltA, prpC) are blocked by mutation (59). Acetate, which can be excreted by P. aeruginosa at the end of growth under oxygen limitation (60), does not appear to act as a metabolic signal here, because the enzymes involved in the utilization of acetate via acetyl-CoA are not involved in this regulation of the TTSS (59). It is known that RsmA is a positive regulator and that GacA is a negative regulator of the TTSS (61, 62). Thus, in P. aeruginosa, the Gac/Rsm system has inverse effects on the TTSS, on the one hand, and on the production of secondary metabolites, biofilm polysaccharides, and toxic exoenzymes, on the other hand (63). Given the high degree of conservation of the Gac/Rsm pathway in P. aeruginosa and P. fluorescens (10), it is tempting to speculate that in P. aeruginosa the observed Krebs cycle-dependent control of TTSS expression (57, 59) could be mediated by RsmY and RsmZ. A reciprocal experiment cannot, unfortunately, be carried out in P. fluorescens, because strain Pf-5 of this species lacks a TTSS (15), and most probably strain CHA0 also does.

In Legionella pneumophila, there is evidence that the intracellular alarmone ppGpp influences the activity of the LetS/LetA two-component system, which is homologous to the GacS/GacA system. Stress conditions, carbon source depletion, and amino acid starvation can trigger the synthesis of ppGpp, via the SpoT and RelA enzymes (64). It is unlikely that under our experimental conditions ppGpp would play a major role in the regulation of secondary metabolism of P. fluorescens, because nutrients were not depleted during the idiophase, not even in the dilute medium used for the metabolic analysis (supplemental Fig. S3). Moreover, we did not find any relA or spoT mutants in our screens for dim mutants, although arguably our Tn5 mutagenesis was not saturating. The question of whether Krebs cycle intermediates might modulate the synthesis of the extracellular signal(s) activating GacS cannot be answered at present, because there is no precise biochemical assay for the signal(s), and signal biosynthetic genes have not yet been identified. The energy status of the P. fluorescens cells was not substantially compromised during the idiophase. This can be seen from the fact that the pool sizes of key metabolites, such as hexose-phosphates, 3-phosphoglycerate, and phosphoenolpyruvate, were only about 2-fold lower in the idiophase than in the trophophase (Table 3), whereas the induction of the rsmX/Y/Z genes was much more pronounced during this transition (Fig. 2).

From a biocontrol point of view, it is interesting to note that major root exudate components, such as succinate and fumarate, were among those carbon sources that favored rsmZ expression (Fig. 7). These carbon sources are also preferred growth substrates of fluorescent pseudomonads in general. Including these inexpensive carbon sources in the growth media and formulation mixtures of fluorescent biocontrol pseudomonads may be of practical interest for achieving optimal suppression of root diseases, and such a strategy should be preferable to genetic manipulation of the biocontrol bacteria. Although it was possible to boost the expression of the GacA-dependent sRNAs and antibiotic secondary metabolites under in vitro conditions by introducing a fumA mutation into strain CHA0, this did not result in improved biocontrol efficacy under natural soil conditions (Table 2). This was not surprising, because we had previously found that a constitutively active form of GacS led to overproduction of antifungal secondary metabolites in vitro but not to better biocontrol of Fusarium crown and root rot of tomato (42).

In conclusion, our study on P. fluorescens CHA0 and P. aeruginosa PAO shows that a regulatory link exists between primary and secondary metabolism, via Krebs cycle function and GacA-dependent sRNAs. This regulation does not involve the accessory sensors RetS and LadS but requires functional GacS and GacA. On the one hand, our findings open up new perspectives in the understanding of biocontrol mechanisms of P. fluorescens and in the application of fluorescent pseudomonads as biocontrol agents. On the other hand, our results also shed new light on the observed link between Krebs cycle function and pathogenicity in P. aeruginosa (59).

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