OPEN ACCESS Freely available online

PLOS ONE

Characterization of the Biocontrol Activity of *Pseudomonas fluorescens* Strain *X* Reveals Novel Genes Regulated by Glucose

Gerasimos F. Kremmydas, Anastasia P. Tampakaki, Dimitrios G. Georgakopoulos*

Department of Agricultural Biotechnology, Agricultural University of Athens, Athens, Greece

Abstract

Pseudomonas fluorescens strain X, a bacterial isolate from the rhizosphere of bean seedlings, has the ability to suppress damping-off caused by the oomycete *Pythium ultimum*. To determine the genes controlling the biocontrol activity of strain X, transposon mutagenesis, sequencing and complementation was performed. Results indicate that, biocontrol ability of this isolate is attributed to *gcd* gene encoding glucose dehydrogenase, genes encoding its co-enzyme pyrroloquinoline quinone (PQQ), and two genes (*sup*5 and *sup*6) which seem to be organized in a putative operon. This operon (named *sup*X) consists of five genes, one of which encodes a non-ribosomal peptide synthase. A unique binding site for a GntR-type transcriptional factor is localized upstream of the *sup*X putative operon. Synteny comparison of the genes in *sup*X revealed that they are common in the genus *Pseudomonas*, but with a low degree of similarity. *sup*X shows high similarity only to the mangotoxin operon of *Ps. syringae* pv. *syringae* UMAF0158. Quantitative real-time PCR analysis indicated that transcription of *sup*X is strongly reduced in the *gcd* and PQQ-minus mutants of *Ps. fluorescens* strain X. On the contrary, transcription of *sup*X in the wild type is enhanced by glucose and transcription levels that appear to be higher during the stationary phase. Gcd, which uses PQQ as a cofactor, catalyses the oxidation of glucose to gluconic acid, which controls the activity of the GntR family of transcriptional factors. The genes in the *sup*X putative operon have not been implicated before in the biocontrol of plant pathogens by pseudomonads. They are involved in the biosynthesis of an antimicrobial compound by *Ps. fluorescens* strain X and their transcription is controlled by glucose, possibly through the activity of a GntR-type transcriptional factor binding upstream of this putative operon.

Citation: Kremmydas GF, Tampakaki AP, Georgakopoulos DG (2013) Characterization of the Biocontrol Activity of *Pseudomonas fluorescens* Strain *X* Reveals Novel Genes Regulated by Glucose. PLoS ONE 8(4): e61808. doi:10.1371/journal.pone.0061808

Editor: Dawn Arnold, University of the West of England, United Kingdom

Received December 17, 2011; **Accepted** March 18, 2013; **Published** April 15, 2013

Funding: This work was supported by standard funds of the Greek government to the Agricultural University of Athens, which are distributed to faculty members. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dgeorga@aua.gr

Introduction

Many bacterial strains from the genus *Pseudomonas* are capable of suppressing a range of plant diseases caused by soil-borne plant pathogenic fungi, due to their ability to biosynthesize antimicrobial metabolites. Antibiotics, cyclic lipopeptides (CLPs) with antimicrobial activity, siderophores and hydrogen cyanide are the main secondary metabolites to which the biological control is attributed [1]. Regulation of the biosynthesis of these antimicrobial metabolites has been extensively studied. A wide range of environmental as well as endogenous factors control the transcription of several genes involved in the biosynthesis of antimicrobial metabolites [2,3].

Glucose is one of the environmental factors which affect the biosynthesis of secondary metabolites such as oomycin A [4], kanosamine [5], DAPG [6], pyoluteorin and pyochelin [7], prodigiosin [8], pyrrolnitrin and phenazine [9]. Recently, it has been proposed that it is gluconic acid, not glucose, that regulates the production of antimicrobial metabolites [10]. Moreover, gluconic acid has been suggested as having a direct inhibitory effect on phytopathogenic fungi sensitive to lower concentrations of the acid [11].

Gluconic acid derives from glucose by an oxidative reaction in the periplasmic space, thus affecting the pH and the availability of soluble phosphates in all glucose-containing media [12]. The oxidation of glucose to gluconic acid is catalysed by membrane-bound quinoprotein glucose dehydrogenases (Gcd) that are involved either in biocontrol of plant pathogens [10,13] or in pathogenicity of bacteria in mammals [14]. Among various quinoprotein dehydrogenase enzymes in bacteria, Gcd uses pyrroloquinoline quinone (PQQ) as an essential cofactor [15]. Genes involved in the biosynthesis of PQQ are organised in a putative gene cluster that is expressed as an operon and its transcription is regulated by various carbon sources [16]. Insertional inactivation of the PQQ biosynthetic genes has proven their significance in biocontrol [17,18,19,13] and in plant growth promotion [20].

A rise of interest in CLP metabolites has been recently noted, due to their biosurfactant, antimicrobial and phytototoxic activity [21]. The biosynthetic model of various CLPs has been fully elucidated, yet regulation for most of them is still under study [22]. Environmental factors such as pH, temperature, carbon and nitrogen sources [23,24], plant signal molecules [25] and protozoan predators [26] affect the production of CLP metabo-

lites. Also, endogenous factors like the two component regulatory system GacA/GacS [27,28,29], quorum sensing [30,31,32,33], sigma factors and Hsp [34] control the expression of several CLP biosynthetic genes.

Pseudomonas fluorescens strain X is a bacterial biocontrol agent able to suppress cucumber and sugar beet damping-off caused by *Pythium ultimum*. Suppression of damping-off by *Ps. fluorescens* strain X has been proved to be more effective over other *Pseudomonas* and *Bacillus* strains [35]. Nevertheless, its biocontrol ability has not yet, been linked to any known antimicrobial metabolites.

The aim of the present study was to elucidate the mechanism by which *Ps. fluorescens* strain X suppresses damping-off. In order to achieve this, transposon mutants of strain X were created (designated sup$^-$) which were impaired in their ability to suppress the *in vitro* radial growth of *P. ultimum*. Sup$^-$ mutants were found to carry the transposon integration in six different genes. Results indicate that genes *sup*5 and *sup*6 have key role in the biosynthesis of a antimicrobial metabolite, while genes *sup*1, *sup*2, *sup*3 and *sup*4 play a secondary role, indirectly controlling the transcription of the first two.

Results

Biochemical Characterization of *Ps. fluorescens* Strain X and Derived Mutants

Nine mutants (k36, W139, R48, B161, B91, A150, ρ26, δ40 and ρ93) were isolated out of a mutant library of 12000 derived from random Tn5 insertion mutagenesis of *Ps. fluorescens* strain X. All mutants had lost the ability to inhibit *P. ultimum* radial growth on Potato Dextrose Agar (PDA) and retained the same growth rate with the wild type (data not shown). When the wild type was incubated on minimal medium (M9) supplemented with glucose (2% w/v) as the sole carbon source, it acidified the substrate, lowering the pH from 6 to 5. On the contrary, mutants k36, W139, R48, B139, B91, A150 and ρ26 increased the pH of the medium from 6 to 8, while δ40 and ρ93 did not alter the pH (Table 1). Furthermore, cell-free filtrates of the wild type strain and the complemented mutants (described in a subsequent section) after growth in either Potato Dextrose Broth (PDB) or Luria Bertani Broth (LB), were treated overnight with proteinase K and pronase and were tested for inhibition of *P. ultimum* radial growth (Table 1). No alterations in the antimicrobial activity for any of the treated filtrates were observed (Table 1). These results indicate that the antimicrobial activity might be attributed to a molecule either of non-peptidic nature or containing non-proteinaceous aminoacids.

Cloning and Sequencing of the Suppression Genes

Southern hybridization confirmed that each of the mutants used in this study contained a single transposon insertion in the chromosome. Mutants containing more than one insertion were not analysed further (data not shown). Subsequent cloning and sequencing of the chromosomal regions flanking the transposon insertion in the selected mutants revealed that the transposon was localized in three different genomic regions. In mutants k36, W139 and R48 the transposon integration was in a 2,418 bp gene, designated *sup*1. The transposon insertion in mutants B163, B91 was in *sup*2, (2,424 bp). In mutants A150 and ρ26 the transposon was in two genes, *sup*3 and *sup*4, (276 bp and 996 bp respectively). Genes *sup*2, *sup*3 and *sup*4 were neighbouring in a genomic region of 7119 bp (Fig. 1). Finally, mutants δ40 and ρ93 had a transposon integration in genes *sup5* (777 bp) and *sup6* (348 bp) respectively. These genes were localized in a third genomic region of 6,782 bp (Fig. 2).

Identification and Characterization of the Chromosome Regions Containing the Suppression Genes

The deduced product of *sup*1 (805 amino acids; 85.9 kDa) exhibits the highest similarity (95% identical) to the putative PQQ-dependent glucose dehydrogenase (Gcd) of the fully sequenced biocontrol strain *Ps. fluorescens* SBW25 encoded by PFLU1086 (GenBank accession no. YP_002870745.1). The product of *sup*1 was 70% identical to the Gcd from the well-characterized strain *Ps. fluorescens* CHA0 (ACN53518.1), encoded by locus FJ694890 (10), as well as to the Gcd from the biocontrol strain *Ps. fluorescens* Pf-5, encoded by PFL_4916 (AAY94145.1).

The proteins encoded by the neighbouring genes *sup*2, *sup*3 and *sup*4, show high similarity to PqqF, PqqD and PqqE respectively, involved in the biosynthesis of pyrroloquinoline quinone (PQQ). Sup2 (807 amino acids; 87.9 kDa) exhibits relatively low similarity (61% identical) to the putative peptidase PqqF of *Ps. fluorescens* CHA0 (CAA60730.1) [17]. The putative product of *sup*3 (91 amino acids; 10.3 kDa) has the highest similarity (96%) with PqqD of *Ps. fluorescens* SBW25 (YP_002875096.1). The deduced product of *sup*4 (331 amino acids; 37.4 kDa) showed 99% identity to the PqqE of *Ps. fluorescens* SBW25 (YP_002875097.1). The genes *sup*5 and *sup*6 are located in a genomic region similar to the *Ps. syringae* pv.*syringae* UMAF0158 mangotoxin biosynthesis region. The deduced product of gene *sup*5 (258 amino acids; 28.7 kDa) is a protein of unknown function with highest similarity (73% identical) to the protein encoded by *orf*3 from *Ps. syringae* pv. *syringae* UMAF0158 (ABG00044.1). Interestingly, the deduced product of *sup*5 is less similar to the proteins encoded from other *Ps. fluorescens* strains. Sup5 is 69% similar to the protein encoded by Pfl01_0128 from *Ps. fluorescens* Pf0–1 (YP_345861.1) and 53% similar to the protein encoded by PFLU_0121 from *Ps. fluorescens* SBW25 (YP_002869817.1). Furthermore, *sup*6 encodes a protein (115 amino acids; 13.3 kDa) that belongs to the cupin superfamily. The cupin superfamily, whose name comes from a conserved β-barrel fold structure (deriving from the Latin term '*cupa*' that stands for a small barrel), is a functionally diverse family that comprises enzymatic and non-enzymatic members [36]. Among all *Pseudomonas* species, the only strain with a gene homologous to *sup*6 is the opportunistic human pathogen *Ps. aeruginosa* PA7. Sup6 is 58% identical to the protein encoded by PSPA7_2111 (YP_001347484.1), which bears two cupin domains (data not shown), thus structurally categorised in the bicupins group of the cupin protein superfamily.

Complementation of Sup$^-$ Mutants

Mutants k36, R48 and W139 were complemented by a 2771 bp fragment amplified with the primers set gcd1-gcd3, which included the gene *sup*1 and a 356 bp region upstream. By promoter prediction analysis of the 356 bp region upstream, a unique promoter site was found ($P=0.92$). Complemented mutants regained the ability to inhibit radial growth of *P. ultimum* on PDA and to acidify the medium like the wild type.

In order to investigate if *sup*2, *sup*3 and *sup*4 were separately involved in biological control and acidification of PDA, several DNA fragments containing parts of the PQQ genomic locus were tested for their ability to complement mutants B91, B163, ρ26 and A150. Among these, the only one which restored the suppressive phenotype in all four mutants was a 6383 bp fragment which included five genes (*sup*2, *orf*1, *orf*2, *orf*3, *sup*3 and *sup*4) and a 504 bp region upstream of *sup*2 (Fig. 1). The 6383 bp region was PCR-amplified with primers FOR2 and pqqE2. Smaller fragments of this area and individual genes tested could not restore the sup phenotype. Complemented mutants had fully restored the ability

Table 1. Characteristics of sup$^-$ mutants and complementation analysis.

Strain	Acidification[a]	*In vitro* inhibition of *P. ultimum* radial growth by PDB filtrates of bacteria	*In vitro* inhibition of *P. ultimum* radial growth by LB filtrates of bacteria	Antimicrobial activity of PDB filtrates after treatment with proteinase K and pronase[b]
X	+	+	−	+
k36/pBBRgcd1	+	+	−	+
k36	−	−	−	−
W139/pBBRgcd1	+	+	−	+
W139	−	−	−	−
R48/pBBRgcd1	+	+	−	+
R48	−	−	−	−
B163/pBBRpqqF-E	+	+	−	+
B163	−	−	−	−
B91/pBBRpqqF-E	+	+	−	+
B91	−	−	−	−
A150/pBBRpqqF-E	+	+	−	+
A150	−	−	−	−
ρ26/pBBRpqqF-E	+	+	−	+
ρ26	−	−	−	−
δ40/pBBRsupD	ND	+	−	+
δ40	ND	−	−	−
ρ93/pBBRsupD	ND	+	−	+
ρ93	ND	−	−	−

ND: Not Detected.
[a]Acidification was observed on solid minimal medium M9 supplemented with 2% w/v glucose, as described before [14].
[b]Enzyme treatment was performed for filtrates from incubation in PDB, as described before [47].
doi:10.1371/journal.pone.0061808.t001

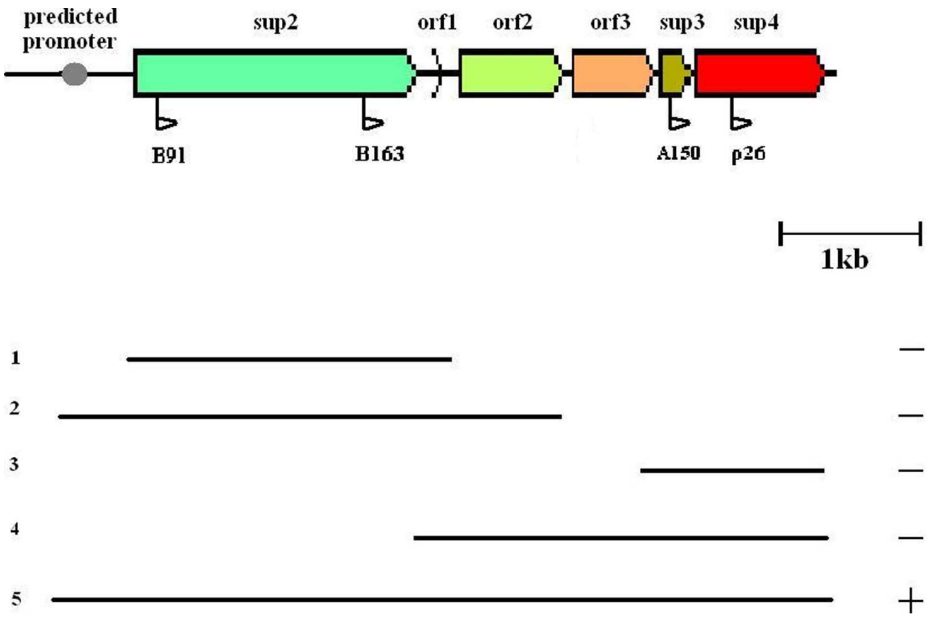

Figure 1. Complementation analysis of the PQQ biosynthesis region in *Ps. fluorescens* strain X. PCR fragments of this region (1–5), with different sets of genes from *Ps. fluorescens* X, and their ability to complement sup$^-$ mutants. Ability to complement is noted with plus (+) or minus (−). The direction of the plasposon Tn*5*-RL27 insertion in the derivative mutants B91, B163, A150 and ρ26 is indicated with a flag beneath the sequence. The predicted site for the unique putative promoter is also marked.
doi:10.1371/journal.pone.0061808.g001

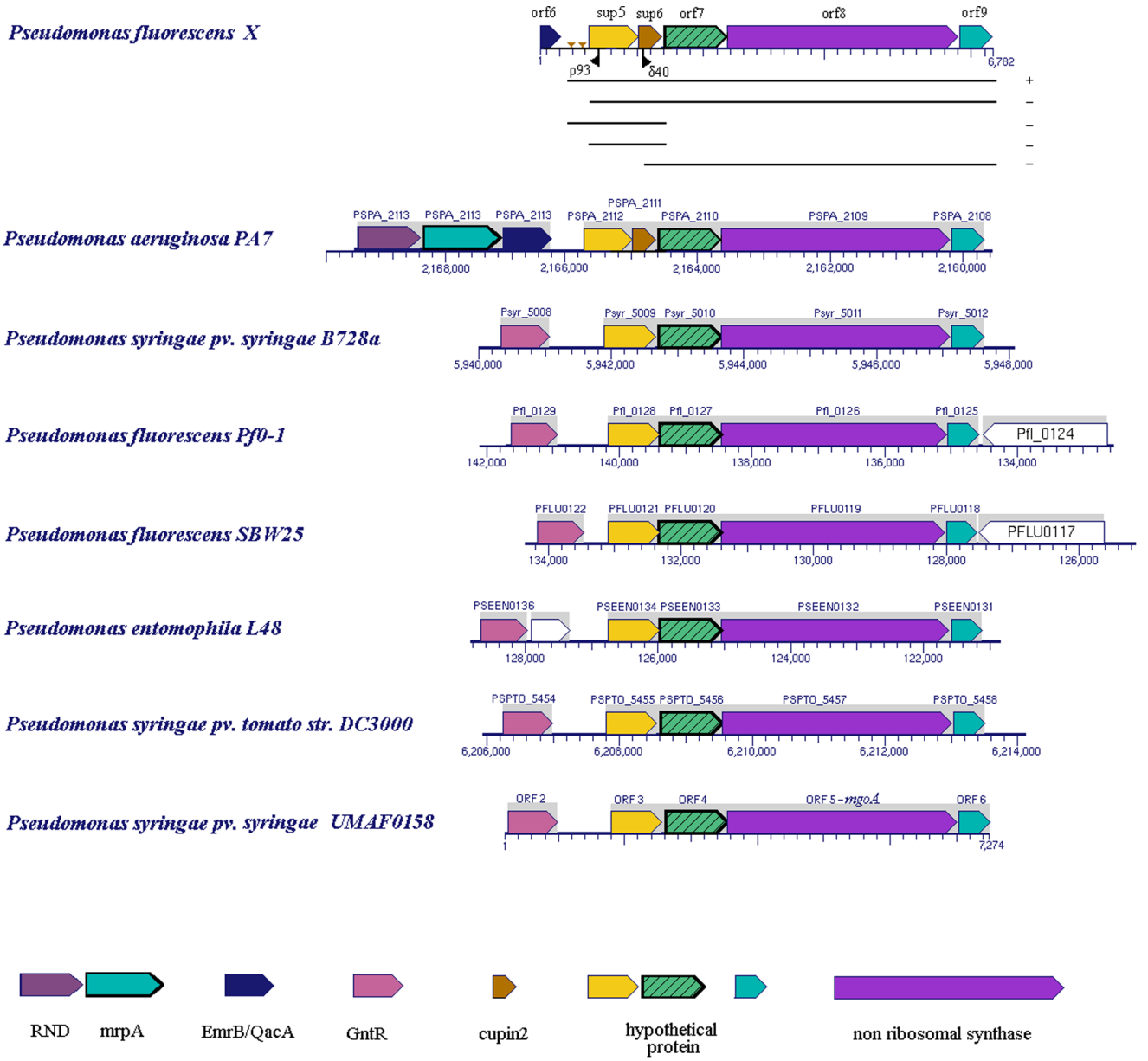

Figure 2. Arrangement of the genes in the genomic locus of *sup*5 and *sup*6, compared to other *Pseudomonas* strains, and complementation analysis of the region. The lines beneath the genomic of *Ps. fluorescens* X represent regions of this locus that were PCR-amplified, cloned into pBBR1MCS5 and tested for complementation. Ability to complement is noted with plus (+) or minus (−). Putative ORFs are indicated by thick coloured arrows on a line. Genes that might be organised in a putative operon are enclosed by a grey frame. The direction of the plasposon Tn*5*-RL27 insertion in mutants δ40 and ρ93 is indicated with a black arrow beneath the sequence (▶). Predicted sites for the unique putative promoter and operator are also marked (▼). Size, genomic location and locus tag of the different ORFs sequenced in *Ps. aeruginosa* PA7, *Ps. fluorescens* Pf0–1, *Ps. fluorescens* SBW25, *Ps. entomophila* L48, *Ps. syringae* pv. *syringae* B728a,*Ps. syringae* pv. *tomato* DC3000 and *Ps. syringae* pv. *syringae* UMAF0158 are indicated.
doi:10.1371/journal.pone.0061808.g002

to inhibit the radial growth of *P. ultimum* on PDA and to acidify the medium.

Complementation of sup$^-$ mutants δ40 and ρ93, which bear a transposon integration in genes *sup*5 and *sup*6 respectively, could only be achieved by a 6,496 bp region which includes five genes (*sup*5, *sup*6, *orf*8, *orf*9 and *orf*7) and the 423 bp region upstream of *sup*5. Smaller DNA fragments containing only parts of this 6,496 bp region did not complement the sup- mutations in mutants δ40 and ρ93 (Fig. 2). The region that complemented the sup phenotype was PCR-amplified from *Ps. fluorescens* strain X genomic DNA with the primer set supFor-supRev.

Analysis of the *sup5* and *sup6* Genomic Locus

The genomic locus containing genes *sup*5 and *sup*6 has not been previously implicated in the biocontrol activity of any bacterial antagonists of plant pathogens. Therefore, we studied this locus in more detail. By primer walking upstream and downstream of the transposon insertion, a total of 6,782 bp was cloned and sequenced from the region flanking these two genes in strain X.

Several ORFs (open reading frames) were identified (Fig. 2), including a non-ribosomal peptide synthase (NRPS), *orf*8; a polyketide cyclase/dehydrase, *orf*9; a protein of unknown function, *orf*7; and a partial sequence of a *Emr*B/*Qac*A family drug resistance transporter, *orf*6 (data not shown). The organization of these genes in strain X is similar to the homologous loci of all fully sequenced *Pseudomonas* strains (Fig. 2) and shows high similarity with the locus attributed to mangotoxin biosynthesis by *Ps. syringae* pv. *syringae* UMAF0158 [37]. A detailed analysis of the 1,158 amino acids from the deduced product of the *orf*8 gene determined to contain a sole module [38,39] consisting of an adenylation, a thiolation, a condensation and a reductase domain (no other domain was detected). The domains and the conserved core motifs of the NRPS encoded by *orf*8 had minimal differences with those from MgoA of the strain UMAF0158 (data not shown).

Prediction analysis of sites for putative promoters resulted in the presence of a unique promoter upstream of *sup*5 ($P = 0.96$). The uniqueness of this putative promoter implies that the genes downstream might be transcribed together. Also, data resulting from the synteny search of this region revealed the existence of an operon consisting of four to five genes (Fig. 2), depending on the strain.

In this genomic locus and in the region upstream of the putative four-gene operon, most *Pseudomonas* strains examined in this study (except *Ps. aeruginosa* PA7) have a gene encoding a putative transcription factor of the GntR family. Since sequencing of the *Ps. fluorescens* strain X chromosome has not been completed for this area, it is not known whether this transcription factor exists in this strain. The potential existence of a gene encoding this transcription factor in strain X may be an indication that this factor may regulate the transcription of *sup*X in *Ps. fluorescens* strain X. Analysis of the region between the site of the unique putative promoter and the gene *sup*5, revealed a putative operator site belonging to transcriptional factors of the *Fad*R subfamily of GntR transcriptional regulators, with the sequence GAGTGGTCAGCGTTAAC. The consensus sequence for *Fad*R operators is AACTGGTCNGACCAGTT [40]. The existence of a putative operator site in the particular region, suggests that a factor of the *Fad*R subfamily might control the transcription of the genes downstream. The region containing the site of the putative promoter and the putative operator site of the transcriptional factor extends 423 bp upstream of the putative operon *sup*X (Fig. 2).

Expression of *sup*5, *sup*6 and *orf*8 in the Wild Type and in Mutants ρ26 and k36

Quantitative gene expression analysis was performed to study the expression levels of genes *sup*5, *sup*6 and *orf*8 during growth of the wild type strain X and two selected mutants (k36, mutation in gene *sup*1 for gcd and ρ26, mutation in gene *sup*4 for PQQ) in two different liquid media (LB and PDB) during the exponential and stationary phases of growth. The media were selected because strain X inhibits *P. ultimum* radial growth on PDA but not on LA. We decided to study the expression of genes *sup*5 and *sup*6 because they are involved in the biocontrol activity of strain X, as shown by mutagenesis and complementation. We also decided to study the expression of *orf*8, a gene in the same putative operon, because it encodes a non-ribosomal peptide synthase, a class of genes responsible for the biosynthesis of antimicrobial compounds which suppress plant pathogens.

RNA from cells of the wild type and mutants was isolated from an identical stage of their growth, based on standard growth curves and a specific optical density at mid-exponential ($OD_{600} = 0.6$) and stationary ($OD_{600} = 1$) phase.

During the mid-exponential phase, transcription levels of *sup*5, *sup*6 and *orf*8 in the wild type were low and very similar in both media (Fig. 3E, 3F). At the stationary phase, transcription of *sup*X genes was low when the wild type strain was grown in LB, but much higher in PDB (Fig. 3A, 3B), in correlation with the production of an antifungal compound in PDA and the resulting reduction of radial growth. These genes exhibit the highest expression levels during the stationary phase in strain X, much like most bacterial genes responsible for the biosynthesis of secondary metabolites known to suppress plant pathogens.

Expression of *sup*5, *sup*6 and *orf*8 in the two mutants compared to the wild type was significantly decreased during the stationary phase, when bacteria were grown in PDB (Fig. 3C, 3D). Mutants ρ26 and k36 exhibited similar expression levels of genes *sup*5, *sup*6 and *orf*8 during stationary and mid-exponential phase in both nutrient media (data not shown). These results indicate that the insertional mutations in genes *gcd* and *pqq*E, which consequently result in an inactive glucose dehydrogenase, decreased the transcription of genes *sup*5, *sup*6 and *orf*8 which are responsible for the biocontrol activity of *Ps. fluorescens* strain X.

Discussion

To elucidate the mechanism of antagonism towards the oomycete *P. ultimum*, nine mutants of the bacterial antagonist *Ps. fluorescens* strain X deficient in the *in vitro* inhibition of *P. ultimum* radial growth were isolated after mutagenesis with the miniplasposon Tn5-RL27. Sequencing of the DNA areas flanking the transposon insertion and complementation of the mutated genes showed that three separate genomic regions are involved in the biological control traits of strain X. The biocontrol ability of *Ps. fluorescens* strain X depends on glucose dehydrogenase, its co-factor PQQ, and the proteins encoded by two additional genes (*sup*5 and *sup*6). A gene for a non-ribosomal peptide synthase (NPRS) which is located together with *sup*5 and *sup*6 in the same putative operon may also be responsible for the biocontrol activity of strain X, since NRPSs are known to synthesize antimicrobial compounds involved in the suppression of plant pathogens *in planta*.

In previous studies it has been reported that biological control traits of bacterial antagonists, such as the production of antifungal compounds, are intimately related to Gcd and its cofactor PQQ, but not in the same way for every strain. A Tn5 insertion mutant of *Ps. fluorescens* CHA0, in which the transposon had integrated in one of the PQQ biosynthetic genes (*pqq*F), showed increased production of pyoluteorin (PLT) and decreased production of 2,4-diacetylphloroglucinol (DAPG) [17]. The authors of this paper concluded that the increase in the PLT production was caused by the Tn5 integration, while the loss of DAPG production was attributed to a second spontaneous mutation that led to subsequent loss of the ability to suppress black root rot in tobacco plants and take-all in wheat. Moreover, a mutant of CHA0 carrying an in-frame deletion in the gene encoding Gcd had lost the ability to produce organic acids and to solubilise inorganic phosphate, but exhibited increased production of the antifungal metabolites DAPG and PLT. Consequently, the Δ*gcd* mutant was more effective in the biological control of *G. graminis* var. *tritici* [10]. A similar study was performed to characterize the antagonistic activity of *Rahnella aquatilis* HX2. Insertional mutations in *gcd* and *pqq*E as well as the Δ*gcd* in-frame deletion mutant were impaired in acidification of the medium and in the production of an antibacterial substance, resulting in reduced biological control of grapevine crown gall [13]. This finding shows a different effect of the same mutations in this strain. In the biocontrol strain *Serratia marcescens* W1 the genomic region of the PQQ biosynthetic genes

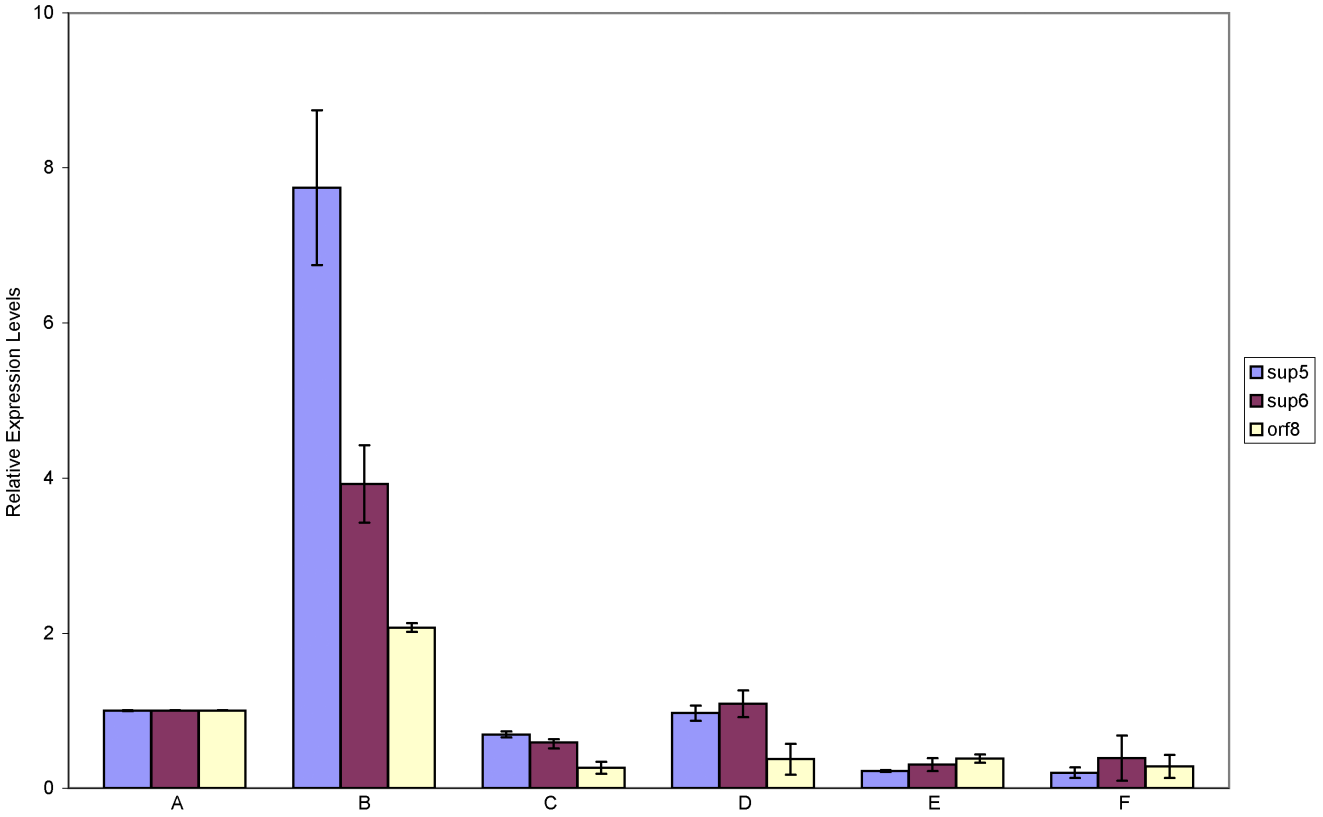

Figure 3. Expression levels of *sup*5, *sup*6 and *orf*8 in cells of *Ps. fluorescens* strain X and the mutants ρ26 and k36. The expression of *sup*5, *sup*6 and *orf*8 was measured using *rpo*D as the housekeeping gene standard. Results are shown as relative expression levels compared to the expression in the wild type in LB during stationary phase. (A) Expression in the wild type grown in LB, at stationary phase; (B) Expression in the wild type grown in PDB, at stationary phase; (C) Expression in mutant k36 grown in PDB, at stationary phase; (D) Expression in mutant ρ26 grown in PDB, at stationary phase; (E) Expression in the wild type grown in LB, at exponential phase; (F) Expression in the wild type strain grown in PDB, at exponential phase. For each time point, mean values of three replicates are given; the error bars represent the standard errors of the mean.
doi:10.1371/journal.pone.0061808.g003

was functionally expressed in *E. coli*. The transformed strain gained the ability to inhibit the *in vitro* radial growth of *Magnaporthe grisea* and *Cercospora citrullina* [18].

In *Ps. fluorescens* strain X, mutational inactivation of genes encoding PQQ biosynthesis proteins or Gcd resulted in loss of the *in vitro* inhibition of *P. ultimum*. This result is similar to the report on *R. aquatilis* HX2 [13] but different from the findings on *Ps. fluorescens* CHA0 [17,10]. In accordance with the results from both HX2 and CHA0, are the results presented for the *Ps. fluorescens* strain X mutants impaired in the acidification of PDA, a glucose-containing medium (Table 1). Loss of the acidification ability in PQQ or Gcd mutants has been attributed to lack of oxidation of glucose to gluconic acid, a reaction catalysed by membrane-bound PQQ-dependant Gcd [10]. Although it has been suggested that gluconic acid is the key molecule for the biocontrol activity of strains AN5 and CHA0 [11,10], the exact mechanism of biocontrol remains undefined in these strains.

The study of the mutants *Ps. fluorescens* δ40 and ρ93 was intriguing due to the similarity of the genes disrupted by the Tn5 insertion (*sup*6 and *sup*5) and the flanking region with the homologous genomic locus in *Ps. syringae* pv. *syringae* UMAF0158, which is involved in the biosynthesis of mangotoxin [37]. The putative product of *sup*5 was found to be similar to ABG00044.1, the product of *orf*3 in the strain UMAF0158, while *sup*6 was absent from the region involved in mangotoxin biosynthesis in the strain UMAF0158 [37]. Among all fully sequenced *Pseudomonas* strains, the only strain with a gene homologous to *sup*6 is the opportunistic human pathogen *Ps. aeruginosa* PA7. However, the putative protein encoded by *sup*6 exhibits relatively low aminoacid similarity (58%) with protein YP_001347484.1 from strain PA7, whose function is unknown.

Interesting findings were obtained from the *in silico* analysis of the genomic region in which *sup*5 and *sup*6 are localized. Synteny search concluded that these genes seem to be organized in a putative operon, designated *sup*X, together with genes *orf*7, *orf*8 (which encodes a NRPS) and *orf*9. The homologous genes in strain UMAF0158 have also been suggested to be organised in an operon [37]. From the synteny search, we also located a gene encoding a transcriptional regulator of the GntR family, upstream of this putative operon in most *Pseudomonas* strains. We do not yet have sequence data of the region upstream of *sup*X in *Ps. fluorescens* strain X, therefore we cannot confirm the presence of a GntR transcriptional regulator in this strain. However, we found a putative FadR operator site in this region, which is recognised by a transcriptional factor of the GntR family. Future work will be to sequence more of the region upstream of *sup*X and to determine the existence of a GntR-type transcriptional regulator, its operator site and the type of regulation on the transcription of the putative operon *sup*X.

The family of GntR transcriptional regulators is divided into four major subfamilies (FadR, HutC, MocR, and YtrA). DNA-binding domains, operator sequences [41,40], as well as functions

and types of regulation [41,42] for each subfamily have been previously described. It has been demonstrated that glucose and gluconic acid hold a key role in the inactivation of regulators belonging to this superfamily [43]. Moreover, glucose inactivates *agl*3R, a GntR transcriptional repressor in *Streptomyces coelicolor*, resulting in the expression of genes involved in the ABC excretion system and antibiotic biosynthesis [44]. On the contrary, in *Serratia sp. ATCC 39006* gluconic acid inactivates the PigT transcriptional regulator, of the GntR superfamily, preventing the expression of the prodigiosin biosynthetic genes [8].

The existence of a putative FadR operator site upstream of the putative operon *sup*X is a possible indication that transcription of *sup*X might also be regulated by a factor of the GntR family. Thus, trascriptional regulation of *sup*X might be subjected to regulation by glucose, possibly through gluconic acid, the product of the catalytic action of Gcd on glucose. In order to investigate whether this hypothesis is valid, we tested the expression of three genes of the putative operon *sup*X in the wild type and in two mutants (ρ26 and k36) with deficient Gcd activity. Transcription of *sup*X correlated with the *in vitro* inhibition data of *P. ultimum* by strain X, since elevated transcription levels of *sup*5, *sup*6 and *orf*8 in the wild type were observed in PDB and not in LB. (Table 1). Expression analysis of *sup*5, *sup*6 and *orf*8 in mutants ρ26 and k36 (impaired in Gcd activity) supports the role of glucose on the antagonistic properties of strain X. Transcript levels of all three genes were significantly decreased in these mutants. The decrease was evident during the mid-exponential and stationary phase in both nutrient media tested. Finally, the expression of *sup*X was elevated during stationary phase, a typical condition during the biosynthesis of secondary metabolites. Together with the mutational and complementation analysis of *sup*X, this demonstrates that the putative operon *sup*X is responsible for the antagonistic activity of *Ps. fluorescens* strain X through the biosynthesis of an antimicrobial secondary metabolite (the chemical characterization of this metabolite is in progress).

The exact role of *sup*5 and *sup*6 in the biosynthesis of the antimicrobial metabolite is currently under investigation. Our primary hypothesis suggests that *sup*6, contributes either to the final folding of the metabolite or in the transcriptional regulation of the genes in the operon *sup*X, due to its high similarity to the functionally diverse cupin superfamily [36]. The product of *sup*5, which exhibits similarity to a heme-oxygenase-like protein, may catalyse the oxidation of the peptide chain synthesized by the NRPS encoded by *orf*8. Since the putative operon *sup*X includes a NRPS, the novel metabolite might be of peptidic nature. The results of the protease treatment of the filtrates (Table 1) do not necessarily contradict this hypothesis. Resistance of a peptidic antimicrobial compound to protease may be due to the presence of non-proteinaceous amino acids and D-amino acids, or the lack of optimum conditions for protease and proteinase cleavage (pH, final concentration of the enzyme or the antimicrobial molecule, denaturing buffer).

The results of this study demonstrated that the putative operon *sup*X is related for the first time with the biological control of a soil-borne phytopathogen by a *Ps. fluorescens* strain. Genes with some homology to those in *sup*X exist in other pseudomonads as well, including known strains with biocontrol activity, as well as plant and human pathogens. However, these genes have never been related to the biocontrol of plant pathogens before, so their role in strain X is a novel finding. Ongoing and future work will focus on the characterization of the antimicrobial compound(s) produced by strain X, the regulation of the biosynthesis of this compound by glucose and the exact role of the genes in *sup*X in the biosynthesis of this antimicrobial metabolite.

Experimental Procedures

Bacterial Strains, Plasmids, and Mutagenesis

All bacterial strains and plasmids used in this study are listed in Table 2. *Ps. fluorescens* strain X was grown at 28°C, and *E. coli* strains was grown at 37°C. All strains were routinely grown on LB agar, unless otherwise stated. A spontaneous derivative of *Ps. fluorescens* strain X resistant to rifampicin was used for transposon mutagenesis. This derivative had identical biochemical, growth and biocontrol characteristics with the wild type (data not shown). Transposon mutagenesis of *Ps. fluorescens* strain Xrif was carried out by triparental mating using the mini plasposon Tn-RL27, as described previously [45] and *E. coli* HB101 carrying plasmid pRK2013 [46] as the helper. This mini plasposon carries the origin of replication from plasmid R6K (*ori*R6K) to allow cloning of transposon insertion sites. Derivative strains were isolated after 48 h at 28°C on King's B medium under selection of kanamycin (100 μg/ml) and rifampicin (40 μg/ml). In total, a library of *Ps. fluorescens* strain Xrif mutants containing over 12000 strains was constructed.

Biochemical Characterization of *Ps. fluorescens* Strain X Mutants

The growth of *Ps. fluorescens* strain Xrif mutants and their ability to suppress fungal growth was tested with dual cultures on PDA at 23°C. After a first screening, potential mutants were further tested in three replicate plates together with the wild-type to confirm the loss of *P. ultimum* growth inhibition. Loss of suppression of fungal growth was assessed 2 days later.

Filtrates of strain X and selected mutants grown in PDB and LB 48 h at 23°C were tested for growth inhibition of *P. ultimum* as previously described [35].

Enzymatic treatment by pronase and proteinase K, of the filtrates was performed according to Arrebola *et al.* [47]. Observations of the culture pH were performed using different pH indicators [14].

DNA Manipulation and Sequencing

DNA digestion, ligation reactions, and transformation of *E. coli* were performed according to standard protocols [48]. Genomic DNA isolation was performed using the GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich Co. LLC., Germany). Plasmid mini-preps were done using the Qiaprep spin miniprep and midiprep kit (Qiagen GmbH, Düsseldorf, Germany). For sequence analysis of the regions flanking the miniplasposon insertions, published primers were used [45]. Automated DNA sequencing of rescue plasmids was carried out by Macrogen Inc. (Korea) and VBC-Biotech Service GmbH (Austria).

Isolation and Characterization of Genomic Loci Carrying a Transposon Insertion

Plasmid rescue was performed to clone the genomic locus of the insertion in every mutant. Genomic DNA was digested with *BamH*I and subsequently treated with T4 DNA ligase. The ligation mix was transformed into *E. coli* DH5α/λpir, where circularized fragments containing the transposon replicate as plasmids, allowing the selection only of chromosomal fragments containing the transposon [45]. Southern blot analysis was performed (DIG High Primer DNA labelling and detection starter kit^{TM}, Roche Applied Science, Germany) in *BamH*I-digested genomic DNA from the selected mutants using the Tn-RL27 as probe to determine the uniqueness of the insertion in the genome and

Table 2. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strain, plasmid, or oligonucleotide	Characteristics or sequence (5′→3′)	Reference and/or source
E. coli		
DH10b	F^-, mcrA, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80d*lac*ZΔM15, Δ*lac*X74, *end*A1 *rec*A1, *deo*R, Δ(*ara*,*leu*)7697 *ara*D139, *gal*U, *gal*K, *nup*G, *rps*L, λ^-	[54]
DH5*α*/λpir	*sup E44*, Δ*lac*U169 (F80*lac*ZDM15), *rec*A1, *end*A1, *hsd*R17, *thi*-1, *gyr*A96, *rel*A1, λpir phage lysogen	[55]
HB101	*hsd*R, recA,proA,leu-0,ara-l4 gaiK2, *lac*Yl, xyl-5, mtl-1 str-2, *thi*-1, *sup*E44	[48]
Ps. fluorescens		
X	wild type	[35]
Xrif	Rif^R (spontaneous mutant)	This study
A150	Xrif derivative, sup3::Tn5-RL27 Km^R, sup^-	This study
B91	Xrif derivative, sup2::Tn5-RL27 Km^R, sup^-	This study
B163	Xrif derivative, sup4::Tn5-RL27 Km^R, sup^-	This study
ρ93	Xrif derivative,, sup5::Tn5-RL27 Km^R, sup^-	This study
ρ26	Xrif derivative, sup4::Tn5-RL27 Km^R, sup^-	This study
k36	Xrif derivative, sup1::Tn5-RL27 Km^R, sup^-	This study
R48	Xrif derivative, sup1::Tn5-RL27 Km^R, sup^-	This study
W139	Xrif derivative, sup1::Tn5-RL27 Km^R, sup^-	This study
δ40	Xrif derivative, sup6::Tn5-RL27 Km^R, sup^-	This study
Plasmids		
pBBRgcd1*	pBBR1MCS5/sup1	This study
pBBRgcd2	pBBR1MCS5/sup1	This study
pBBRpqqF	pBBR1MCS5/sup2	This study
pBBRpqqE	pBBR1MCS5/sup3	This study
pBBRpqqD	pBBR1MCS5/sup4	This study
pBBRpqqDE	pBBR1MCS5/sup3–4	This study
pBBRpqqFA*	pBBR1MCS5/sup2-orf1	This study
pBBRpqqFAB*	pBBR1MCS5sup2-orf1-orf2	This study
pBBRpqqABCDE	pBBR1MCS5/orf1–3,sup3–4	This study
pBBRpqqF-E*	pBBR1MCS5/sup2–4	This study
pBBRsup5–6	pBBR1MCS5/sup5–6	This study
pBBRsupA	pBBR1MCS5/sup6-orf7-orf8-orf9	This study
pBBRsupD*	pBBR1MCS5/sup5-sup6-orf7-orf8-orf9	This study
pRK2013	IncP-I, *tra*RK2+, *rep*RK2, *rep*E1 Km^R	[46]
pRL27	vector of Tn5-RL27 (Km^R-oriR6 K)	[45]
Oligonucleotides		
gcd1**	TCGggtaccTGAGCATTGCGTTCGCGTGAC; *Kpn*I	This study
gcd3	TCGtctagaCGCCAGCGTTGCTTAATCTG; *Xba*I	This study
FOR2	TTTGGgaatccTGACCACTCGATGTTCAGC; *Eco*RI	This study
pqqE2	GTACATCATCgaatccCGTTGAGGCGCTCA; *Eco*RI	This study
supFor	CAGCtctagaGGGAACTTGATGG; *Xba*I	This study
supRev	GCCTCCGCCTGCtctagaTATGTC; *Xba*I	This study
δ40α	CAGTGcGTGCGTcatatgAACTTCGAAGTG; *Nde*I	This study
δ40β	CCTTtctagaTGATACTCAGTAGTAGTGC; *Xba*I	This study
ρ93α	GATAAGGAGCGCcatatgGAAGATAAAAAG; *Nde*I	This study
rpoDf	GATTCGTCAGGCGATCAC	This study
rpoDr	AATACGGTTGAGCTTGTTGA	This study
rpoBf	ATCCGCAAGGACTTTAGC	This study
rpoBr	GGATAGCCAGAAGGTACG	This study
sup6f	ACGGTAGTTACTTCTTCAG	This study

Table 2. Cont.

Strain, plasmid, or oligonucleotide	Characteristics or sequence (5′→3′)	Reference and/or source
sup6r	CTTCAAACAACAGGCATC	This study
sup5f	AAATCATCCTGGGCGAAG	This study
sup5r	CGAAGTGGCTGTAGTGAC	This study
orf8f	ACTATCCGTCGTGTCATCA	This study
orf8r	AAACATCACTCGCATCGTTA	This study

*includes the sequence of the putative predicted promoter.
**In the nucleotide sequences, restriction enzyme sites are shown in lowercase letters.
doi:10.1371/journal.pone.0061808.t002

determine the size of the genomic fragments containing the transposon element.

Complementation of Mutants

Complementation of the wild type phenotype was accomplished for all isolated strains. Specific primers designed for PCR amplification of the genes and loci in which the transposon had integrated, are listed in Table 2. Amplification of genomic loci from genomic DNA of the wild type, in which the insertion was localized in the sup$^-$ mutants, was possible using the Expand Long Range dNTPack (Roche Diagnostics Gmbh, Germany). The broad host range vector pBBR1MCS5, carrying the gene for gentamycin resistance, was used for cloning single genes or genomic loci [49]. The plasmids that were constructed were transformed into *E. coli* DH10b and were subsequently inserted into the respective mutants by triparental conjugation.

Transcriptional Analysis

For the transcriptional analyses, RNA was isolated from bacterial cells according to standard procedures [48], followed by DNase I (Promega) treatment. Total RNA was extracted from *Ps. fluorescens* strain Xrif and two mutant strains k36 and ρ26 grown in PDB and LB medium, at midlog and stationary phase. The concentration and purity of the RNA samples were measured by using an ND100 spectrophotometer (Nanodrop Technologies, USA) according to the manufacturer's protocols. RT-PCR was performed by the SYBR® FAST One-Step qRT-PCR Kit Universal (Kapa Biosystems, USA). In every reaction, 50 ng of total RNA was used, according to the manufacturer's protocol. Real-time quantitative PCR (Q-PCR) was conducted with the Mx3005P^{TM} system from Stratagene (USA). The concentration of the primers was optimized (200 nM final concentration for the all genes analysed) according to the manufacturer's technical data sheet. The primers used for the Q-PCR were chosen with the help of the Beacon Designer v 9.1software and are listed in Table 2. Control reactions in which cDNA synthesis was circumvented, ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination.

Normalization of the results was performed by using *rpo*D as the housekeeping gene [50]. The *rpo*D gene was used to provide an internal control cDNA that was amplified with oligonucleotides rpoDf/rpoDr (Table 2) and used to normalize the sample data. After the PCR, a melting curve was generated to confirm the amplification of a single product. The cycle in which the SYBR green fluorescence crossed a manually set cycle threshold (CT) was used to determine transcript levels. For each gene, the threshold was fixed based on the exponential segment of the PCR curve. Relative transcript levels of genes of interest were analyzed using the 'Delta-delta method' as described previously [51]. Q-PCR analysis was performed in three (technical) replicates on two independent RNA isolations (biological replicates).

Bioinformatics

Database searches were performed with the BLAST 2.0 service of the National Center for Biotechnology Information (Bethesda, MD, U.S.A.). Amino acid sequences were aligned with MultAlin [52]. Synteny analysis was performed by using the BioCyc Database [53]. The putative promoter site prediction was performed with two bioinformatic applications, BPROM software (Softberry Inc., Mount Kisco, NY, U.S.A.) and NNPP 2.2 software (Berkeley Drosophila Genome Project-BDGP, Berkeley, CA, U.S.A.). Analysis of the cores and domains of NRPS, translated by *orf*8, was accomplished according to previous work [38,39]. Primer design for PCR and RT-PCR was carried out using DNASTAR and BeaconDesign, respectively.

Accession Numbers

The nucleotide and deduced amino acid sequences for the *sup*1 gene, encoding the PQQ-dependent Gcd in *Ps. fluorescens* strain *X*, has been deposited in the GenBank database under accession no. HQ383687. The nucleotide and deduced amino acid sequences for the *sup*2, *sup*3 and *sup*4 gene, encoding the PQQ biosynthesis proteins F, D and E, as parts of the flanking region in *Ps. fluorescens* strain X, have been deposited in the GenBank database under accession no. JQ039398. The nucleotide and deduced amino acid sequences of the genes located in the *sup*X putative operon, as well as parts flanking this region, have been deposited in the GenBank database under accession no. JQ039399.

Acknowledgments

We thank Christos Fotiadis, Chrysanthi Kalloniati, Athina Andriopoulou, Nikos Delibaltadakis, Christina Merakou and Tina Semenic for valuable technical assistance. We are grateful to Steve Lindow (UC Berkeley, USA) and the Benaki Phytopathological Institute (Kifissia, Greece) for providing some of the microorganisms and plasmids used in this study. This work is dedicated to the memory of Professor Nikos Malathrakis (TEI Crete, Greece).

Author Contributions

Conceived and designed the experiments: DGG GFK. Performed the experiments: GFK. Analyzed the data: GFK. Contributed reagents/materials/analysis tools: APT. Wrote the paper: GFK DGG.

References

1. Gross H, Loper EL (2009) Genomics of secondary metabolite production by *Pseudomonas spp*. Nat Prod Rep 26: 1408–1446.
2. Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81: 537–547.
3. Ramos JL (2004) *Pseudomonas* (volume 3): Biosynthesis of macromolecules and molecular metabolism. NewYork: Kluwer Academic/Plenum Publishers. 740 p.
4. Gutterson NI, Layton TJ, Ziegle JS, Warren GJ (1986) Molecular cloning of genetic determinants for inhibition of fungal growth by a fluorescent pseudomonad. J Bacteriol 165: 696–703.
5. Milner JL, Silo-Suh L, Lee JC, He HY, Clardy J, et al. (1996) Production of kanosamine by *Bacillus cereus* UW85. Appl Environ Microbiol 62: 3061–3065.
6. Shanahan P, O'Sullivan DJ, Simpson P, Glennon JD, O'Gara F (1992) Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl Environ Microbiol 58: 353–358.
7. Duffy BK, Défago G (1999) Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl Environ Microbiol 65: 2429–2438.
8. Fineran PC, Everson L, Slater H, George PC, Salmond GPC (2005) A GntR family transcriptional regulator (PigT) controls gluconate-mediated repression and defines a new, independent pathway for regulation of the tripyrrole antibiotic, prodigiosin, in *Serratia*. Microbiology 151: 3833–3845.
9. Park JY, Oh SA, Anderson AJ, Neiswender J, Kim JC, et al. (2011) Production of the antifungal compounds phenazine and pyrrolnitrin from *Pseudomonas chlororaphis* O6 is differentially regulated by glucose. Lett Appl Microbiol 52: 532–537.
10. de Werra P, Pechy-Tarr M, Keel C, Maurhofer M (2009) Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol 75: 4162–4174.
11. Kaur R, Macleod J, Foley W, Nayudu M (2006) Gluconic acid: An antifungal agent produced by *Pseudomonas* species in biological control of take-all. Phytochemistry 67: 595–604.
12. James DW Jr, Gutterson NI (1986) Multiple antibiotics produced by *Pseudomonas fluorescens* HV37a and their differential regulation by glucose. Appl Environ Microbiol 52: 1183–1189.
13. Guo YB, Li J, Li L, Chen F, Wu W, et al. (2009) Mutations that disrupt either the pqq or the gdh gene of *Rahnella aquatilis* abolish the production of an antibacterial substance and result in reduced biological control of grapevine crown gall. Appl Environ Microbiol 75: 6792–6803.
14. Pujol CJ, Kado CI (1999) *gdh*B, a gene encoding a second quinoprotein glucose dehydrogenase in *Pantoea citrea*, is required for pink disease of pineapple. Microbiology 145: 1217–1226.
15. Felton LM, Anthony C (2005) Biochemistry: Role of PQQ as a mammalian enzyme cofactor?. Nature 433: E10–11.
16. Gliese N, Khodaverdi V, Gorisch H (2010) The PQQ biosynthetic operons and their transcriptional regulation in *Pseudomonas aeruginosa*. Arch Microbiol 192: 1–14.
17. Schnider U, Keel C, Voisard C, Défago G, Haas D (1995) Tn5-directed cloning of pqq genes from *Pseudomonas fluorescens* CHA0: mutational inactivation of the genes results in overproduction of the antibiotic pyoluteorin. Appl Environ Microbiol 61: 3856–3864.
18. Kim YH, Kim CH, Han SH, Kang BR, Cho SM, et al. (2006) Expression of pqq genes from *Serratia marcescens* W1 in *Escherichia coli* inhibits the growth of phytopathogenic fungi. Plant Pathol J 22: 323–328.
19. Han SH, Kim CH, Lee JH, Park JY, Cho SM, et al. (2008) Inactivation of pqq genes of *Enterobacter intermedium* 60–2G reduces antifungal activity and induction of systemic resistance. FEMS Microbiol Lett 282: 140–146.
20. Choi O, Kim J, Kim JG, Jeong Y, Moon JS, et al. (2008) Pyrroloquinoline quinone is a plant growth promotion factor produced by *Pseudomonas fluorescens* B16. Plant Physiol 146: 657–668.
21. Raaijmakers JM, de Bruijn I, de Kock MJ (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. Mol Plant Microbe Interact 19: 699–710.
22. Nybroe O, Sorensen J (2004) Production of cyclic lipopeptides by fluorescent pseudomonads. In: Ramos JL, editor. *Pseudomonas*, Biosynthesis of Macromolecules and Molecular Metabolism. NewYork: Kluwer Academic/Plenum Publishers. 147–172.
23. Bender CL, Alarcon-Chaidez F, Gross DC (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev 63: 266–292.
24. Mukherjee AK, Das K (2005) Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. Fems Micriobiol Ecol 54: 479–489.
25. Mo YY, Geibel M, Bonsall RF, Gross DC (1995) Analysis of sweet cherry (*Prunus avium* L.) leaves for plant signal molecules that activate the *syrB* gene required for synthesis of the phytotoxin syringomycin, by *Pseudomonas syringae* pv. *syringae*. Plant Physiol 107: 603–612.
26. Mazzola M, de Bruijn I, Cohen MF, Raaijmakers JM (2009) Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*. Appl Environ Microbiol 75: 6804–6811.
27. Koch B, Nielsen TH, Sorensen D, Andersen JB, Christophersen C, et al. (2002) Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system. Appl Environ Microbiol 68, 4509–4516.
28. Nielsen TH, Nybroe O, Koch B, Hansen M, Sorensen J (2005) Genes involved in cyclic lipopeptide production are important for seed and straw colonization by *Pseudomonas* sp. strain DSS73. Appl Environ Microbiol 71: 4112–4116.
29. D'aes J, Hua GKH, De Maeyer K, Pannecoucque J, Forrez I, et al. (2011) Biological control of *Rhizoctonia* root rot on bean by phenazine and cyclic lipopeptide producing *Pseudomonas* CMR12a. Phytopathology 101: 996–1004.
30. Lu SE, Scholz-Schroeder BK, Gross DC (2002) Characterization of the *sal*A, *syr*F, and *syr*G regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. *syringae*. Mol Plant Microbe Interact 15: 43–53.
31. Cui X, Harling R, Mutch P, Darling D (2005) Identification of N-3-hydroxyoctanoyl-homoserine lactone production in *Pseudomonas fluorescens* 5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing. Eur J Plant Pathol 111: 297–308.
32. de Bruijn I, Raaijmakers JM (2009) Regulation of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens* by the ClpP Protease. J Bacteriol 190: 1910–1923.
33. de Bruijn I, Raaijmakers JM (2009) Diversity and functional analysis of LuxR-type transcriptional regulators of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens*. Appl Environ Microbiol 75: 4753–4761.
34. Dubern JF, Lagendijk EL, Lugtenberg BJJ, Bloemberg GV (2005) The heat shock genes *dna*K, *dna*J, and *grp*E are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. J Bacteriol 187: 5967–5976.
35. Georgakopoulos DG, Fiddaman P, Leifert C, Malathrakis NE (2002) Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. J Appl Microbiol 92: 1078–86.
36. Dunwell JM, Purvis A, Khuri S (2004) Cupins: the most functionally diverse protein superfamily?. Phytochemistry 65: 7–17.
37. Arrebola E, Cazorla FM, Romero D, Pérez-García A, de Vicente A (2007) A Nonribosomal peptide synthetase gene (mgoA) of *Pseudomonas syringae* pv. *syringae* is involved in mangotoxin biosynthesis and is required for full virulence. Mol Plant Microbe Interact 20: 500–509.
38. Marahiel MA, Stachelhaus T, Mootz DH (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97: 2651–2673.
39. Schwarzer D, Finking R, Marahiel MA (2003) Nonribosomal peptides: from genes to products. Nat Prod Rep 20: 275–287.
40. Kazakov AE, Rodionov DA, Alm E, Arkin AP, Dubchak I, et al. (2009) Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in Proteobacteria. J Bacteriol 191: 52–64.
41. Rigali S, Derouaux A, Giannotta F, Dusart J (2002) Subdivision of the helix-turn-helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies. J Biol Chem 277: 12507–12515.
42. Gorelic M, Lunin VV, Skarina T, Savcheinko A (2006) Structural characterization of GntR/HutC family signaling domain. Protein Sci 15: 1506–1511.
43. Tong S, Porco A, Isturiz T, Conway T (1996) Cloning and molecular genetic characterization of the *Escherichia coli gnt*R, *gnt*K, and *gnt*U Genes of GntI, the Main System for Gluconate Metabolism. J Bacteriol 178: 3260–3269.
44. Hillerich B, Westpheling J (2006) A New GntR Family Transcriptional Regulator in *Streptomyces coelicolor* is required for morphogenesis and antibiotic production and controls transcription of an ABC transporter in response to carbon source. J Bacteriol 188: 7477–7487.
45. Larsen RA, Wilson MM, Guss AM, Metcalf WW (2002) Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch Microbiol 178: 193–201.
46. Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77: 7347–7351.
47. Arrebola E, Cazorla FM, Durán VE, Rivera E, Romero OF, et al. (2003) Mangotoxin: a novel antimetabolite toxin produced by *Pseudomonas syringae* inhibiting ornithine/arginine biosynthesis. Physiol Mol Plant Path 63: 117–127.
48. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1659 p.
49. Kovach ME, Elzer PH, Hill SD, Robertson GT, Farris MA, et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175–176.
50. de Bruijn IM, de Kock JD, de Waard P, van Beek TA, Raaijmakers JM (2008) Massetolide A biosynthesis in *Pseudomonas fluorescens*. J Bacteriol 190: 2777–2789.
51. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29: e45.
52. Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16: 10881–10890.

53. Caspi R, Altman T, Dale JM, Dreher K, Fulcher CA, et al. (2009) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 38: D473–9.
54. Durfee T, Nelson R, Baldwin S, Plunkett G 3rd, Burland V, et al. (2008) The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. J Bacteriol 190: 2597–2606.
55. Miller VL, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *tox*R. J Bacteriol 170: 2575–2583.