Investigation of the quorum-sensing regulon of the biocontrol bacterium *Pseudomonas chlororaphis* strain PA23

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**Abstract**

*Pseudomonas chlororaphis* strain PA23 is a biocontrol agent capable of protecting canola from stem rot disease caused by the fungal pathogen *Sclerotinia sclerotiorum*. PA23 produces several inhibitory compounds that are under control of a complex regulatory network. Included in this cascade is the PhzRI quorum sensing (QS) system, which plays an essential role in PA23 biocontrol, as well as CsaRI and AurRI, which have not yet been characterized in PA23. The focus of the current study was to employ RNA sequencing to explore the spectrum of PA23 genes under QS control. In this work, we investigated genes under the control of the main QS transcriptional regulator, PhzR, as well as those differentially expressed in an AHL-deficient strain, PA23-6863, which constitutively expresses an AiiA lactonase, rendering the strain QS defective. Transcriptomic profiling revealed 545 differentially expressed genes (365 downregulated; 180 upregulated) in the *phzR* mutant and 534 genes (382 downregulated; 152 upregulated) in the AHL-deficient PA23-6863. In both strains, decreased expression of phenazine, pyrrolnitrin, and exoprotease biosynthetic genes was observed. We have previously reported that QS activates expression of these genes and their encoded products. In addition, elevated siderophore and decreased chitinase gene expression was observed in the QS-deficient stains, which was confirmed by phenotypic analysis. Inspection of the promoter regions revealed the presence of “phz-box” sequences in only 58 of the 807 differentially expressed genes, suggesting that much of the QS regulon is indirectly regulated. Consistent with this notion, 41 transcriptional regulators displayed altered expression in one or both of the QS-deficient strains. Collectively, our findings indicate that QS governs expression of approximately 13% of the PA23 genome affecting diverse functions ranging from secondary metabolite production to general metabolism.

**Introduction**

Certain pseudomonads are able to inhibit fungal pathogens via the production of secondary metabolites through a process known as biological control. *Pseudomonas chlororaphis* strain...
PA23 is one such organism that suppresses canola stem rot caused by the fungal pathogen *Sclerotinia sclerotiorum* [1,2]. We have established that biocontrol by this bacterium occurs through direct and indirect mechanisms. Direct pathogen inhibition results from exposure to secreted bacterial products including the antibiotics pyrrolnitrin (PRN) and phenazine (PHZ), together with HCN, chitinases, proteases, lipases and siderophores [1,3]. PA23 also exerts its effects indirectly through priming the plant defense response, enabling the plant to more effectively fend off pathogen attack [4].

A complex regulatory network governs expression of PA23 antifungal (AF) compounds. At the top of this hierarchy sits the GacS-GacA two component signal transduction system that is essential for PA23 biocontrol [5,6]. Working in concert with Gac is the Rsm system, which consists of RsmA-like translational repressor proteins and small regulatory RNAs [7]. Additional regulators overseeing production of PA23 biocontrol metabolites include the stationary phase sigma factor RpoS [8], PsrA (Pseudomonas Sigma Regulator A) [6], the stringent response (SR) [8], the anaerobic regulator ANR [9], and a novel LysR-type regulator called PtrA [10].

Adding to this complexity, PA23 AF compounds are expressed in a population-density-dependent fashion through quorum sensing (QS) [11]. Like other gram-negative bacteria, PA23 uses N-acylhomoserine lactones (AHLs) as indices of population density [11–13]. The first QS system identified in PA23 consists of the transcriptional regulator PhzR and the AHL synthase PhzI. The genes encoding these elements, *phzR* and *phzI*, are situated upstream of the *phzABCDEFG* biosynthetic locus responsible for PHZ production [11]. Characterization of a *phzR* mutant (PA23*phzR*) revealed a lack of fungal inhibition, which was attributed to reduced PHZ, PRN and protease production [11]. Likewise, a strain carrying pME6863, which encodes an AiiA lactonase [14], exhibited a similar phenotype. A second QS system called CsaRI (Cell Surface Alteration) has been identified in the closely related *P. chlororaphis* 30–84 [15]. In this strain, the Csa system is not involved in the regulation of secondary metabolites or biocontrol genes, instead, it controls cell surface properties and biofilm formation [15]. While homologs of *csaI* and *csaR* are present in the PA23 genome, the role of this QS system in PA23 remains unknown. PA23 also contains homologs of a third QS system, AurRI, which has been reported in *P. chlororaphis* subsp. *aurantiaca* PB-St2; however, it has not been characterized [16].

In *Pseudomonas aeruginosa*, global transcriptomic analysis using microarrays revealed that over 300 genes are under QS control [17,18], greatly exceeding the number identified through more targeted approaches. For the first time, the magnitude of QS control was recognized to extend well beyond virulence, regulating diverse aspects of *P. aeruginosa* physiology. In organisms that employ AHL-based QS, control is mediated in one of two ways: directly through interaction with the promoter regions of target genes, or indirectly through other regulators. For several genes in the former category, consensus sequences have been identified that are required for LuxR-AHL complex binding [19]. These “lux box-like” sequences are located in different positions depending on the gene in question [18,19]. In strain PA23, a “phz-box” sequence was identified upstream of the PHZ-biosynthetic locus as well as other genes under QS control [6,11]. We have previously established that the Phz QS system is deeply enmeshed in the complex hierarchy of gene regulation in strain PA23. Analysis of *gac* mutants revealed a lack of AHL production; consequently QS appears to be under control of this global regulatory system [5]. The PhzRI QS system is also interconnected with RpoS [11], ANR [9], and the transcriptional regulator PtrA [20]. As such, a large number of QS-regulated genes are expected to be indirectly regulated in this bacterium.

The focus of the current study was to explore the scope of genes under QS control in strain PA23 through RNA sequencing (RNA-seq). Analysis of a *phzR* mutant and an AHL-deficient derivative revealed that QS regulates approximately 13% of the PA23 genome, impacting diverse aspects of physiology ranging from secreted exoproducts to central metabolism.
Moreover the vast majority of genes in the QS regulon appear to be indirectly controlled as very few contained phz boxes in their promoter regions.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are outlined in Table 1. E. coli strains were cultured on Lennox Luria Bertani (LB) media (Difco Laboratories, Detroit, MI) at 37˚C. P. chlororaphis PA23 was cultured and maintained on LB media (Difco) at 28˚C. S. sclerotiorum was cultured and maintained on Potato Dextrose Agar (PDA; Difco) at 22˚C. Antibiotics were purchased from Research Products International Corp. (Prospect, IL) and supplemented at the following concentrations: gentamicin (Gm; 20 μg mL⁻¹), tetracycline (Tc; 15 μg mL⁻¹) for P. chlororaphis PA23 and Gm (15 μg mL⁻¹) and Tc (15 μg mL⁻¹) for E. coli. For phenotypic assays and cDNA library synthesis, strains were cultured in M9 Minimal Salts Media (M9; Difco) supplemented with 1 mM MgSO₄ and 0.2% glucose, from here on referred to as M9-Glc.

Nucleic acid manipulations

Isolation, purification, endonuclease digestion and all other manipulation of DNA was performed according to the protocols described in Sambrook et al. [21].

Table 1. Bacterial strains, plasmids and oligonucleotides sequences.

| Strains                  | Relevant genotypes                                  | Source or reference |
|-------------------------|-----------------------------------------------------|---------------------|
| **Pseudomonas chlororaphis** |                                                      |                     |
| PA23                    | PHZ⁺, PRN⁺; Rif⁺; WT (soy bean root tip isolate)    | [2]                 |
| PA23phzR                | Gm marker inserted into phzR gene                   | [11]                |
| PA23-6863               | PA23 carrying pME6863 (AHL-deficient)⁷              | [11]                |

**Oligonucleotide sequences**

| phzA RT-PCR FWD         | 5’- GACTGGCAATGGCACACAC-3’                           | [19]                |
| phzA RT-PCR REV         | 5’- GCAAATAACCTTCCGGATAACC-3’                       | [19]                |
| prnA RT-PCR FWD         | 5’- CGTGCACTGGTCTTCTG-3’                            | [19]                |
| prnA RT-PCR REV         | 5’- GTACTCGGCTGTAATCGG-3’                           | [19]                |
| hacC-FOR                | 5’- AATCTGCTCAAACGCGTCCG-3’                         | [9]                 |
| hacC-REV                | 5’- TGCCTATGTCGAGCCCTTG-3’                          | [9]                 |
| phzI RT-PCR FWD         | 5’- GCGATGCCGTTGTTCTGG-3’                           | [19]                |
| phzI RT-PCR REV         | 5’- AGCGATCTGTGAGTGGACTG-3’                         | [19]                |
| phzR RT-PCR REV         | 5’- GAATCCCTGAGCTCAACCC-3’                          | [19]                |
| rpoB RT-PCR REV         | 5’- ATCAGCGGCCCTAACTACG-3’                          | [19]                |
| rpoB RT-PCR REV         | 5’- CTGTTCCTGTCGCTATG-3’                            | [19]                |
| csaI-qpcr-F             | 5’- GCCGCAACCAATACCTAC-3’                           | This study          |
| csaI-qpcr-R             | 5’- GCGGCAACCAATACCTAC-3’                           | This study          |
| csl-qpcr-F              | 5’- CGTGCACTGGTCTTCTG-3’                            | This study          |
| csl-qpcr-R              | 5’- GCTGCACTGGTCTTCTG-3’                            | This study          |
| aurR-qpcr-F             | 5’- TTGGGCTGAGGACCTG-3’                             | This study          |
| aurR-qpcr-R             | 5’- ATGGGCTGAGGACCTG-3’                             | This study          |
| aurI-qpcr-F             | 5’- TGGTACGACAGCCATG-3’                             | This study          |
| aurI-qpcr-R             | 5’- ATGGGCTGAGGACCTG-3’                             | This study          |

Rif; rifampicin; Gm; gentamicin.
⁷pME6863 constitutively expresses an aiiA lactonase from Bacillus sp. A24 [14].

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Reaction (PCR) was conducted following standard conditions outlined by New England Biolabs (NEB) (Ipswich, USA) data sheets supplied with their buffer system and Taq polymerase.

**RNA extraction and cDNA library synthesis**

cDNA libraries were generated for PA23, PA23phzR, and PA23-6863. Three biological replicates of each strain were cultured in 30 ml of M9-Glc. Cells were harvested at early stationary phase (OD₆₀₀ 1.20–1.50) by pelleting for 10 min at 6000 rpm at 4°C followed by flash freezing in liquid nitrogen. Pellets were stored at -80°C for up to one week. Total RNA was extracted using the Fermentas Plant RNA extraction kit (Waltham, USA) per manufacturer’s instructions. Residual genomic DNA was removed by treatment with TURBO RNAase-free DNase I (Ambion, Carlsbad, USA) according to manufacturer’s instructions. RNA concentration was verified using a NanoVue spectrophotometer (GE Healthcare), and quality was measured using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Pico and Nano Chips (Agilent Technologies; Santa Clara, CA, USA). cDNA libraries were constructed using the alternative HTR protocol described by Kumar et al. [22] adapted for bacterial RNA. Ribosomal RNA was depleted using the MicroExpress kit (Ambion) as per manufacturer’s instructions. Fragmentation time was reduced to 10 min and the number of cycles for final PCR amplification of the libraries was adjusted to 12 [22]. Final cDNA libraries with ligated adaptors were size-selected to fall between 250 and 500 bp using the E-Gel® electrophoresis system (Invitrogen). cDNA quantity was measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher, Rockford, USA) with a Nanodrop 3300 (Thermo Fisher). cDNA was validated using Agilent Bioanalyser High Sensitivity DNA Chips (Agilent Technologies) at three points: i) after first and second strand cDNA synthesis; ii) after the final PCR amplification of the libraries; and iii) after size selection with the E-Gel® electrophoresis system. 100-bp single-end RNA-sequencing was carried out at Genome Québec (Montreal, Canada) on the Illumina HiSeq 2000 platform with a multiplex value of 9. The sequencing reads analyzed in this publication have been deposited in NCBI’s Gene Expression Omnibus [23], GEO Series accession number GSE114924 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114924).

**Data analysis**

Sequenced reads were analysed by FastQC to determine quality (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the Trimomatic tool [24] enabled removal of low quality reads (Q < 30) and barcode adapters. Reads mapping to each gene was counted after mapping raw reads to the genome via the Rockhopper program [25] using the *P. chlororaphis* PA23 reference annotation from NCBI (gi: accession no. NZ_CP008696). Of the total reads, 93–96% mapped to the *P. chlororaphis* PA23 genome across samples (S1 Table). Genes belonging to the same transcriptional unit were predicted based on the number of nucleotides between genes and similarity in the level of expression of each gene using Rockhopper software. The R Bioconductor package DESeq2 [26] was employed to normalise raw read counts from each replicate and to determine significantly differentially expressed genes (adjusted p-value ≤ 0.01). This output enabled generation of gene expression profiles for each strain. The log₂ fold change values ≥1.5 or ≤ -1.5 were selected based on previous analysis carried out by Shemesh et al., [27] on the *Streptococcus mutans* transcriptome. Functional analysis was carried out via Cluster of Orthologous Groups (COG) analysis. COG categories were assigned to the translated transcript sequences through the Conserved Domain Database (CDD) and the batch web-CD search tool [28,29].
Autoinducer assay
The amount of AHL produced by each strain was approximated by spotting 5 μl of an M9-Glc culture, grown in for 24 h and adjusted to an OD<sub>600nm</sub> of 1, onto Chromobacterium violaceum CVO26 seeded LB agar plates. The radius of the purple zone surrounding each colony was measured after 24 h of incubation at 28°C.

Siderophore assay
To analyze siderophore production, a 5 μl volume of overnight bacterial culture grown in M9-Glc was spotted onto Chrome Azurol S (CAS) media [30]. The diameter of the yellow zone surrounding the colonies, indicative of siderophore production, was measured following 24 hours of incubation at 28°C.

Chitinase assay
Strains were tested for their ability to produce chitinase according to the protocol outlined by Wirth & Wolf [31]. Strains were cultured in M9-Glc broth until they reached early stationary phase (OD<sub>600</sub> 1.20–1.50). A 250 μl aliquot of cell free supernatant was incubated with equal parts of 0.1M NaOAc, pH 5.2 (250 μL) and carboxymethyl-chitin-Remazol brilliant violet aqueous solution (250 μL) (Blue Substrates, Göttingen, Germany). After incubation for 1 hour at either 28°C or 37°C, reactions were stopped by the addition of 250 μl of 1M HCl. Mixtures were cooled on ice for 10 min, spun at 20,000xg for 10 min and absorbance readings were taken at 550 nm. Three replicates were analysed for each strain and experiments were performed in triplicate.

Motility assays
Swimming motility assays were carried out by growing cultures of PA23 and derivative strains to early stationary phase (24 h) in 3 ml of M9-Glc at 28°C. The cultures were then standardized to an OD<sub>600nm</sub> of 1 and stab-inoculated (half-way down) into the centre of M9-Glc media solidified with 0.3% agar. Plates were incubated for 72 h at 28°C and the diameter of the swim zone was measured every 24 h. Five replicates were inoculated for each strain and three independent experiments were performed.

To assess swarming motility, fresh colonies of PA23 and derivative strains were gently inoculated using an applicator stick onto the surface of a swarm media plate (0.5% peptone, 0.3% yeast extract solidified with 0.8% agar). Plates were incubated for 96 h at 28°C, pictures of each plate were taken every 24 h and the area of the swarming colony was measured using ImageJ software [32]. Five plates were analyzed for each strain and three independent experiments were performed.

Quantitative PCR (qPCR)
Quantitative PCR (qPCR) was used to monitor expression of metabolite and regulatory genes involved in biocontrol. Triplicate cultures of PA23 and derivative strains were grown to early stationary phase in a 3 ml volume of M9-Glc. Cells were harvested by incubating 500 μl of culture with 2x volume of RNAprotect reagent (QIAGEN, Valencia, USA) for 5 min followed by centrifugation for 10 min at 6000 rpm. Pellets were stored at -20°C for up to one week. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Residual genomic DNA was removed by treatment with TURBO RNAsafe-free DNAsafe I (Ambion). RNA concentrations were measured at absorbance 260 and 280 nm; only RNA samples with A260/A280 between 1.8 and 2.0 were used in subsequent steps. cDNA was generated by reverse transcription using
the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) and random hexamer primers in a 20 μL total reaction volume. The following conditions were employed: initial heating at 25˚C for 10 min, reverse transcription at 50˚C for 15 min, and enzyme denaturation at 85˚C for 5 min. Sequences for the PA23 genes of interest were obtained from GenBank (gi: NZ_CP008696) and the primer sequences are listed in Table 1. PCR was performed using a CFX96 Connect™ Real-Time PCR Detection System (Bio-rad, Hercules, USA) and SsoFast™ EvaGreen® Supermix (Bio-rad). The final 10 μL volume mixture in each well contained 0.4 μL of both forward and reverse primers (12 μM), 1 μL of 1:20 diluted cDNA, 5 μL of SsoFast™ EvaGreen® Supermix and 3.4 μL of nuclease-free water. PCR reaction conditions included an initial denaturation at 98˚C for 2 min, followed by 39 cycles of 98˚C for 5 s, 60˚C for 30 s, and 60˚C for 5 s. Melt-curve analysis was performed to evaluate the formation of primer dimers and other artefacts to validate results. Each reaction was performed in triplicate and experiments were repeated three times with three biological replicates. Relative gene expression was calculated using the ΔΔCt method as described by Livak & Schmittgen [33], with rpoB as the reference gene and the CFXManager™ software (Bio-rad).

**phz-box identification**

The presence of putative phz-boxes upstream of differentially expressed genes determined through RNAseq were identified using the MEME suite Motif Alignment and Search Tool (MAST) algorithm (http://meme-suite.org/tools/mast) [34,35]. A phz-box consensus sequence VCKRCHWGHKYKBSHWRK (where V = A, C or G; K = G, T; R = A, G; H = C, T or A; W = A, T; Y = C, T; B = C, G or T; S = G, C; N = A, T, G or C) was generated using previously identified phz-boxes in PA23 [9,11]. Only consensus sequences located within 500 basepairs upstream of the translational start site were considered significant.

**Results and discussion**

**Identification of genes under QS control**

In PA23, the Phz QS system is essential for biocontrol as strains lacking this regulatory circuitry no longer produce the antibiotics and degradative enzymes required for fungal antagonism [11]. To fully appreciate the global effect of QS on PA23 gene expression, RNAseq was conducted on two QS-deficient strains. The phzR mutant, PA23phzR, was generated through allelic exchange and exhibits reduced AHL levels compared to wild type (S1 Fig) [11]. Conversely, PA23-6863 contains the *Bacillus* sp. A24 AHL lactonase-encoding aiiA gene on a plasmid and is AHL-deficient (S1 Fig) [11]. Because the PA23 genome encodes three QS systems including PhzRI, CsaRI and AurRI, PA23-6863 is equivalent to a triple mutant. This is relevant because in the closely related *P. chlororaphis* subsp. *aurantiaca* strain PB-St2, the three AHL synthases, PhzI, CsaI and AurI, produce structurally similar molecules, all of which have a lactone ring with varying acyl chain lengths and substitutions [16]. While the AHLS produced by PA23 have not yet been characterized, we speculate that they have a similar profile to those of PB-St2. Because AiiA lactonase cleaves the lactone ring which is common to AHLS, and this activity is independent of the length or substitutions of the acyl chain, all AHLS are degraded by this enzyme [36–38]. Thus, the presence of the lactonase ensures that there is no cross-activation by non-cognate AHLS.

We chose M9-Glc media to simulate the nutrient-limiting conditions present in the environment and cells were harvested at early stationary phase (OD$_{600}$ 1.2–1.5) when secondary metabolite production occurs. Five hundred and forty-five differentially expressed genes were identified in the phzR mutant background (365 downregulated; 180 upregulated),
corresponding to 8.8% of the genome (S2 Table). In the AHL-deficient PA23-6863, a total of 534 genes showed altered expression (382 downregulated; 152 upregulated) representing 8.6% of the genome (S3 Table). In total, 807 of the 6,179 coding sequences in the PA23 genome showed differential expression in one or both of the QS-deficient strains.

The degree of overlap between differentially expressed genes in the phzR mutant and in the AHL-deficient strain is quite low; 36.8% of downregulated and 26.2% of upregulated genes were conserved between them (Fig 1). Genes showing altered expression exclusively in PA23-6863 is likely due to the fact that in this strain, all signal molecules are degraded leading to a total loss of QS; conversely in PA23 phzR, only the Phz system is affected. It is also possible that QS signals are regulating gene expression through receptor-independent mechanisms, as demonstrated in \textit{P. aeruginosa} [39]. Intriguingly, 275 genes showed differential regulation in the phzR mutant but not the AHL-deficient strain. There are examples of LuxR proteins that regulate gene expression in the absence of AHL; for instance in \textit{Pectobacterium atrosepticum}, VirR binds to the promoter of the \textit{rsmA} gene activating transcription. Upon binding to 3-oxo-C6-HSL, VirR undergoes a conformation change that results in dissociation from the promoter and \textit{rsmA} is no longer expressed [40–42]. The \textit{P. aeruginosa} transcriptional regulator RhlR was recently reported to regulate gene expression in a C4-HSL-dependent and C4-HSL-independent manner [43]. In the absence of the AHL synthase (RhlI), RhlR directs expression of genes involved in biofilm formation and virulence factors [43]. Finally, AHL-deficient strains of \textit{Pseudomonas corrugata} and \textit{Pseudomonas brassicacearum} are phenotypically similar to wild type; whereas loss of the LuxR homolog results in dramatically altered traits [44] (Saikai and de Kievit, unpublished data). Collectively, these findings support a regulatory role for LuxR proteins in the absence of AHL binding.

While the global impact of QS in a biocontrol pseudomonad has yet to be undertaken, microarray studies have been performed on two pathogenic pseudomonads, namely \textit{P. aeruginosa} PAO1 and \textit{P. syringae} B728a [17,18,46,47]. Results from the current study show that expression of 13.06% of the PA23 genome is governed by QS. In \textit{P. aeruginosa} PAO1, QS-modulated genes were reported to account for 6–10% of the genome and the majority of genes were positively regulated [17,18]. We discovered that in PA23phzR, 67% of the differentially expressed genes
regulated genes showed decreased expression, while 33% were upregulated. Similarly, in PA23-6863, 71.5% and 28.5% of genes displayed decreased and increased expression, respectively. Thus, for both PAO1 and PA23, QS is largely serving as a positive regulator of gene expression. In stark contrast to these large regulons, only 9 genes were found to be controlled by the *P. syringae* QS circuitry [47]. All nine genes were located near the *ahlR* locus and all were positively regulated by QS.

Functional characterisation of differentially expressed genes in PA23*phzR* and PA23-6863

Next, we sought to predict the functional role of the PA23 QS-controlled genes through Cluster of Orthologous Group (COG) analysis. COG clusters are constructed using functional characterization based on prokaryotic genomes [48]. According to their predicted function, the 545 differentially expressed genes in the *phzR* mutant could be divided into 21 COG categories (Fig 2A, S2A Fig, S2 Table). Similarly, the 534 genes identified in PA23-6863 could be grouped into 22 COG categories (Fig 2B, S2B Fig, S3 Table). Several of the more relevant categories are discussed in detail in the following sections.

Role of QS in regulation of PA23 secondary metabolites. In both QS-deficient strains, a number of genes involved in secondary metabolite production were significantly downregulated. For example, genes required for synthesis of PHZ (EY04_RS25715-45), hydrogen cyanide (EY04_RS11540-50), and exoprotease (EY04_RS11085) exhibited $\geq 3.42 \log_2$ fold decreased expression compared to the WT. Similarly, expression of the *prn* biosynthetic genes (EY04_RS17650-35) was reduced 1.93- and 4.17-log$_2$ fold in PA23*phzR* and PA23-6863, respectively. In a previous study, PA23 QS-deficient strains exhibited decreased *phzA-lacZ* and *prnA-lacZ* transcription and reduced PHZ, PRN and protease production [11], consistent with our RNAseq data. When qPCR was used to validate our global transcriptomic findings, expression of the *phz*, *prn* and *hcn* genes in PA23*phzR* and PA23-6863 was decreased (Fig 3).

Other exoproduct genes that were differentially expressed in both QS derivatives include chitinase-encoding genes (EY04_RS16020, EY04_RS09705; downregulated $\geq 6.91 \log_2$ fold) and siderophore biosynthetic genes (EY04_RS15355-410; upregulated $\geq 2.61 \log_2$ fold). When we employed end-product analysis to support these findings, chitinase activity was completely abolished in both PA23*phzR* and PA23-6863 (0.00 ± 0.001) compared to the wild type (0.20 ± 0.006). On CAS agar, orange halos indicative of siderophore production, were significantly larger around colonies of PA23*phzR* (8.0 ± 0.3 mm) and PA23-6863 (7.5 ± 0.1 mm) compared to the PA23 parent (3.5 ± 0.2 mm). Collectively, the RNAseq findings are in keeping with phenotypic characteristics of the QS-deficient strains, validating the robustness of this approach for defining the PA23 QS regulon [3,11].

Role of QS in motility. Several motility genes were significantly upregulated in the QS-deficient strains (S3 Fig, S2 Table and S3 Table). In addition to a number of chemotaxis signal transduction genes being induced, genes predicted to modulate cellular levels of cyclic di-GMP (EY04_RS05905, EY04_RS09010, EY04_RS10080, EY04_RS15460, EY04_RS24510) were upregulated in one or both of the QS-deficient strains. Notably, flagellin (EY04_RS07580), a diguanylate phosphodiesterase (EY04_RS05905), as well as the alternative stators, MotD (EY04_RS07755) and MotY (EY04_RS24205) were uniquely upregulated in PA23*phzR* (S3 Fig). The MotCDY stators along with low levels of c-di-GMP are required to provide higher torque for flagellar movement through high agar concentrations in *P. aeruginosa* [50,51]. Such findings suggest that PA23*phzR* and PA23-6863 are more motile. Consistent with this, the swim zone of PA23*phzR* was significantly larger than the WT and PA23-6863, while PA23-6863 was more motile than the WT strain in 0.3% agar (Table 2, S3 Fig). Since the swarming
Quorum sensing regulon of *Pseudomonas chlororaphis* PA23

A

B

Genes

PA23pxfR

Energy production and conversion
Cell cycle control, cell division, chromosome partitioning
Amino acid transport and metabolism
Nucleotide transport and metabolism
Carbohydrate transport and metabolism
Coenzyme transport and metabolism
Lipid transport and metabolism
Translation, ribosomal structure and biogenesis
Transcription
Replication, recombination and repair
Cell wall/membrane/envelope biogenesis
Cell motility
Posttranslational modification, protein turnover, chaperones
Inorganic ion transport and metabolism
Secondary metabolites biosynthesis, transport and catabolism
General function prediction only
Function unknown
Signal transduction mechanisms
 Intracellular trafficking, secretion, and vesicular transport
Defence mechanisms
Mobiles: prophages, transposons

PA23-6963

Energy production and conversion
Cell cycle control, cell division, chromosome partitioning
Amino acid transport and metabolism
Nucleotide transport and metabolism
Carbohydrate transport and metabolism
Coenzyme transport and metabolism
Lipid transport and metabolism
Translation, ribosomal structure and biogenesis
Transcription
Replication, recombination and repair
Cell wall/membrane/envelope biogenesis
Cell motility
Posttranslational modification, protein turnover, chaperones
Inorganic ion transport and metabolism
Secondary metabolites biosynthesis, transport and catabolism
General function prediction only
Function unknown
Signal transduction mechanisms
 Intracellular trafficking, secretion, and vesicular transport
Defence mechanisms
Mobiles: prophages, transposons
pattern of PA23 is irregular [5], the area of the swarming colony on 0.8% agar was measured using ImageJ software [32]. After four days, PA23*phzR* swarmed significantly more than the WT strain (Table 2, S3 Fig). PA23-6863 showed more variation between replicates and the area of swarming was not significantly different from the WT, but was less than PA23*phzR* (Table 2, S3 Fig). These results are consistent with findings that QS suppresses motility in other rhizobacteria, such as *P. syringae* and *Sinorhizobium meliloti* [52–54]. Since the switch from a sessile to a motile cell is energetically costly and flagella can induce plant defenses, it is beneficial for bacteria to tightly regulate motility until a lack of nutrients necessitate movement to a more nutritive environment [55].

**Characterization of QS-regulated genes involved in other physiological processes.** The largest percentage of QS regulated transcripts encoded proteins of unknown function (35.8% AHL; 29.7% phzR), which was also observed by Wagner et al. [18]. Other major categories found to be QS regulated include those associated with cellular energetics and metabolism, such as energy production and conversion (category C; 3.4% AHL; 5.5% phzR), amino acid transport and metabolism (category E; 5.4% AHL; 6.2% phzR), carbohydrate transport and metabolism (category G; 5.4% AHL; 4.6% phzR), and lipid transport and metabolism (category I; 3.6% AHL; 4.4% phzR). Consistent with these findings, PAO1 genes linked to amino acid biosynthesis and metabolism, carbon compound catabolism, energy metabolism, and

![Fig 2](https://doi.org/10.1371/journal.pone.0226232.g002)

**Fig 2**. Differentially expressed genes in (A) PA23*phzR* and (B) PA23-6863 when compared to PA23 wild type. Significantly differentially expressed genes are divided into functional categories based on Cluster of Orthologous Groups (COGs) [49]. Genes important for the synthesis and regulation of biocontrol products are marked.

![Fig 3](https://doi.org/10.1371/journal.pone.0226232.g003)

**Fig 3**. qPCR fold change in gene expression in PA23*phzR* and PA23-6863 compared to PA23 wild type. Analyzed genes were compared against *rpoB* as a reference gene. Gene expression in the wild type was normalized to 1.0. For strains that differ significantly from the wild type, columns have been marked with an asterisk (* < 0.01; **p < 0.001).
fatty acid and phospholipid metabolism were differentially expressed in the absence of QS [18]. Genes involved in translation, ribosomal structure and biogenesis were also found to be under QS control (category J; 3.0% AHL; 9.4% phzR), including a large number of 50S and 30S ribosomal proteins as well as the 16S rRNA processing protein, RimM. Similar results were demonstrated for the PfsI/R QS regulon of the rice pathogen *Pseudomonas fuscovaginae*, where the majority of genes positively regulated by PfsI/R are involved in translation, ribosomal structure and biogenesis, including 20 ribosomal proteins and RimM [56].

In *P. aeruginosa*, QS controls expression of not only virulence factors but the secretion systems required for export [18]. Our transcriptomic analysis revealed decreased expression of genes encoding type IV (EY04_RS00545) and VI (EY04_RS29490, EY04_RS29495, EY04_RS29520) secretion systems in one or both of the PA23 QS-deficient strains. Moreover, expression of resistance-nodulation-cell division (RND) efflux transporters including MexE homologues (EY04_RS19235, EY04_RS17230, EY04_RS17225, EY04_RS00160) was reduced in the absence of QS. Similarly in strain PAO1, three RND efflux systems were found to be under QS control [18]. At present, the mechanism by which PA23 biocontrol metabolites are transported outside of cells has not been elucidated. We hypothesize that at least some of these compounds are exported via these secretion systems and/or active efflux.

### Table 2. Motility of PA23 and derivative strains.

| Strain   | Swim 0.3% agar<sup>*</sup> | Swarm 0.8% agar<sup>†</sup> |
|----------|---------------------------|-----------------------------|
|          | 24 h                      | 48 h                        | 72 h                        | 96 h<sup>†</sup> |
| PA23 WT  | 7.2 (0.8)                 | 10.4 (1.1)                  | 12.4 (1.3)                  | 162.8 (18.6)  |
| PA23-phzR| 23.0 (2.1)<sup>§</sup>    | 46.6 (2.6)<sup>§</sup>      | 69.8 (3.6)<sup>§</sup>      | 815.2 (304.4)<sup>||</sup> |
| PA23-6863| 11.3 (1.5)<sup>||</sup>    | 24.0 (1.0)<sup>§</sup>      | 35.1 (2.9)<sup>§</sup>      | 83.2 (50.1)<sup>#</sup> |

<sup>*</sup>Mean (SD) diameter (mm) of the swim zone obtained from three independent experiments.

<sup>†</sup>Mean (SD) area (mm<sup>2</sup>) of swarming colony obtained from three independent experiments.

<sup>§</sup>Significantly different from WT (P < 0.001).

<sup>||</sup>Significantly different from WT (P < 0.05).

<sup>#</sup>Not significantly different from WT.

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QS directly and indirectly regulates gene expression in PA23

For genes under QS control, regulation may occur in one of two ways: directly through PhzR-AI binding to the promoter or indirectly through control of other regulators. For those in the former category, an activated PhzR dimer is believed to bind to a highly-conserved consensus sequence known as the *phz*-box, previously identified upstream of the PA23 *phzA* and *phzl* genes [11]. In *P. aeruginosa*, 7% of QS-regulated genes reportedly contain *las*- or *rhl*-boxes in the promoter region [17,18]. To determine if a similar trend is observed in PA23, we analysed the genome for *phz*-boxes using the Motif Alignment and Search Tool (MAST) algorithm [34,35]. In PA23, 545 and 534 genes were differentially regulated in the PhzR- and AHL-deficient backgrounds, respectively. However, only 58 transcriptional units containing 99 genes contained *phz*-boxes within the 500-bp promoter region, suggesting indirect regulation of the majority of genes (Table 3). Consistent with this, a large number of transcriptional regulators display altered expression in the QS-deficient strains (Table 4). Seventeen percent of these differentially expressed transcriptional regulators contain a *phz*-box sequence in the promoter region, namely *csaI*, *csaR*, *phzl*, *phzR*, *rpoS* (EY04_RS20250), *mvaV* (EY04_RS16500) and an AraC-type transcriptional regulator gene (EY04_RS01040) (Table 4). It is also possible...
Table 3. Transcriptional units containing a phz-box sequence in the promoter region.

| Putative phz-box sequence | Function COG category | Locus tag | log2 fold change | Distance between phz-box and ATG start |
|---------------------------|-----------------------|-----------|-----------------|--------------------------------------|
| gccgCCGCTTGTCGGCACAATcggc| Hypothetical protein  | -         | -               | 386                                  |
| tgcgCTCTACAGCTTTCGGCCGcagc| Hypothetical protein  | -         | -               | 537                                  |
| ttcgCCGCAAGATCTGCGTGcagg| R body protein RebB-like protein | - | I | -2.57 | 346 |
| gaggACGCGCTGATGCCTAGGcctc| ATP-dependent protease ATP-binding subunit HslU | COG1220 | O | 3.83 | - 218 |
| tccgCCGCGATGCGCGCGcctc| Pyruvate dehydrogenase | COG2609 | C | 3.18 | - 286 |
| gagtCCGCCTGGCTAGGcctc| Dihydrolipoamide acetyltransferase | COG2207 | C | 2.83 | - 226 |
| tahgACGGCGCTGCCTAGGcctc| Histidine kinase | cl27674 | K | 2.31 | - 231 |
| ttcgCCGGCTGTGCCTGGGcctc| Carbon-nitrogen hydrolase | COG0388 | R | 3.48 | - 341 |
| ttcgCTCTGCTGACGGCCTAGGcctc| N-acetylmuramoyl-L-alanine amidase | COG3023 | M | 3.21 | - 321 |
| taccACGGCGCTGCCTAGGcctc| Acyl-homoserine-lactone synthase CsaI | cl17182 | T | 3.13 | - 313 |
| cacgGGGCGATCCGCGCGCAAGTcgtt| Hypothetical protein  | -         | -               | 265                                  |
| atcgCCGCGCTGCCTAGGcctc| Methyltransferase | cl28097 | S | 3.12 | - 280 |
| tggcACGCGCTGCTAGGcctc| Transcriptional regulator CsaR | COG2771 | K | 3.11 | - 277 |
| gaggCCGCGCTGCCTAGGcctc| Chitinase | COG3469 | G | 3.10 | - 346 |
| atcgCCGCGCTGCCTAGGcctc| 2Fe-2S ferredoxin | cl28397 | - | -2.53 | - 283 |
| gaggCCGCGCTGCCTAGGcctc| ATPase | EY04_RS1890 | 5 | -2.20 | - 189 |
| tatcCCGCGCTGCCTAGGcctc| ADP-glucose transferase | COG0534 | G | 3.11 | - 534 |
| gaggCCGCGCTGCCTAGGcctc| POTRA domain protein | cl28555 | - | -1.86 | - 285 |
| atcgCCGCGCTGCCTAGGcctc| 2Fe-2S ferredoxin | cl28397 | - | -2.53 | - 283 |
| gaggCCGCGCTGCCTAGGcctc| ATPase | EY04_RS1890 | 5 | -2.20 | - 189 |
| atcgCCGCGCTGCCTAGGcctc| POTRA domain protein | cl28555 | - | -1.86 | - 285 |
| gaggCCGCGCTGCCTAGGcctc| 2Fe-2S ferredoxin | cl28397 | - | -2.53 | - 283 |

(Continued)
| Putative phz-box sequence^1 | Function | COG category | Locus tag^2 | log2 fold change | Distance between phz-box and ATG start^3 |
|-----------------------------|----------|--------------|-------------|-----------------|-------------------------------------|
| atggTCTGCTGATCTGGCCAGTtcac | Sugar ABC transporter ATPase | COG3839 G | EY04_RS22875 | -4.48 | -3.63 |
| tggTGCCAGCTTGGTCTATGTgtag | Glyceraldelyde-3-phosphate dehydrogenase | COG0057 G | EY04_RS22920 | -3.03 | -4.91 |
| catgCCTACCTCTCCGTGCAGTtccaa | Amino acid APC transporter | COG0531 E | EY04_RS22975 | -3.82 | - |
| Arginine deiminase | COG2235 E | EY04_RS22980 | -4.54 | - |
| Ornithine carbamoyltransferase | COG0078 E | EY04_RS22985 | -4.38 | - |
| Carbamate kinase | COG0549 E | EY04_RS22990 | -3.22 | - |
| Acetyl-CoA carboxylase | - - | EY04_RS22995 | - - | - |
| ctggCCGGCCAGATTGGCCAGTggcc | Hypothetical protein | - - | EY04_RS2543S | -3.00 | 316 |
| agccCTACCGATCCGTGAGTggcc | Acyl-homoserine-lactone synthase PhzI | cl17182 T | EY04_RS25705 | -5.53 | -5.15 |
| tggaACTACGATCTGATGTgatt | Transcriptional regulator PhzR | COG2771 K | EY04_RS25710 | -4.12 | -2.71 |
| atccACTACAGATCTGAGTtccaa | Phenazine biosynthesis protein PhzA | cl09109 - | EY04_RS25715 | -10.14 | -6.68 |
| PhzB | - - | EY04_RS25720 | -10.68 | -6.21 |
| PhzC | cl03230 - | EY04_RS25725 | -8.25 | -6.59 |
| Isochorismatase | COG1535 Q | EY04_RS25730 | -8.91 | -7.61 |
| PhzE | cl27696 - | EY04_RS25735 | -7.24 | -6.27 |
| 2,3-dihydro-3-hydroxyanthranilate isomerase | COG0384 R | EY04_RS25740 | -8.34 | -6.28 |
| PhzG | cl25685 - | EY04_RS25745 | -8.71 | -6.21 |
| ctggAGCCAGGTCGCGTCGggcg | Gamma-glutamyl kinase | COG0263 E | EY04_RS26495 | -1.52 | 254 |
| gacaCCGGCTGTTTCAAGGacaa | Lipoate-protein ligase | COG0321 H | EY04_RS27530 | -1.71 | 181 |
| lipoyl synthase | EY04_RS27525 | - - | - |
| ttcgCCGGCTGAATCGCAAATgcttt | 305 ribosomal protein S14 | COG0199 J | EY04_RS28075 | -2.00 | 199 |
| agccCCGGCTGTCGGCATGaggcc | Hypothetical protein | cl01888 - | EY04_RS28670 | -3.29 | - |
| gactGGTCTCCAGGCTGCCAaaca | Zinc ABC transporter permease | COG1108 P | EY04_RS29155 | 6.22 | 6.52 |
| Metal ABC transporter substrate-binding protein | COG0803 P | EY04_RS29150 | 7.57 | 7.82 |
| Phosphoribosyl-AMP cyclohydrolase | COG0139 E | EY04_RS29145 | 5.91 | 6.40 |
| Carbonate dehydratase | COG0663 R | EY04_RS29140 | 7.03 | 7.63 |
| Dihydroorotase | COG0044 F | EY04_RS29135 | 6.65 | 7.32 |
| Threonyl-tRNA synthetase | COG0411 J | EY04_RS29130 | 4.87 | 5.46 |
| gtcgGGGCAAGAGAGGTGGgctta | Molecular chaperone DnaK | COG1734 J | EY04_RS29180 | 7.05 | 8.16 |
| Hypothetical protein | COG0523 R | EY04_RS29185 | 7.37 | 8.07 |
| Hypothetical protein | - - | EY04_RS29190 | 6.47 | 7.52 |
| ggcgCCGACCAGCTGGCAGGGgaga | Hypothetical protein | cl27250 - | EY04_RS29455 | -2.26 | - |
| tgcaCAGACCAAAACCGCCGgcgg | Type VI secretion protein | COG3520 U | EY04_RS29525 | -3.13 | -1.78 |
| CaggCGGCATGCATCTGAGGgagg | Cystathionine gamma-synthase | COG0672 P | EY04_RS30070 | 1.55 | 2.10 |
| ttaaGCAGATGGATTAAAAGacca | sn-glycerol-3-phosphate transporter | CG02271 G | EY04_RS30160 | -2.86 | 113 |
| ggcgCCGACCTGCCTGCCAGTggtt | Polysaccharide deacetylase | COG0726 GM | EY04_RS15615 | 4.97 | - |
| atggTCTGCTGACGGCTTATGTgtag | GTPase CgtA | COG0536 DL | EY04_RS26500 | -1.60 | 94 |
| cggCGGCGCGATGCCTGTCGAggg | Kinase | COG1947 I | EY04_RS25860 | -1.58 | 317 |
| atacCGGACCAGGCGTGGCTGactg | ATPase | COG4962 UW | EY04_RS20750 | -2.22 | 331 |

Type II secretion system protein F | cl19503 - | EY04_RS20745 | -2.41 | - |

(Continued)
that genes lacking canonical phz-boxes are still directly regulated; while R proteins generally have a high affinity to their cognate lux-like boxes, PhzR, CsaR or AurR may be capable of binding non-canonical sequences in promoter regions, as has been demonstrated for LasR from *P. aeruginosa* [57]. In total, 41 genes encoding transcriptional regulators showed differential expression in one or both of the QS-deficient strains. The expression of 11 genes encoding regulators was altered in both strains, while 18 and 12 genes exhibited altered expression in PA23-6863 and PA23*phzR*, respectively (Table 4). These findings support the hypothesis that a substantial proportion of the QS regulon is subject to indirect control. While a detailed discussion of all 41 regulators is not feasible, examples of prominent genes classified into each of these categories are provided below.

**Regulatory genes showing differential expression in both QS-deficient strains.** *phzl* and *phzR* are among the 11 regulatory genes that showed differential expression in PA23*phzR* and PA23-6863 (Table 4). As expected, both genes were positively regulated consistent with the paradigm of autoinduction (Table 4). The gene encoding RpoS showed 2.13- and 3.28-log2-fold lower expression in PA23*phzR* and PA23-6863 respectively. Cross-regulation between QS and RpoS has been previously demonstrated [11]. *rpoS*, was found to be positively regulated by the Phz QS system [11], in keeping with findings presented herein.

Our RNAseq analysis revealed a connection between the Csa and Phz QS systems. In PA23*phzR* and PA23-6863, *csaR* was downregulated by a factor of 2.64 and 3.24, whereas *csaI* was downregulated 5.74 and 6.06, respectively. Through qPCR analysis, we discovered that *csaR* and *csaI* were both downregulated at least 10-fold in PA23-6863 and PA23*phzR* (Fig 3).
In the case of *aurI* and *aurR*, differential expression could not be determined from the RNAseq data due to low read coverage of this region. Nevertheless, qPCR analysis revealed that *aurI* was upregulated, while *aurR* was downregulated in PA23-6863 (Fig 3). A similar trend was seen in

### Table 4. Regulatory genes under quorum-sensing control in *Pseudomonas chlororaphis* PA23.

| Strain          | Regulator                                      | Locus Tag        | PA23-6863 | PA23phzR |
|-----------------|------------------------------------------------|------------------|-----------|----------|
| PA23phzR        | ArsR family transcriptional regulator          | EY04_RS05450     | -         | -4.12    |
|                 | MarR family transcriptional regulator          | EY04_RS19240     | -         | -4.12    |
|                 | Transcriptional regulator                      | EY04_RS22860     | -         | -4.12    |
|                 | Transcriptional regulator                      | EY04_RS20895     | -         | -4.12    |
|                 | AraC family transcriptional regulator          | EY04_RS15230     | -         | -4.12    |
|                 | GntR family transcriptional regulator          | EY04_RS32820     | -         | -4.12    |
|                 | MarR family transcriptional regulator          | EY04_RS07295     | -         | -4.12    |
|                 | Transcriptional regulator                      | EY04_RS28835     | -         | -4.12    |
|                 | DeoR family transcriptional regulator          | EY04_RS24510     | -         | -4.12    |
|                 | AraC family transcriptional regulator          | EY04_RS15465     | -         | -4.12    |
|                 | Transcriptional regulator                      | EY04_RS14845     | -         | -4.12    |
|                 | LysR family transcriptional regulator          | EY04_RS14120     | -         | -4.12    |
| PA23-6863 & PA23phzR | Acyl-homoserine-lactone synthase CsaI*          | EY04_RS11850     | -9.06    | -5.74    |
|                 | Acyl-homoserine-lactone synthase PhzI*          | EY04_RS25705     | -5.53    | -5.15    |
|                 | Transcriptional regulator                      | EY04_RS25710     | -4.12    | -2.71    |
|                 | Fis family transcriptional regulator           | EY04_RS29535     | -4.04    | -1.87    |
|                 | LysR family transcriptional regulator          | EY04_RS27730     | -3.78    | -1.69    |
|                 | PadR family transcriptional regulator          | EY04_RS24465     | -3.57    | -4.12    |
|                 | Cro/CI family transcriptional regulator        | EY04_RS18125     | -3.52    | -1.68    |
|                 | RNA polymerase sigma factor RpoS               | EY04_RS05305     | -3.28    | -2.13    |
|                 | LuxR family transcriptional regulator          | EY04_RS11855     | -3.24    | -2.64    |
|                 | GntR family transcriptional regulator          | EY04_RS23170     | -2.98    | -3.18    |
|                 | Transcriptional regulator                      | EY04_RS29740     | 1.85     | 1.92     |
| PA23-6863        | LuxR family transcriptional regulator          | EY04_RS06740     | -4.47    | -       |
|                 | Transcriptional regulator                      | EY04_RS27335     | -4.13    | -       |
|                 | TetR family transcriptional regulator          | EY04_RS01505     | -3.20    | -       |
|                 | AraC family transcriptional regulator          | EY04_RS23115     | -2.96    | -       |
|                 | Transcriptional regulator                      | EY04_RS16500     | -2.92    | -       |
|                 | AraC family transcriptional regulator          | EY04_RS01040     | -2.70    | -       |
|                 | LuxR family transcriptional regulator          | EY04_RS23625     | -1.77    | -       |
|                 | H-NS histone MvaT                               | EY04_RS23865     | -1.73    | -       |
|                 | LuxR family transcriptional regulator          | EY04_RS02735     | -1.71    | -       |
|                 | Crp/Fnr family transcriptional regulator       | EY04_RS06720     | -1.70    | -       |
|                 | GntR family transcriptional regulator          | EY04_RS07920     | -1.66    | -       |
|                 | AraC family transcriptional regulator          | EY04_RS19450     | 1.60     | -       |
|                 | AraC family transcriptional regulator          | EY04_RS04305     | 1.81     | -       |
|                 | Transcriptional regulator                      | EY04_RS01770     | 2.16     | -       |
|                 | RNA polymerase sigma factor                    | EY04_RS00050     | 2.19     | -       |
|                 | RNA polymerase subunit sigma-70               | EY04_RS10675     | 2.22     | -       |
|                 | RNA polymerase sigma factor*                   | EY04_RS20250     | 2.66     | -       |
|                 | GntR family transcriptional regulator          | EY04_RS12060     | 3.76     | -       |

*Contains a phz-box sequence in the promoter region.

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However, expression of aurI and aurR was not significantly different from PA23 (Fig 3). While the regulatory network governing aurIR and csaIR expression has not yet been determined, this hierarchical arrangement is similar to that observed in P. aeruginosa which employs two AHL-based QS systems called Las and Rhl. In this bacterium, both the transcriptional activator (rhlR) and the AHL synthase (rhlI) genes are under control of the Las system [58,59].

**Regulatory genes showing differential expression in PA23phzR or PA23-6863.** Twelve genes encoding regulators belonging to diverse families including AraC, ArsR, DeoR, GntR, LysR and MarR, exhibited altered expression exclusively in PA23phzR (Table 4). The latter group includes two homologues of MvaT and MvaV, which are functionally and structurally similar to the H-NS family of regulators reported to play a role in exoproduct secretion by biocontrol and pathogenic pseudomonads [12,60]. In PA23-6863, mvaT and mvaV exhibited a 1.73- and 2.92-log₂ fold reduction in gene expression, respectively. In P. protegens CHA0, biocontrol activity against Pythium ultimum was virtually abolished in mvaV mvaT double mutants, and reduced in mvaT and mvaV single mutants [60]. Surprisingly, MvaT and MvaV are repressors of most genes encoding exoproducts such as DAPG, HCN and exoproteases in CHA0, while positively modulating the production of PLT and siderophores. In P. aeruginosa PAO1, MvaT is a global regulator of virulence factors and biofilm formation, and is involved in transcriptional repression of QS [61]. At present, the role of the aforementioned regulators, including MvaT and MvaV, in PA23 physiology has yet to be defined.

**Summary**

Exploration of the QS regulon of biocontrol strain PA23 has revealed that approximately 13% of the genome is under QS control. This circuitry regulates diverse aspects of PA23 physiology that extend well beyond the secreted factors required for fungal antagonism. We believe that much of the QS regulon is subject to indirect control as phz-box elements were identified upstream of only a small percentage of QS-regulated genes. The fact that numerous transcriptional regulators show altered expression in the absence of QS further supports this notion. The number of differentially expressed genes that are unique to the AHL-deficient strain compared to the phzR mutant suggests that the Csa and/or Aur regulons are quite expansive and likely govern more than cell surface properties [15]. Future transcriptomic analysis of csaRI- and aurRI-mutants should be conducted to reveal the scope of genes under CsaRI and AurRI QS control. Such studies will undoubtedly uncover interactions with other regulators, adding another layer to the increasingly complex cascade governing expression of PA23 biocontrol factors.

**Supporting information**

S1 Fig. Autoinducer produced by PA23 and derivative strains, assessed using the AHL biosensor strain, Chromobacterium violaceum CVO26. Picture is representative of five biological replicates obtained from three independent experiments. (PDF)

S2 Fig. Functional analysis of differentially expressed genes in (A) PA23phzR and (B) PA23-6863 in comparison to wild type using Cluster of Orthologous Group (COG) analysis. The red bars indicate percent of differentially regulated genes that are downregulated, and the blue bars indicate percentage of genes that are upregulated in each category. (TIFF)
S3 Fig. Motility of PA23, PA23phzR and PA23-6863. A) Motility genes upregulated in PA23phzR and PA23-6863 compared to PA23. B) Swim plates (0.3% agar) after 24, 48, and 72 h of incubation; Swarm plates (0.8% agar) after 96 h incubation. Pictures are representative of five biological replicates obtained from three independent experiments.

(PDF)

S1 Table. RNA-sequencing library reads mapped to the Pseudomonas chlororaphis PA23 genome.

(DOCX)

S2 Table. Differentially expressed genes in PA23phzR compared to PA23 wild type.

(DOCX)

S3 Table. Differentially expressed genes in PA23-6863 compared to PA23 wild type.

(DOCX)

S1 File. Expression level of all genes in Pseudomonas chlororaphis PA23phzR and PA23-6863 relative to WT (first tab of xlsx). Rockhopper prediction of genes transcribed in the same transcriptional unit (second tab of xlsx).

(XLSX)

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