The Role of the Cytoplasmic Heme-binding protein (PhuS) of *Pseudomonas aeruginosa* in Intracellular Heme Trafficking and Iron homeostasis. A. P. Kaur, I. B. Lansky and A. Wilks

Supplementary Materials

Extraction of 4-hydroxy-2- alkyl quinolines (HAQs) and analysis by HPLC and mass spectrometry- HAQs were extracted and analyzed from cultures at the time of sampling for microarray i.e. $O.D._{600}$ of 1.5 and 3.0 as described previously (1). Briefly, 10 ml of culture was centrifuged at 15,000 × g for 15 min, the supernatant passed through a 0.2 μm filter and extracted three times with 5 ml of acidified ethyl acetate. Following extraction, the solvent was evaporated, and the resulting residue was dissolved in 200 μl of acetonitrile containing 0.1% acetic acid. Reverse phase HPLC (Waters Associates) was performed on a 3.0 x 250 mm C18 column (Atlantis™ dC18 5μm) using a modification of a previously described procedure (2). Samples were analyzed over a 60 min linear 30 to 100% gradient of acetonitrile containing 0.1% acetic acid. The column was re-equilibrated with 100% acetonitrile containing 0.1% acetic acid for 3 min and returned to 30% acetonitrile containing 0.1% acetic acid for a further 10 min at a constant flow rate of 0.3 ml/min. The elution profile was monitored at 330 nm and the major peaks collected and analyzed on a Finnigan LCQ classic ion-trap Mass Spectrometer (Finnigan MAT, San Jose, CA) equipped with an ESI source. The MS instrument was operated in a positive-ion mode with Finnigan Xcalibur software 1.1. The spectra were obtained in full scan mode (150-350 *m/z*). The collision energy for collision induced dissociation was set to 35 eV. As a control, a PQS standard (a gift from Dr. Susanne Häussler) was subjected to the same separation and analysis to confirm the presence of PQS as the major product.

*Real-time PCR assays-*To determine the validity of the microarray data, changes in transcript level observed on microarray analysis were validated by quantitative real-time PCR (qRT-PCR). Genes and primer sequences utilized for the analysis are listed in the Supplementary data (Table S1). The RT-PCR was performed on a Biorad iCycler with the iScript cDNA Synthesis Kit and iQ SYBR Green Supermix kit (BioRad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions. All reactions were normalized to the *rpoD* gene encoding the principle sigma factor, σ^{70} (3). The relative change in transcript levels were determined according to the $2^{-\Delta\Delta C_T}$ procedure (4). The melt-curve analysis was performed to evaluate PCR specificity.

Supplementary Table S1. List of primers used for RT-PCR.

Gene	Sense Primer	Anti-sense Primer
lecB	TTCGCCCTGGTCGGCTC	CCGAGCGGCCAGTTGATC
mexG	CTGGACCGCAAGCTATGGC	AGAAGGTGTGGACGATGAGGAT
mexR	AACTACCCCGTGAATCCCGA	GCTCGCTCTGGATGCGC
pchR	GCGATGCAGGCGTTGGC	CTCGCCGGAAAGGAACTGTG
phnA	CGTTGAGCGAGGACTGTCC	CCCAGCGTTCCGAGCAC
phzA1	TCGAGGACGGCTGCGC	CGTTGTGCCACTCCCAGTC
phzG2	GCCGCCCGTACCAGACC	TCGTGCAGCCGCTCGG
pqsA	GCCATTCCCGCCGTGATC	CGCCAAAGGACCGCTCAG
pqsE	GGATGATGACCTGTGCCTGTT	CCTTCAACCAGCGCCCAG
pvdS	CTTCGAGATGTACCGCCTGC	CGTCGCGGATCATGAAGTTGA
tonB	CCGCCGAAGATCGAGGAAC	GGCTGGGCTGGCTGGAT

Supplementary Table S2. Validation of the microarray analysis by RT-PCR.

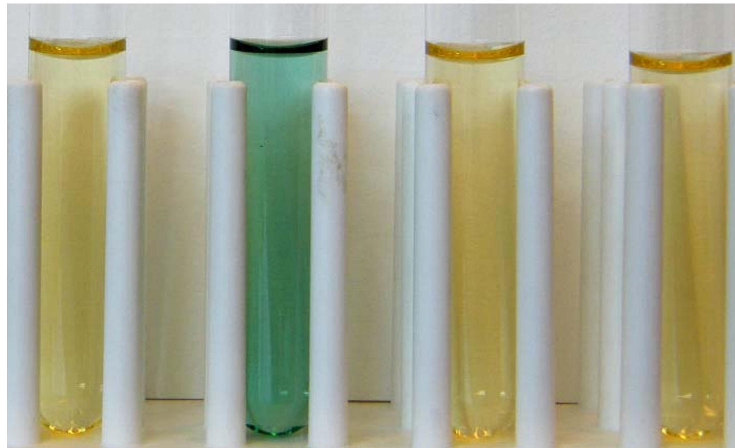
Gene	Fold change by RT-PCR[a]	Fold change by microarray[b]
lecB	9.2 ± 1.6	15.6 ± 0.03
mexG	89.5 ± 3.1	81.9 ± 0.03
mexR	4.3 ± 0.04	4.90 ± 0.04
pchR	6.3 ± 0.7	4.02 ± 0.03
phnA	13.7 ± 1.9	10.0 ± 0.26
phzA1	34.9 ± 3.6	40.3 ± 0.05
phzG2	22.0 ± 2.3	23.1 ± 0.20
pqsA	12.8 ± 0.9	10.2 ± 0.23
pqsE	7.3 ± 0.05	6.9 ± 0.29
pvdS	20.05 ± 4.8	13.5 ± 0.05
tonB	2.89 ± 0.59	4.2 ± 0.05

(a) The fold change values were calculated according to the $2^{-\Delta\Delta C_T}$ procedure (4). Values represent the mean of three experiments ± standard errors.

(b) The fold change was calculated as described in materials and methods. A positive value indicates the up-regulation and a negative value the down-regulation of a transcript in the *phuS* mutant. The values are shown with standard errors. Each mean was calculated from three separate microarray experiments for the wild type and *phuS* mutant.

A

IA614 **IA614 -Δ*phuS*** **IR1648** **IR1648 -Δ*phuS***



B

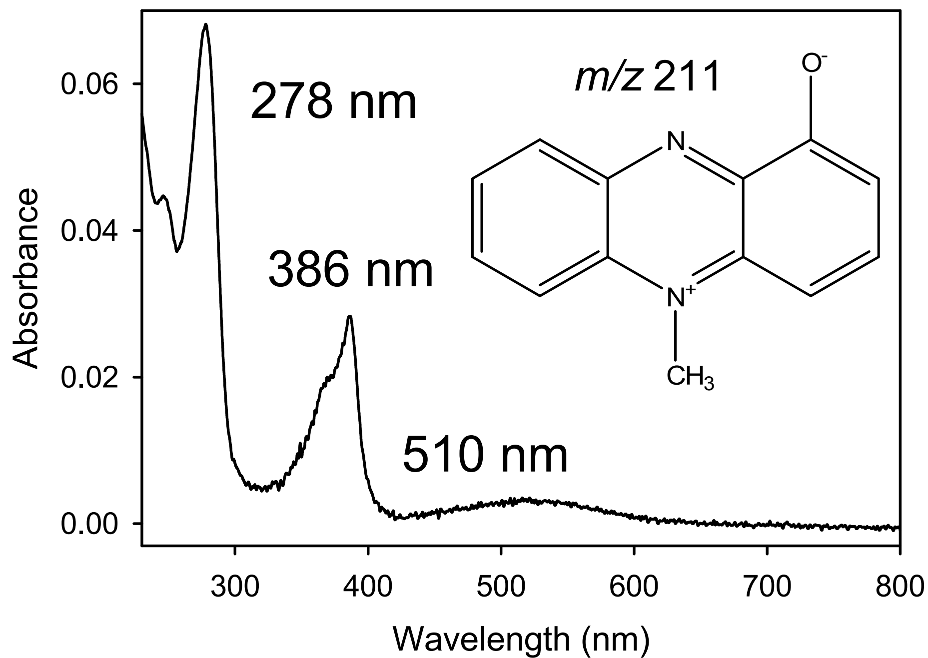


Fig. S1. *Pyocyanin production in the P. aeruginosa isogenic wild type IA614 and mutant strains*. (A) Cell free LB-media from 24 hour cultures. (B) UV-visible spectrum of pyocyanin in 0.2 N HCl. Inset; chemical structure of pyocyanin and the mass as calculated by ESI-MS.

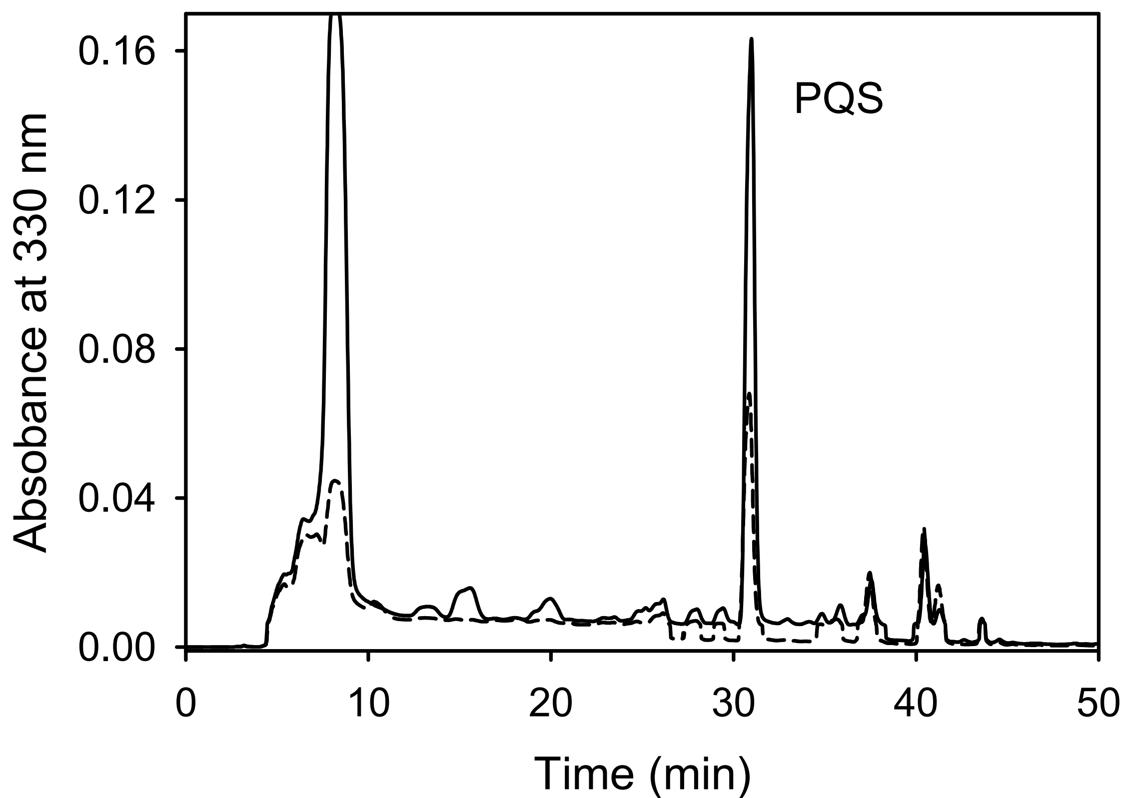


Fig. S2. *HPLC analysis of extracted 4-hydroxy-2-alkyl quinolines from the wild type MPAO1. (----) and the 7520 phuS::Tn (——) mutant.* The samples were extracted from media following pelleting of the cells from cultures grown to an O.D.$_{600}$ of 1.5.

REFERENCES

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