



SawR a new regulator controlling pyomelanin synthesis in *Pseudomonas aeruginosa*



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ABSTRACT

Surface Acoustic Waves (SAW) were previously shown to inhibit biofilm formation, increase bacterial susceptibility to antibiotic treatment and alter the transcription pattern of *Pseudomonas aeruginosa*. Here we characterize one gene, *sawR* (PA3133), that is highly overexpressed when *P. aeruginosa* is exposed to SAW. SawR is a putative transcription factor belonging to the TetR regulator family. When overexpressed *sawR* causes numerous phenotypes, including the accumulation of a brown pigment which we identified as pyomelanin. In this study we describe how *sawR* regulates pyomelanin synthesis. We show that *sawR* down-regulates the expression levels of *hmgA* and this causes the accumulation of homogentisic acid which in turn undergoes oxidation and polymerization to pyomelanin. Using bioinformatics, we were able to identify a specific amino acid, arginine 23, which is found within the *sawR* DNA binding domain and is crucial for its regulatory activity. Our results indicate that *sawR* does not affect any other genes in the phenylalanine/tyrosine metabolic pathway and its repressive ability on *hmgA* is not mediated by the *hmgA* repressor PA2010 (i.e. *hmgR*). Taken together, our results shed light on the regulatory cascade controlling pyomelanin synthesis and uncover yet another unknown regulator involved in its regulation.

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that enacts diverse metabolic functions and complex regulatory cascades. One of the most visible phenotypes of *P. aeruginosa* is its ability to produce a range of pigments under certain growth conditions. These include: (i) Pyocyanin, which gives *P. aeruginosa* its characteristic blue-to-green color and has been determined to display antibiotic, antifungal and cytotoxic properties which are thought to contribute to *P. aeruginosa* pathogenesis (Jayaseelan et al., 2014). (ii) Pyoverdine, which is associated with a yellow-green and fluorescent color and acts as a siderophore and as an important virulence factor (Schalk and Guillon, 2013). (iii) Pyorubin which is a red-to-pink pigment that can protect the bacterium from oxidative stress (Ogunnariwo and Hamilton-Miller, 1975). And finally (iv) the brown pigment pyomelanin.

Pyomelanin is a dark colored pigment that belongs to the melanin pigment family (Schmaler-Ripcke et al., 2009). Melanins are produced by a range of organisms including: animals, plants, humans and bacteria (Solano, 2014). Pyomelanin has been reported to be associated with many functions including: resistance to oxidative stress (Ahmad et al., 2016), iron uptake (Chatfield and Cianciotto, 2007) and virulence (Hunter and Newman, 2010). In *P. aeruginosa*, over production of

pyomelanin has been reported to be common in strains recovered from cystic fibrosis and urinary tract infection patients (Rodríguez-Rojas et al., 2009).

The synthesis of pyomelanin is a byproduct of the phenylalanine/tyrosine metabolic pathway and involves five different proteins, PhhR that transcriptionally regulates the metabolic pathway and four enzymes: phenylalanine-4-hydroxylase (PhhA), pterin-4- α -carbinolamine dehydratase (PhhB), aromatic amino acid aminotransferase (PhhC), and 4-hydroxyphenylpyruvate dioxygenase (Hpd) (Arias-barrau et al., 2004; Palmer et al., 2010). Homogentisic acid (HGA) is the product of Hpd and the substrate of homogentisate 1, 2-dioxygenase (HmgA) (Chatfield and Cianciotto, 2007). Under normal conditions, HmgA degrades HGA to maleylacetoacetate. However, when the levels of HmgA are low (e.g. repression of *hmgA* expression), accumulation of HGA causes its secretion out of the cell where it undergoes oxidation and self-polymerization into pyomelanin (Ketelboeter et al., 2014). In *P. putida*, it was previously shown that *hmgA* is repressed by HmgR (Arias-barrau et al., 2004). HmgR is a member of the TetR family of transcription regulators (Ramos et al., 2005) and it binds directly to the *hmgA* promoter and represses *hmgA* expression. In fact, strains that over express *hmgR* are known to accumulate pyomelanin (Arias-barrau et al., 2004).

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Our laboratory, has been investigating the biological effect of low-frequency surface acoustic waves (SAW). SAW have the ability to travel through solid material creating soft vibrations (Wohltjen and Dessy, 1979). Previous studies have demonstrated that SAW can successfully prevent microbial biofilm formation on medical devices and catheters (Hazan et al., 2006; Kopel et al., 2011). We have shown that in addition to its ability to prevent biofilm formation, SAW also affects the resistance of biofilms to antibiotics (Kopel et al., 2011). We have also discovered that SAW triggers a specific bioacoustic response and can alter the transcription pattern of *P. aeruginosa*. One of the genes that was most highly up-regulated upon exposure to SAW was PA3133 (Kopel et al., 2011), herein termed *sawR* (i.e. surface acoustic wave regulator). *SawR* shares homology to the TetR transcription regulator family (Ramos et al., 2005). In the current study we begin to characterize the role of *SawR* and we demonstrate its involvement in pyomelanin production by inhibiting *hmgA* expression. We also identify PA2010 as the HmgR homolog in *P. aeruginosa*. Finally, we present data that suggest that the regulation of *SawR* is indirect but not mediated through HmgR. Taken together our results provide important insight into pyomelanin biosynthesis and highlight a new regulator, *SawR*, that regulates pyomelanin production.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Supplementary Table S1. *P. aeruginosa* strains were grown at 37 °C in Luria-Bertani medium (LB, Difco) with agitation. Solid medium was prepared by adding 1.5% agar (BA, Difco) to LB.

Antibiotics were added to the media as required for selection: for *P. aeruginosa*, 300 µg/ml carbenicillin (Carb) and 100 µg/ml gentamicin (Gm); for *Escherichia coli*, 100 µg/ml ampicillin (Amp) and 10 µg/ml gentamicin (Gm). Tyrosine 10 mM (Sigma) was added to the media to achieve elevated activity of the phenylalanine/tyrosine metabolic pathway. Ascorbic acid 10 mM (Sigma) was added to media to inhibit pyomelanin production.

The following growth media were used during the process of constructing deletion *P. aeruginosa* mutant strains: Vogel Bonner Minimal Medium (VBMM), *Pseudomonas* Isolation Agar (PIA, Difco) and No salt Luria-Bertani (NSLB) containing 10% sucrose.

2.2. Molecular methods

DNA fragments were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo). Amplified products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen). DNA and plasmids were digested using FastDigest restriction enzymes (Thermo Scientific). Ligation was done using T4 DNA Ligase (Fermentas). All of these processes were carried out according to the manufacturer's instructions.

2.3. Plasmid construction

To create the *sawR* and PA2010 overexpressing strains, both genes were amplified using PCR (primers 21–24 listed in Supplementary Table S2). The amplified fragments were then digested by BamHI and HindIII and inserted into pUCP18 (Schweizer, 1991) digested by the same enzymes. The plasmids were then introduced into DH5α (NEB5α, Invitrogen) by electroporation and the bacteria were plated on LB agar containing Amp. The plasmids were extracted and sequenced for sequence verification. The plasmids were then inserted into PAO1 by electroporation (Smith and Iglewski, 1989) and the bacteria were plated on LB agar containing Carb.

To create histidine tagged *sawR* and PA2010 over expressing strains (His-tag), both genes were PCR-amplified using a reverse primer

containing six histidine codons (primers 25–28 listed in Supplementary Table S2). The amplified fragments were then digested by BamHI and EcoRI and ligated into pUCP18 which was cleaved by the same enzymes. The plasmids were then inserted into DH5α by electroporation and the bacteria were plated on LB agar containing Amp. The plasmids were extracted and sequence-verified, and then inserted into PAO1 by electroporation and plated on LB agar containing Carb.

For the construction of the point mutated *sawR* (R23E), the mutated *sawR* sequence was synthesized by hylabs® and PCR-amplified (primers 29–30 listed in Supplementary Table S2). The amplified fragment was then digested by BamHI and EcoRI and ligated into pUCP18 which was cleaved by the same enzymes. The plasmid was then inserted into DH5α (NEB5α) by electroporation and the bacteria were plated on LB agar containing Amp, followed by sequence verification. The plasmid was then inserted into PAO1 by electroporation and the bacteria were plated on LB agar containing Carb.

2.4. Strain construction

To construct the *sawR* mutant strain, fragments upstream and downstream to the gene were PCR-amplified (primers 1–4 listed in Supplementary Table S2). The upstream fragment was then digested by HindIII and XbaI and ligated into pEX18-Gm (Choi and Schweizer, 2005) to create *p-UpsawR*. The downstream fragment was digested by BamHI and SacI and ligated into *p-UpsawR* to create *p-UDsawR*. The plasmid was then inserted into *E. coli* strain DH5α by electroporation and plated on gentamicin (Gm) plates for selection. The plasmid was extracted, sequenced and inserted into *E. coli* strain S17 (Thoma and Schobert, 2009) for conjugation.

To construct the *hpd*, *hmgA*, PA2010 and *kata* mutant strains, fragments upstream and downstream to the genes were amplified using PCR (primers 5–20 listed in Supplementary Table S2). The two PCR products (upstream and downstream for each gene) were then joined by amplification using primers that targeted the adjacent regions upstream and downstream, via splicing by overlapping extension (SOE) PCR (Anton and Ichiro, 2011). We then used Gateway recombination (Hmelo et al., 2015) to insert the PCR product into pEX18GmGW (Hmelo et al., 2015) using BP clonase (Invitrogen) according to the manufacturer's instructions. The plasmids were then inserted into *E. coli* strain DH5α by electroporation and plated on Gm plates for selection. The plasmids were extracted, sequence-verified and inserted into *E. coli* strain S17 for conjugation.

For the conjugation from S17 (donor strain) into PAO1 (acceptor), strains were grown separately in LB medium at 37 °C overnight. The acceptor strain was then diluted (1:2) into 6 ml LB medium and grown for an additional 3 h at 42 °C without agitation. Donor strains (S17/p-UDsawR, S17/p-UDhpd, S17/p-UDhmgA, S17/p-UDPA2010 and S17/p-UDkata) were diluted 1:100 into 6 ml and grown for an additional 3 h at 37 °C with agitation. After incubation, 1 ml of the acceptor strain and 3 ml of the donor strain were transferred onto durapore membrane filters (0.45 µm HV, Millipore). The membrane, holding the strains, was transferred onto LB agar plate and incubated overnight at 30 °C. Strains were then scraped off the membrane, suspended in Phosphate buffered saline (PBSX1, Sigma) and plated on VBMM plates containing 100 µg/ml Gm and incubated at 37 °C for 36 h for selection. Strains were then streaked on NSLB plates containing 10% sucrose and incubated at 30 °C for 24 h. Colonies were then plated on LB, PIA and LB containing 100 µg/ml Gm. Finally, PCR was carried out to verify the mutation was conferred on colonies that did not grow on LB plates containing Gm.

2.5. Bioinformatics analysis

Protein blast was used in order to find the *sawR* DNA binding site. In order to identify the most conserved amino acids at the DNA binding site a multiple sequence alignment was performed comparing the DNA binding site of the *sawR* homologs from *Pseudomonas* and non-

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