Pseudomonas aeruginosa MifS-MifR Two-Component System Is Specific for α-Ketoglutarate Utilization

Gorakh Tatke1,2, Hansi Kumari2*ab, Eugenia Silva-Herzog2*ab, Lourdes Ramirez1, Kalai Mathee2*aa

1 Department of Biological Sciences, College of Arts & Sciences, Florida International University, Miami, Florida, United States of America, 2 Department of Molecular Microbiology and Infectious Diseases, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida, United States of America

* Current address: Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida, United States of America

Abstract

Pseudomonas aeruginosa is a Gram-negative, metabolically versatile opportunistic pathogen that elaborates a multitude of virulence factors, and is extraordinarily resistant to a gamut of clinically significant antibiotics. This ability, in part, is mediated by two-component regulatory systems (TCS) that play a crucial role in modulating virulence mechanisms and metabolism. MifS (PA5512) and MifR (PA5511) form one such TCS implicated in biofilm formation. MifS is a sensor kinase whereas MifR belongs to the NtrC superfamily of transcriptional regulators that interact with RpoN (σ54). In this study we demonstrate that the mifS and mifR genes form a two-gene operon. The close proximity of mifSR operon to poxB (PA5514) encoding a β-lactamase hinted at the role of MifSR TCS in regulating antibiotic resistance. To better understand this TCS, clean in-frame deletions were made in P. aeruginosa PAO1 creating PAOΔmifS, PAOΔmifR and PAOΔmifSR. The loss of mifSR had no effect on the antibiotic resistance profile. Phenotypic microarray (BioLOG) analyses of PAOΔmifS and PAOΔmifR revealed that these mutants were unable to utilize C5-dicarboxylate α-ketoglutarate (α-KG), a key tricarboxylic acid cycle intermediate. This finding was confirmed using growth analyses, and the defect can be rescued by expressing mifR or mifSR expressed in trans. These mifSR mutants were able to utilize all the other TCA cycle intermediates (citrate, succinate, fumarate, oxaloacetate or malate) and sugars (glucose or sucrose) except α-KG as the sole carbon source. We confirmed that the mifSR mutants have functional dehydrogenase complex suggesting a possible defect in α-KG transport. The inability of the mutants to utilize α-KG was rescued by expressing PA5530, encoding C5-dicarboxylate transporter, under a regulatable promoter. In addition, we demonstrate that besides MifSR and PA5530, α-KG utilization requires functional RpoN. These data clearly suggests that P. aeruginosa MifSR TCS is involved in sensing α-KG and regulating its transport and subsequent metabolism.
Introduction

*Pseudomonas aeruginosa* is a metabolically versatile, Gram-negative opportunistic pathogen that is well known for its extensive spatio-temporal distribution [1]. It is a dominant nosocomial pathogen capable of causing acute and chronic infections in immunocompromised and immunosuppressed patients [2,3]. In particular, patients with AIDS, severe burn wounds, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis and neutropenia are predisposed to *P. aeruginosa* infections [1,4–7]. *P. aeruginosa* chronic pulmonary infections are characterized by intensive bronchial neutrophilic inflammation resulting in respiratory failure [8,9], a major cause of fatality in CF patients [10]. Moreover, *P. aeruginosa* is associated with keratitis [11] and chronic suppurative otitis media [12] leading to visual impairment and deafness [13,14]. *P. aeruginosa* possess numerous virulence factors, both cell-surface associated and secretory, which significantly contribute to its pathogenesis [15]. Effective treatment of *P. aeruginosa* infections is impeded by its extraordinary intrinsic and acquired resistance to numerous clinically important antibiotics [16]. Thus, antibiotic resistance and expression of multi-determinant virulence factors are two critical hallmarks in *P. aeruginosa* infections that make it an intimidating pathogen.

Successful infection and disease progression depends significantly on the ability of any pathogen to effectively utilize available nutrients that are essential for its growth and survival. *P. aeruginosa* is renowned for its extraordinary ability to utilize wide range of organic compounds such as carbohydrates, amino acids, fatty acids, mono- and polyalcohols, di- and tri-carboxylic acids as sources of carbon, nitrogen and energy [1]. However, unlike other bacteria where glucose is the preferred carbon source [17,18], *P. aeruginosa* preferentially utilizes tricarboxylic acid (TCA) cycle intermediates [19,20], specifically, C₄-dicarboxylates of the TCA cycle such as malate, fumarate and succinate [19–21].

The TCA cycle is an amphibolic pathway that serves two main purposes: energy-generation in aerobic organisms (catabolism), and the generation of intermediates to serve as biosynthetic precursors for fatty acid, amino acid and carbohydrate synthesis (anabolism) [22]. The metabolic intermediates of the TCA cycle consist of a group of organic anions that include C₄-dicarboxylates (succinate, fumarate, malate and oxaloacetate), C₅-dicarboxylates (α-ketoglutarate (α-KG)) and C₆-tricarboxylates (citrate, isocitrate) [23,24]. However, the role of TCA cycle intermediates is not restricted to energy metabolism or to serve as biosynthetic precursors. In the recent years, TCA cycle intermediates, in-particular, succinate and/or α-KG have gained significant importance as biological signaling molecules in variety of organisms including, bacteria [25], animals [26] and plants [27].

Sensing the available nutrients is a prerequisite for mobilizing the uptake systems. Bacterial two-component systems (TCSs), involving a membrane-bound histidine sensor kinase (HK) and a cytoplasmic response regulator (RR) play an integral part in bacteria’s ability to sense physiological cues. In response to stimuli, the sensor autophosphorylates at a conserved histidine residue at the C-terminus, and subsequently the phosphate is transferred to an aspartate residue at the N-terminus of the RR [28–30]. TCSs in *Bacillus subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Klebsiella pneumoniae*, *Rhizobium meliloti* and *Rhizobium leguminosarum* have been shown to regulate extracellular C₄-dicarboxylates and tricarboxylates transport [28,31–36]. Of these, DctB-DctD in *R. meliloti* is an extensively studied TCS, which in coordination with sigma factor RpoN(σ⁵⁴) regulates the extracellular transport of C₄-dicarboxylates succinate, fumarate and malate [37,38].

Three TCS protein pairs in *P. aeruginosa* namely, PA5165/PA5166 (DctB/DctD), PA5512/PA5511 (MifS/MifR) and PA1336/PA1335 have been identified to be homologous to the *Rhizobium* C₄-dicarboxylate transport regulatory DctB/DctD [39]. Amongst the three, very little is
known of PA1336/PA1335. The PA5165/PA5166 (DctB/DctD) TCS has been demonstrated to regulate the transport of C₄-dicarboxylates, succinate, fumarate and malate in coordination with the sigma factor RpoN ($\sigma^{54}$) [39]. The SK MifS (65.3 kDa) and RR MifR (49.6 kDa) share 51% and 69% sequence identity to the $R$. meliloti DctB and DctD, respectively [40]. The RR MifR is involved in regulating the maturation stage of $P$. aeruginosa biofilm formation as mifR deficient mutants fail to form microcolonies [41]. Later studies reported the interdependence of pyruvate fermentation and functional MifR in supporting microcolony formation [42]. However, the mechanism by which MifR is activated in this process remains obscure and no relation with HK MifS has been established. Using clean in-frame deletion mutants of the mifS, mifR and mifSR genes we show that MifSR TCS regulates $P$. aeruginosa $\alpha$-KG transport and requires functional RpoN.

**Results**

*mifS and mifR are a part of a two-gene operon*

In eubacteria, the genes that encode a HK and its cognate RR are often linked and are co-transcribed [30]. Our sequence analysis of $P$. aeruginosa PAO1 genome revealed that mifS (PA5512) and mifR (PA5511) are adjacent to each other, in the same orientation. The predicted translation start site of mifR ORF overlaps with mifS translation termination codon indicating that they are cotranscribed (Fig 1A and 1B). To determine if these two genes form an operon, cDNA across the intergenic regions spanning mifS and mifR was amplified using GDT_co-transF1-R1 and GDT_cotransF2-R2 primers (see Materials and Methods). As expected, 200 bp and 100 bp products were detected when using primers that span the overlapping region (Fig 1C, Lane 3 and Lane 4). These results confirm that mifS and mifR are a part of a two-gene operon. As controls, the mifSR genes were also amplified (Fig 1C, Lane 2).

**Loss of mifS and mifR did not affect antibiotic resistance**

To identify the role of MifSR TCS, clean in-frame deletion mutants of mifS, mifR and mifSR were constructed in the prototypic $P$. aeruginosa PAO1. Henceforth they will be referred to as PAOΔmifS, PAOΔmifR and PAOΔmifSR, respectively. For complementation studies, recombinant plasmids containing the entire mifR, mifS and mifSR genes were constructed. The complementing plasmids with the genes are called pMifS, pMifR and pMifSR. These plasmids were introduced into the respective mutant strains.

Previous studies in our lab postulated that the MifSR TCS system, found 81-bp upstream of the poxB operon, may contribute to $P$. aeruginosa B-lactam resistance [43] as the genes regulated by TCS tend to be co-located on the chromosome [30]. However, MIC analyses using E-test and micro-dilution methods showed that the loss of these genes did not affect the antibiotic resistance profile when compared to the parent strain, $P$. aeruginosa PAO1 (Data not shown). Further, qRT-PCR studies showed that deletion of mifS, mifR and mifSR had no effect on the expression of poxB compared to the parent PAO1 (Fig 2).

*mifS, mifR and mifSR mutants failed to grow in the presence of $\alpha$-KG*

The PAOΔmifS, PAOΔmifR and PAOΔmifSR mutants exhibited no discernible phenotype compared to the parent PAO1 when tested for growth, swimming, swarming, twitching motility (LB media), pyocyanin production (LB & King’s A media), pyoverdine production (LB & King’s B Media), congo red binding assay (CR media) and antibiotic resistance (MH media) (Data not shown). Hence, a comparative phenotypic microarray analysis was performed with the wild-type PAO1, PAOΔmifR and PAOΔmifS mutants (BioLOG Inc.). Out of approximately
2000 metabolic and chemical sensitivity assays tested, PAOΔmifR exhibited four gain-of-function and 29 loss-of-function phenotypes whereas PAOΔmifS exhibited two gain-of-function and 23 loss-of-function phenotypes (Fig 3A). A single gain of function phenotype shared

Fig 1. Genome organization of the mifSR gene locus. In P. aeruginosa PAO1 the mifR (PA5511) ORF has a translation start codon (ATG) overlapping the mifS (PA5512) termination codon (TGA), denoted in red (B), suggesting that the mifS and mifR genes are physically linked. The cDNA amplification of the intergenic region spanning the mifS and mifR genes using GDT_cotrans F1-R1 and GDT_cotrans F2-R2 primers (Table 1) confirm that the two genes mifS and mifR are co-transcribed and form an operon (C).

doi:10.1371/journal.pone.0129629.g001
between PAOΔmifS and PAOΔmifR, was the ability to utilize L-methionine. When metabolism and chemical sensitivity were compared, the mutants appear more sensitive to various antibiotics (Fig 3B). However, none of these were reproducible in the lab in the MH media. The loss of mifS and mifR resulted in differential phenotype in the presence of six metabolites, amongst which, two were common to both mifS and mifR mutants (Fig 3B). The shared metabolic phenotypes involved the utilization of L-methionine and α-KG (Fig 3C). Compared to the parent PAO1, the mutants did not exhibit any growth increase when provided with L-methionine (Fig 4). This could be simply due to the difference in culture conditions and BioLOG proprietary media.

![Fig 2. Expression of poxB (PA5514) in mifSR mutants.](image)

Expression of poxB (PA5514) was tested in mifSR mutants relative to PAO1. Data was normalized to expression in PAO1. Bars above or below the line represents up- and down-regulation, respectively and the bars indicate standard errors. The clpX gene (PA1802) was used as the housekeeping control. There was no statistically significant difference (p-value > 0.05) between the wild type PAO1 and mifSR mutant strains as determined by one-way ANOVA and student’s unpaired t test.

doi:10.1371/journal.pone.0129629.g002
The inability to utilize \(\alpha\)-KG by PAO\(\Delta mifS\) (Fig 5A) and PAO\(\Delta mifR\) (Fig 5B) in the BioLOG assay was reproduced in M9 minimal media supplemented with 30 mM \(\alpha\)-KG (Fig 5C). In fact, all three mutant strains, PAO\(\Delta mifR\), PAO\(\Delta mifS\) and PAO\(\Delta mifSR\) failed to grow in the presence of \(\alpha\)-KG (Fig 5C). To rule out potential toxicity, the wild-type \(P.\ aeruginosa\) PAO1 and the mutants were cultured in M9 minimal media with varying concentrations of \(\alpha\)-KG, ranging from 1 to 80 mM (Fig 6). The mutants exhibited no growth in the presence of \(\alpha\)-KG after 24 h at 37°C, whereas the wild-type PAO1 exhibited an increase in growth that was proportional to \(\alpha\)-KG concentration (Fig 6B). All subsequent experiments were done with 30 mM \(\alpha\)-KG.

![Fig 3. mifS and mifR dependent phenotypes.](image)

Fig 3. mifS and mifR dependent phenotypes. To identify the role of \(P.\ aeruginosa\) mifSR TCS, comparative phenotypic microarray of PAO\(\Delta mifS\), PAO\(\Delta mifR\) mutants and wild-type PAO1 strain was performed at BioLOG Inc. (Hayward, CA, USA). Venn diagram of differentially regulated phenotypes of the mutants compared to their isogenic parent PAO1, showing gain of function or loss of function phenotypes (A). Phenotypic differences were further classified based on metabolic and chemical sensitivity properties (B). The phenotypes common to both mifS and mifR mutants are listed (C).

doi:10.1371/journal.pone.0129629.g003

| Substrate                  | Nature of substrate                                                                 | Role |
|----------------------------|-------------------------------------------------------------------------------------|------|
| L-Methionine               | S-source, Nutritional supplement                                                    |      |
| a-Ketoglutaric acid        | C-Source, carboxylic acid                                                           | M    |
| Vancomycin                 | Wall                                                                                |      |
| Sulfadiazine               | Folate antagonist, PABA analog                                                      |      |
| Ribostamycin               | Protein synthesis, 30S ribosomal subunit, aminoglycoside                            |      |
| 5- Fluorouracil            | Nucleic acid analog, pyrimidine                                                     |      |
| Sodium metavanadate        | Toxic anion, PO4 analog                                                            |      |
| Sodium orthovanadate       | Toxic anion, PO4 analog                                                            |      |
| 2- Nitroimidazole          | Nitro compound, oxidizing agent, ribonucleotide DP reductase inhibitor             |      |
| Cefotaxime                 | Wall, cephalosporin                                                                |      |
| Norfloxacin                | DNA topoisomerase                                                                   |      |
| Streptomycin               | Protein synthesis, 30S ribosomal subunit, aminoglycoside                            |      |
| Coumarin                   | DNA intercalator                                                                   |      |
growth defect exhibited by PAOΔmifS, PAOΔmifR and PAOΔmifSR could be restored to the wild-type levels by introducing mifR and mifSR genes into the respective mutants (Figs 5D and 7A).

 α-KG is a key TCA cycle intermediate (Fig 8) and plays an important role in regulating carbon and nitrogen metabolism [44]. It has been previously shown that P. aeruginosa preferentially utilizes TCA cycle intermediates as a carbon source over other compounds [20,21,45]. To test if the growth defect exhibited by the loss of mifS and mifR is restricted to α-KG utilization, the mutants and the complementing strains were grown in the presence of TCA cycle intermediates citrate, succinate, fumarate, malate and oxaloacetate at 30 mM each. No difference in growth was observed between wild type PAO1 and its isogenic mutants in the presence of other TCA cycle intermediates except for α-KG (Table 1). This is not surprising as P. aeruginosa can use the glyoxylate shunt pathway to bypass the need for α-KG (Fig 8) [46]. Furthermore, no difference in the growth profile of the wild type PAO1 and mifSR mutants was
observed when grown in the presence of sugars, glucose and sucrose (30 mM each) (Data not shown). To reconfirm that the presence of α-KG is not toxic, the cells were grown in the presence of citrate and succinate combined in equal concentration with α-KG. The mutants and the wild type shared similar early exponential growth (Fig 9). However, the mutants reached stationary phase earlier as compared to the parent strain PAO1. This suggests that the presence of excess carbon source in the form of α-KG further contributes to the growth of PAO1. These analyses indicate that mifSR mutants are only defective in α-KG utilization.

mifSR mutants are defective in α-KG transport

The absence of growth in the presence of exogenous α-KG could be due to either failure to enter the cells or loss of the mutants’ ability to convert α-KG to succinate. The latter is likely if the mutants failed to express a functional α-KG dehydrogenase complex. The ability of mifSR mutants to grow effectively in the presence of citrate and succinate suggests that these mutants are likely to harbor a functional α-KG dehydrogenase complex, unless the mutants bypass it using the glyoxylate shunt (Fig 8). The former is likely as qPCR analysis of genes encoding...
isocitrate dehydrogenase \((idh, icd)\) and \(\alpha\)-KG dehydrogenase complex \((sucA, sucB, lpd3)\) revealed no difference in the expression levels in the wild-type PAO1 and \(mifSR\) mutants (Fig 10).

\(\alpha\)-KG is a hub for anaplerotic reactions, a process for replenishing TCA cycle intermediates. In this process glutamate, glutamine, proline and arginine act as precursor molecules for \(\alpha\)-KG synthesis [47]. Growth studies in the presence of these amino acids would serve as another indirect measure to test the functionality of \(\alpha\)-KG dehydrogenase complex in \(mifSR\) mutants. To test this hypothesis, PAO1, \(PAO\Delta mifR\), \(PAO\Delta mifS\) and \(PAO\Delta mifSR\) mutants were cultured in the presence of glutamate, glutamine, proline and arginine (Table 2). The parent PAO1 and the isogenic mutants exhibited similar growth phenotype. From the expression studies and growth analyses we deduce that the \(mifSR\) mutants are impaired in \(\alpha\)-KG transport.

**mitSR TCS genes regulate extracellular \(\alpha\)-KG transport**

In a recent study using transposon mutagenesis; PA5530 was identified as the functional \(\alpha\)-KG transporter [48]. To confirm the role of \(P.\ aeruginosa\) PA5530 in \(\alpha\)-KG uptake and identify the role of \(mitSR\) genes, the gene was amplified and subcloned downstream of the inducible \(P_{lacUV5}\) promoter. The plasmid pPA5530 was introduced into PAO1 and the \(mitSR\) mutants. Expression of PA5530 in \textit{trans} in \(PAO\Delta mifS\), \(PAO\Delta mifR\), \(PAO\Delta mifSR\) mutants restored their growth to a level similar to the wild-type PAO1 in M9 minimal media with \(\alpha\)-KG (30 mM) as the sole carbon source (Fig 7B). Expression of an extra copy of PA5530 gene in the wild-type PAO1 did not affect its growth (Fig 5E). This finding suggests that expression of PA5530 is likely regulated by MifSR and/or \(\alpha\)-KG. In fact, expression of PA5530 is regulated by \(\alpha\)-KG, as seen in qRT-PCR analysis when PAO1 was grown in M9 media with varying amounts \(\alpha\)-KG.
The loss of mifS, mifR and mifSR results in a significant decrease in PA5530 expression as compared to the wild type PAO1 in the presence of α-KG (Fig 11B). Thus, α-KG-dependent PA5530 expression requires MifS and MifR.

RpoN (σ^{54}) is required for α-KG utilization

The closest P. aeruginosa MifS and MifR homologs are R. meliloti DctB and DctD [40]. In fact, MifR is 69% similar to R. meliloti DctD that belongs to the Sigma 54 (σ^{54}) dependent NtrC family of transcriptional regulators [39,40]. Thus, it is likely that MifR has the conserved domains found among NtrC family of regulators, an N-terminal regulatory, a central σ^{54} activation and a C-terminal DNA binding domains [49,50]. MifR analysis using the simple modular architecture research tool (SMART) [51] and InterPro [52] revealed the presence of three domains: CheY-homologous receiver/regulatory, a central AAA+ region required for σ^{54} activation, and the DNA binding helix-turn-helix domains (Fig 12A). The central AAA+ domain contains seven conserved regions designated C1 to C7 [50] that are characteristic of σ^{54}-dependent transcriptional regulators. Sequence analysis of MifR revealed the presence of all the seven conserved regions in the AAA+ domain between amino acid residues 144 to 373 (Fig 12B).

Since MifR exhibits high identity to σ^{54}-dependent transcriptional regulators, we hypothesized that P. aeruginosa rpoN mutants should exhibit a α-KG-dependent phenotype, similar to the mifSR mutants. To verify this hypothesis, we tested the ability of PAOΔrpoN mutant to grow in the presence of α-KG (30 mM) (Table 3). As expected, PAOΔrpoN failed to grow in
Fig 8. Tricarboxylic acid (TCA) cycle and its related reactions. Enzymes converting iso-citrate to α-KG (iso-citrate dehydrogenase: Icd, Idh), α-KG to succinyl-coA (α-KG dehydrogenase complex: SucA, SucB, Lpd3) and those involved in the glyoxylate shunt (isocitrate lyase (AceA) and malate synthase G (GlnB)) are shown in bold. Green boxes indicate the amino acid biosynthetic precursors of α-KG involved in the anaplerotic reaction.

doi:10.1371/journal.pone.0129629.g008

Table 1. Growth properties of mifSR mutants in presence of TCA cycle intermediates.

| Carbon Source       | PAO1 | ΔmifR | ΔmifS | ΔmifSR |
|---------------------|------|-------|-------|--------|
| Pyruvate            | +++  | +++   | +++   | +++    |
| Oxaloacetate        | +++  | +++   | +++   | +++    |
| Citrate             | +++  | +++   | +++   | +++    |
| Succinate           | +++  | +++   | +++   | +++    |
| Fumarate            | +++  | +++   | +++   | +++    |
| Malate              | +++  | +++   | +++   | +++    |
| α-Ketoglutarate     | +++  | ---   | ---   | ---    |

++, growth; ---, no growth

Growth of the wild type PAO1 and mifSR mutants was tested in M9 minimal media supplemented with different TCA cycle intermediates at 30 mM each, as the sole carbon source. Cells were cultured for 18 to 24 h at 37°C and their growth was monitored by measuring the absorbance at 600 nm. No difference was observed in the growth rate of mifSR mutants compared to the parent PAO1 strain. Data is represented in terms of growth and no growth phenotype.

doi:10.1371/journal.pone.0129629.t001
the presence of α-KG (Table 3). The growth of the rpoN mutant was restored in PAOΔrpoN::rpoN complementing strain. Further, in trans expression of mifR and mifSR in PAOΔrpoN mutant failed to restore their growth in the presence of α-KG (Table 3). This data confirms that MifR regulatory function requires functional RpoN (σ54).

The small 81-bp mifSR promoter has no obvious RpoN sigma factor -12/-24 consensus sequence: 5’-TGGCACG-N4-TTGW-3’ in which W stands for either A or T (Fig 13A) [53]. In fact, it appears to have a potential -10 (consensus: TATAAT) but lacked -35 (consensus: TTGACA) for sigma-70 promoter (Fig 13A) [54]. On the other hand, the promoter region of PA5530 is 315-bp long with strong -12 and -24 boxes upstream of the predicted transcription start site (Fig 13B). We hypothesized that the inability of rpoN mutant to utilize α-KG can be rescued by expressing PA5530 under a regulatable promoter PlacUV5. As expected, the growth of the rpoN mutant was restored when the plasmid harboring the transporter PA5530 was expressed in trans (Table 3). This suggests that expression of PA5530 requires both MifSR TCS and RpoN.

The presence of a common motif, GATCGCGGATt/gTCC, in the PmifS and PA5530 (Fig 13A and 13B) suggest that these two operons share some common regulatory mechanism. In addition, both promoters possess multiple motifs: PmifS has two sets of large overlapping inverted repeats, and PA5530 has three sets of direct repeats (Fig 13A and 13B). However, the role of these motifs remains to be elucidated.

**Discussion**

*P. aeruginosa* pathogenicity relies significantly on its metabolic flexibility. However, establishment of successful infection and its progression requires more than just meeting nutritional...
demands. Precision in sensing environmental signals concomitant with a quick and appropriate response is the key to efficient bacterial adaptation and survival. An arsenal of TCSs encoded in its genome has furnished \textit{P. aeruginosa} with a sophisticated capability to regulate diverse metabolic and virulence processes, ensuring its success as a pathogen [55–57]. \textit{P. aeruginosa} genome encodes one of the largest groups of TCS proteins identified in any sequenced bacterial species [57,58]. Bacterial TCS’s sense and respond to a variety of external cues such as 

![Graph showing quantification of rpoN, acnA, idh, icd, sucA, and lpd3 mRNA by qRT-PCR.](image)

**Fig 10.** Quantification of \textit{rpoN}, \textit{acnA}, \textit{idh}, \textit{icd}, \textit{sucA}, and \textit{lpd3} mRNA by qRT-PCR. RNA was isolated from cells grown in M9 minimal media supplemented with citrate (30 mM), reverse transcribed to cDNA and the presence of specific transcripts was analyzed by qPCR using gene-specific primers (Table 5). The expression of genes encoding aconitate hydratase 1 (acnA (PA1562)) isocitrate dehydrogenase (idh (PA2623)) isocitrate dehydrogenase, α-KG dehydrogenase complex (icd (PA2623)), sucA (PA1585) and lpd3 (PA4829), and σ54 (rpoN (PA4462)) were analyzed in \textit{mifSR} mutants relative to PAO1 (log10 RQ = 1). Bars above or below the line represents up- and down-regulation, respectively and the bars are standard errors. The \textit{clpX} (PA1802) gene was used as the housekeeping control. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni’s post-hoc test. Difference in the expression levels of genes is not statistically significant at \(p\)-value < 0.05.

doi:10.1371/journal.pone.0129629.g010

**Table 2.** Growth profile analysis of the \textit{mifSR} mutants in presence of amino acids.

| Carbon Source | PAO1 | ΔmifR | ΔmifS | ΔmifSR |
|---------------|------|-------|-------|--------|
| Glutamate     | +++  | +++   | +++   | +++    |
| Glutamine     | +++  | +++   | +++   | +++    |
| Proline       | +++  | +++   | +++   | +++    |
| Arginine      | +++  | +++   | +++   | +++    |

+++ , growth; ---, no growth

Cells were grown in the M9 minimal media with the indicated amino acids (30 mM each). Data is represented in terms of growth and no growth phenotype.

doi:10.1371/journal.pone.0129629.t002
nutrient availability, osmolarity, redox state, temperature, and concentrations of other extracellular molecules [59]. However, very few TCS signaling molecules have been identified to date. In this study we suggest that the P. aeruginosa MifSR TCS exclusively senses α-KG, a C₅ dicarboxylate and a key component of TCA cycle. P. aeruginosa antibiotic resistance is independent of MifSR TCS

A common feature of bacterial genomes is a close association between the functionally related genes and their location on the chromosome [60,61]. Typically, genes encoding functionally related HKs and RRs are often physically linked and are co-transcribed as an operon [30,62]. Indeed, our in silico analysis (Fig 1A and 1B) and cDNA amplification (Fig 1C) revealed that mifS-mifR genes are co-transcribed and form an operon. This also suggests that HK-MifS and RR-MifR are functionally related and work as a TCS pair. In addition, TCS proteins are known to regulate expression of genes in their immediate vicinity [30]. The mifSR genes are 81 bp upstream of the two-gene poxAB (PA5513-5514) operon. Due to the proximity of mifSR to poxB which encodes for a β-lactamase, we postulated that mifSR TCS regulates antibiotic resistance. However, our initial results nullified this hypothesis in which comparative MIC's (Data
and qRT-PCR data (Fig 2) showed no difference in antibiotic resistance profiles or poxB expression between the wild-type PAO1 and mifSR single and double deletion mutants.

MifSR TCS regulates *P. aeruginosa* α-KG utilization

A previous transcriptome study of the wild-type PAO1 and a mifR deletion mutant cultivated under biofilm-specific conditions showed significant alteration in the expression of genes involved in regulating *P. aeruginosa* metabolism, small molecule transport and amino acid biosynthesis [42]. The majority of the changes observed in phenotypic microarrays of the mifS and mifR mutant strains cultivated under planktonic conditions were associated with chemical
sensitivity and not with metabolism (Fig 3B). Only 12–16% of phenotypic changes were associated with metabolism. This confirms the significant metabolic differences in the rich planktonic versus anaerobic mode of biofilm growth in *P. aeruginosa* [63].

Petrova *et al.* (2012) have also demonstrated that genes involved in energy metabolism, including anaerobic metabolism and fermentative pathways using arginine (*arcDABC*) and pyruvate, were expressed significantly less in ΔmifR mutant biofilms as compared to its parent PAO1 [42]. Though pyruvate is needed for biofilm formation, it cannot compensate for the loss of *mifR* [42]. Interestingly, the biofilm phenotype associated with the loss of *mifR* can be complemented by *ldhA* encoding D-lactate dehydrogenase to wild type levels of biomass accumulation and microcolony formation [42]. These findings suggest that MifR somehow regulates expression of *ldhA*, a second gene in a three-gene operon gacS-*ldhA*-PA0926 [57]. Importantly, analyses of the promoters reveal the presence of a shared motif in *PmifS* (GATCCGCCGATGTCC) and *PA5530* (GATCGGCGGATTTCC) (Fig 13) and *gacS* (AATCCGCCGGGCTGC) suggesting a possible coordinate regulation, and that need to be verified.

Our phenotypic microarray analyses and growth experiments suggested that *P. aeruginosa* α-KG utilization requires MifS and MifR (Figs 5 and 7A). The ability of PAOΔmifR, PAOΔmifS and PAOΔmifSR to grow in the presence of α-KG was restored by in trans expression of *mifR* and *mifSR* (Fig 7A). Interestingly, the PAOΔmifS was complemented by pMifR and pMifSR (Fig 5D) but not by pMifS alone. To rule out that gene expression may have been compromised, the *mifS* gene was cloned downstream of the inducible P_{lacUV5} promoter. Though the expression of stable protein was visible in a protein gel, it failed to complement PAOΔmifS mutant (data not shown). This suggests that cis-expression of *mifS* and *mifR* is critical for MifS-function. Other researchers have encountered similar problems involving histidine kinases [64]. Moreover, complementation of the PAOΔmifS with pMifR suggests that either phosphorylation is not required or there is a potential crosstalk between MifR and other non-cognate HKs. Alternatively, phosphorylation of MifR can occur through small molecule phosphor-donors, like acetyl phosphate, carbamoyl phosphate and phosphoramidate [65]. Such phenomenon is observed with other TCS RRs [66–68]. However, this has to be verified.

The C_5-dicarboxylate α-KG is an important intermediate in the energy-generating TCA cycle (Fig 8) and plays a key role in regulating carbon and nitrogen metabolism [44]. Similar to other bacteria [69], TCS’s in *P. aeruginosa* have been reported to regulate transport and utilization of TCA cycle intermediates such as succinate, fumarate, malate and citrate [39,56]. The *R. meliloti* DctB/DctD system is a well-characterized TCS that controls the transport of TCA cycle C_4-dicarboxylates succinate, fumarate and malate [69]. Though *P. aeruginosa* MifS/MifR proteins are homologous to *R. meliloti* DctB/DctD TCS proteins, the *mifSR* mutants efficiently

### Table 3. Growth properties of PAO1ΔrpoN and its derivatives in the presence of α-KG and LB.

| Strain        | Plasmid | α-KG  | LB    |
|---------------|---------|-------|-------|
| PAO1ΔrpoN     | -       | ---   | +++   |
| Vector        | ---     | +++   | +++   |
| pRpoN         | ++      | +++   | +++   |
| pMifR         | ---     | +++   | +++   |
| pMifSR        | ---     | +++   | +++   |
| pPA5530       | +++     | +++   | +++   |

+++ , growth; --- , no growth

Growth of PAO1ΔrpoN mutant and its derivatives was tested in the M9 minimal media supplemented with α-KG (30 mM) and in the LB media at 37°C for 24h.

doi:10.1371/journal.pone.0129629.t003
utilized citrate, succinate, fumarate, malate, oxaloacetate, sucrose and glucose but exclusively failed to grow in the presence of $\alpha$-KG (Table 1). This was further supported by another parallel study that shows that $\alpha$-KG utilization requires MifR [48]. Thus, the *P. aeruginosa* MifSR TCS is specifically and uniquely involved in C5-dicarboxylate $\alpha$-KG utilization.

MifSR TCS modulates *P. aeruginosa* $\alpha$-KG transport

The inability to utilize $\alpha$-KG suggested that the mifSR mutants either have a defective $\alpha$-KG dehydrogenase complex (inability to convert $\alpha$-KG to succinyl-coA, Fig 8), or they are deficient in the transport of $\alpha$-KG into the cell. The former was ruled based upon multiple findings: unchanged expression levels of genes encoding $\alpha$-KG dehydrogenase, *lpd3* (*PA4829*) and *sucA* (*PA1585*) (Fig 10); the ability to use C4 and C6 dicarboxylates (Table 1) and C5 family of amino
acids such as arginine, proline, glutamine, and histidine (Table 2). The C5 family of amino acids act as biosynthetic precursors of glutamate that ultimately are converted to α-KG by a transamination reaction or through the action of glutamate dehydrogenase [70]. These findings strongly argued that the mifSR mutants were defective in their ability to transport α-KG into the cell.

To date, among the identified carboxylate transporters, the C4-dicarboxylate transporters have been reasonably well characterized. Based on protein sequence similarity analysis, bacterial C4-dicarboxylate transporters are classified into five families, namely, dicarboxylate transport (DctA); dicarboxylate uptake (DcuAB), (DcuC) and (CitT) and the tripartite ATP-independent periplasmic (TRAP) families [69]. Amongst these, DctA transporters, a subgroup of the dicarboxylate/amino acid:cation symporter (DAACS) family [71–73], are extensively studied and are implicated in the transport of C4-dicarboxylates in *Echerischia coli* [74], *Bacillus subtilis* [28], *Rhizobium meliloti* [38,73], *Rhizobium leguminosarum* [37,76] and *Corynebacterium glutamicum* [77]. As we were trying to identify the MifSR-dependent transporter, Lundgren et al., reported that PA5530 is involved in α-KG transport [48]. As predicted, in trans expression of PA5530 was able to restore the ability of mifR, mifS and mifSR mutants to grow in α-KG (Fig 5E). This is further confirmed by the increase in PA5530 expression in PAO1 in the presence of α-KG (Fig 11A). PA5530 shares no homology with the *P. aeruginosa* C4-dicarboxylate transporter PA1183 (DctA). However, it does have conserved protein domain family PRK10406 implicated in α-KG transport and shares ~70% homology to *E. coli* and *Erwinia* spp. α-KG permease Kgp [78,79]. A common feature in the transport of C4-dicarboxylates and other carbon sources in different bacteria is the involvement of TCS mediated regulatory mechanism. Involvement of TCSs, a stimulus-response coupled mechanism, in the transport of C5-dicarboxylates suggests a more profound role of α-KG as a signaling molecule.

**P. aeruginosa** α-KG transport requires functional RpoN (σ54)

*P. aeruginosa* RpoN (σ54) is involved in a myriad of functions including expression of virulence factors and nutrient uptake [80]. Functional RpoN is reported to be critical for maintaining a carbon-nitrogen balance in *Pseudomonas* [56,81–84]. Sequence analysis of MifR indicated a requirement of functional RpoN in modulating *P. aeruginosa* α-KG utilization. Our study confirms that α-KG utilization in *P. aeruginosa* PAO1 requires functional RpoN (Table 3). This phenotype is not strain-specific as phenotypic microarray profiling (BioLOG) of *P. aeruginosa* PA14 rpoN mutant exhibited a similar phenotype, a significant difference in the ability to utilize α-KG as a carbon source as compared to the wild-type PA14 [85]. An RpoN-dependent phenotype was also observed with citrate and 4-hydroxyphenylacetate utilization [85]. Similarly, utilization of C4-dicarboxylates succinate, fumarate and malate in *R. meliloti* and *P. aeruginosa* also requires the sigma factor RpoN (σ54)[ 37,39,86].

The need for RpoN (σ54) to utilize α-KG in *P. aeruginosa* can be bypassed by expressing PA5530 encoding for the transporter under a regulatable promoter but not MifS and MifR. Consistent with the need for RpoN (σ54), the promoter for PA5530 has the requisite signature sequences (Fig 13). Like most complex RpoN-dependent promoters [87], the region is long with multiple motifs that include a signature sequence (AAC/uAAC/uAA) for catabolite repression control (Crc) protein, a post-transcriptional inhibitor that binds the mRNA preventing translation [88–90]. Expression of crc is in-turn regulated by RpoN-dependent non-coding RNA CrcZ [90] whose absence in rpoN mutant can also lead to reduced expression of PA5530. Also, analysis of *P. aeruginosa* PA14 transcripts indicates that the PA5530 promoter is under a small non-coding antisense RNA (asRNA) regulation [91]. Though the role of Crc, CrcZ and the asRNA in α-KG transport has to be verified experimentally, it suggests an additional layer.
of regulation superimposed on the need for MifS and MifR on the expression of the C₅-dicarboxylate transporter PA5530.

**Conclusion**

In eukaryotic cells, the mitochondria serve as a hub and reservoir of the TCA cycle and its intermediates, respectively. Bacterial pathogens can be highly virulent intruders of the host tissue, causing significant damage leading to cellular aberrations and injury. Mitochondrial dysfunction, a consequence of cell injury, results in efflux of TCA cycle intermediates leading to an increase in their extracellular concentrations [92]. It is known that TCA cycle intermediates (C₄, C₅, and C₆ dicarboxylates) are present at micromolar (μM) concentrations in blood that increase with tissues damaged [26,92]. α-KG can also act as a reactive oxygen species scavenger, especially for hydrogen peroxide, protecting both host and pathogen [93]. For pathogenic bacteria such as *P. aeruginosa*, efficient uptake of TCA intermediates from the host is crucial for its survival, especially when it is bombarded with host reactive oxygen species, and requires the activity of bacterial carboxylate transport proteins. The transport proteins could be specific for C₄, C₅, and C₆ intermediates and may use a cognate TCS. This study suggests a complex regulatory cascade in modulating *P. aeruginosa* C₅-dicarboxylate, α-KG uptake involving the PA5530 transporter, the MifS/MifR TCS and the sigma factor RpoN (Fig 14). It appears that MifS senses the presence of α-KG and signals MifR. The activated MifR in concert with RpoN initiates the transcription of α-KG-specific transporter gene PA5530. Analyses of the published data suggests that the PA5530 promoter is under several layers of regulation including catabolite repression mediated by Crc/CrcZ [90] and the small non-coding asRNA [91]. Though the asRNA has been identified [91], it has not been characterized. It is not surprising that the PA5530 expression is potentially regulated by Crc, as it would allow control of transporter(s) in response to the presence of carbon sources in the environment.

In addition to MifSR (PA5512/PA5511), PA1336/PA1335 have been identified to be homologous to the *Rhizobium* C₄-dicarboxylate transport regulatory DctB/DctD TCS [39,40]. However, the role of PA1336/PA1335 remains to be elucidated. The *P. aeruginosa* genome also encodes 19 other paralogs of PA5530 dicarboxylate transporters, most of which have share less than 50% similarity except for PA0229 (PcaT). PA0229 and PA5530 have 73% similarity. Future studies will determine if the transporters are preferentially or hierarchically upregulated depending on the carbon source. It is also important to note that much of bacterial physiology, particularly of pathogens such as *P. aeruginosa* remains a mystery. Metabolic versatility, expression of virulence factors and antibiotic resistance together makes *P. aeruginosa* an portentous pathogen. Thus, understanding the physiological cues and regulation would provide a better stratagem to fight the often indomitable infections.

**Materials and Methods**

**Strains, media and growth conditions**

*P. aeruginosa* wild-type PAO1 [40] and its derivatives PAOΔmifS, PAOΔmifR, PAOΔmifSR and PAOΔrpoN or *Escherichia coli* strain DH5α were used in this study (Table 4). *Saccharomyces cerevisiae* strain InvSC1 (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used for *in vivo* homologous recombination [94]. Briefly, all bacterial cultures were grown in Luria Bertani (LB) broth (5 g tryptone, 10 g sodium chloride, and 5 g yeast extract per liter) or agar (LB broth with 1.5% agar) (Difco, N, USA) or M9 minimal Media (64 g Na₂HPO₄·7H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5.0 g NH₄Cl, 20 mM MgSO₄, 1 mM CaCl₂ per liter) [95] at 37°C, unless specified otherwise. Yeast extract-peptone-dextrose media (YPED: 20 g Bacto Peptone, 10 g yeast extract, 20 g dextrose per liter) was routinely used to culture *S. cerevisiae* and synthetic
Fig 14. Proposed model for $\alpha$-KG utilization in *P. aeruginosa*. HK-MifS senses the extracellular $\alpha$-KG to undergo phosphorylation. The phosphate is transferred to the RR-MifR. The phosphorylated MifR in coordination with RpoN ($\sigma^54$) activates the expression of $\alpha$-KG specific transporter gene PA5530. PA5530 thus enables the influx of $\alpha$-KG to meet the metabolic and energy demands of the cells. PA5530 promoter ($P_{PA5530}$) region has a Crc binding site (Fig 13), suggesting that it is under the catabolite repression control by Crc/CrcZ. The $P_{PA5530}$ also shows the presence of another uncharacterized small non-coding asRNA indicating a multilayered and complex regulation of the $\alpha$-KG transport system.

doi:10.1371/journal.pone.0129629.g014
define agar-uracil media was used as selection media for pMQ30 yeast transformants [96]. P. aeruginosa competent cells were prepared as previously described [97]. For growth curve and complementation studies M9 minimal media supplemented with glucose, sucrose or TCA cycle intermediates including citrate, α-KG, succinate, fumarate, malate or oxaloacetate were used as a sole carbon source at 30 mM each unless specified otherwise. Motility assays were performed in LB media (Difco, NJ, USA). For pyocyanin and proverdine production strains were cultivated in King’s A medium (Difco, NJ, USA) and King’s B medium [98]. Cation-adjusted Mueller Hinton broth and agar (Difco, NJ, USA) was used in MIC assays. For plasmid maintenance, antibiotics were added to growth media when appropriate, at the specified concentrations: E. coli: ampicillin (Ap) 100 µg/ml, gentamycin (Gm) 15 µg/ml, kanamycin (Km) 20 µg/ml, P. aeruginosa: Gm 75 µg/ml.

Table 4. Strains and plasmids used in this study.

| Strain ID | Strain/Plasmid Background | Relevant characteristics | Source |
|-----------|---------------------------|-------------------------|--------|
| **Escherichia coli** | | | |
| DH5α | E. coli F- Φ80lacZΔM15Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rk- mk-) phoA supE44 k' thi-1 gyrA96 relA1 | | New England Biolabs |
| **Saccharomyces cerevisiae** | | | |
| INVSc1 | S. cerevisiae MATa his3D1 leu2 trp1-289 ural3-52 | | Invitrogen |
| **Pseudomonas aeruginosa** | | | |
| PAO1 | Prototypic wild type | | [40] |
| PKM900 | PAO1 ΔmifS (PA5512) | | PAOΔmifS; This study |
| PKM901 | PAO1 ΔmifR (PA5511) | | PAOΔmifR; This study |
| PKM902 | PAO1 ΔmifSR (PA5511-PA5512) | | PAOΔmifSR; This study |
| PAOΔΔpoN | PAO1 ΔpoN (PA4462) | | [99] |
| PAO1ΔΔpoN::rpoN | PAO1 ΔpoN att Tn7::rpoN_aacC1 | | [99] |
| **Plasmids** | | | |
| pCR2.1 TOPO | ApR, KmR; colE1 11 ori lacZa | | Invitrogen |
| pRK600 | CmR; colE1 tra’ RK2 mob+ | | [100] |
| pRK2013 | KmR; colE1 tra’ RK2 mob+ | | [101] |
| pEXG2 | GmR; colE1, oriT mob+ sacB+ | | [102] |
| pMQ30 | GmR; colE1, oriT | | [96] |
| pPSV37 | GmR; colE1 oriT lacZα PlacUV5 | | [103] |
| pGDT001 | pCR2.1 TOPO ApR, A ~1.7-kb Nhel-Xbal fragment containing mifS ORF (PA5512) amplified from PAO1 genome using HK_mifS F1 and HK_mifS R1 primers and cloned into pCR 2.1 TOPO | | This study |
| pGDT002 | pCR2.1 TOPO ApR, A ~1.3-kb Nhel-Sacl fragment containing mifR ORF (PA5511) ORF amplified from PAO1 genome using GDT_mifR F1 and GDT_mifR R1 primers and cloned into pCR 2.1 TOPO | | This study |
| pGDT003 | pPSV37 GmR; The mifS ORF subcloned from pGDT001 as an Nhel-Xbal fragment into pPSV37 | | pMifs: This study |
| pGDT004 | pPSV37 GmR; The mifR ORF subcloned from pGDT002 as an Nhel-Sacl fragment into pPSV37 | | pMiR: This study |
| pGDT005 | pPSV37 GmR; A ~3.0-kb Nhel-Sacl fragment containing mifSR (PA5511-PA5512) ORFs amplified from PAO1 genome using HK_mifSR F1 and GDT_mifR R1 primers and cloned directly into Nhel-Sacl-cut in pPSV37 | | pMiMRS: This study |
| pGDT006 | pPSV37 GmR; A ~1.3-kb Nhel-Sacl fragment containing PA5530 ORF amplified from PAO1 genome using GDT_PA5530F1 and GDT_PA5530R1 primers and cloned directly into Nhel-Sacl-cut in pPSV37 | | pPA5530: This study |

doi:10.1371/journal.pone.0129629.t004

PLOS ONE | DOI:10.1371/journal.pone.0129629 June 26, 2015 21/31
Genetic manipulations

Genetic manipulations were carried out using standard techniques [95]. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) and are listed in Table 5. Plasmid DNA isolation was carried out using PureLink Hipure Plasmid Miniprep Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and agarose gel fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). RNA and cDNA was made using RNeasy Mini Kit (Qiagen Inc. Venlo, Limburg, Netherlands) and SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA). Restriction endonucleases were from New England Biolabs (Ipswich, MA, USA) and DNA sequencing was carried out at Florida International University (FIU) DNA core and at GENEWIZ Inc (South Plainfield, NJ, USA). All other chemicals were purchased from SIGMA-ALDRICH (St. Louis, MO, USA), AMRESCO (Solon, OH, USA) and Fisher Scientific (Waltham, MA, USA).

Construction of \( P. \) aeruginosa \( \Delta mifR \) mutant

An unmarked \( mifR \) clean in-frame deletion mutant of \( P. \) aeruginosa was generated by gene splicing [104]. Upstream and downstream flanking regions of \( mifR \) were amplified by PCR (GC Rich PCR System, Roche, Indianapolis, IN, USA), using primers listed in Table 5. A 754-bp P1 and a 720-bp P2 were amplified using upstream primers \( mifR \)UF1-EcoRI and \( mifR \)UR1-NheI and the downstream primers \( mifR \)DF1-NheI and \( mifR \)DR1-HindIII (Table 5), respectively from PAO1 genomic DNA. After sequencing to ensure fidelity, P1 and P2 were spliced together to obtain a 1474-bp deletion fragment with a deletion of \( mifR \) containing stop codons at its junction (inserted as part of \( NheI \) site in the primer). This was then sequenced and subcloned into a \( P. \) aeruginosa non-replicative plasmid pEXG2 [102] a \( E\)coRI-\( Hind\)III fragment and moved into the wild-type PAO1 strain by allelic replacement [105] using pRK600 and pRK2013 as the helper plasmids [100,101]. Clones were screened for Gm sensitivity (75 \( \mu \)g ml\(^{-1}\)) and sucrose resistance (8% sucrose) corresponding to a double cross-over recombination event and replacement of the target gene with the deletion product. The presence of the deletion in PAO\( \Delta mifR \) (PKM901) was confirmed by PCR amplification and sequencing of the deletion product (data not shown).

Construction of \( P. \) aeruginosa \( mifS \) and \( mifSR \) mutants

The unmarked \( mifS \) and \( mifSR \) deletion in PAO1 was generated by using the yeast system of double-stranded gap repair and homologous recombination [106]. Briefly, the \( mifS \) and \( mifSR \) upstream and downstream flanking regions were amplified by PCR using primers listed in Table 5. To create a \( mifS \) deletion, an upstream 933-bp P1 and a downstream 1115-bp P2 were amplified using primer pairs \( mifSR \)UF1-\( mifSR \)DF1 and \( mifSR \)UR1-\( mifSR \)DR1, respectively. Similarly, to create \( mifS \) deletion, an upstream 703-bp P1 and a downstream 653-bp were amplified using primer pairs HK\( mifS \)UF-HK\( mifS \)DF and HK\( mifS \)UR-HK\( mifS \)DR, respectively. HK\( mifS \)UF and \( mifSR \)UF1 primers had stretches of homologous DNA, 5\(^{\prime}\)-GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT-3\(^{\prime}\) and 5\(^{\prime}\)-CCAGGCAAATTCTGTTTATCAGACCGCTTCTGCGTTCTGAT-3\(^{\prime}\), respectively, to target recombination of the amplicons with pMQ30 vector. These primer pairs also had complementing sequences at the 3\(^{\prime}\) end to facilitate joining to create the P3 fragment, as well as stop codons (CTAGTTAGCTAG) to prevent any run off translation. The pMQ30 vector has double selection markers \( URA3 \) for yeast and gentamycin for \( E. \) coli [96]. Yeast cells were transformed with the P1, P2 and linearized pMQ30 (\( BamH\)I digested) using standard protocols [106] and colonies were selected on sucrose-uracil plates.
Table 5. Primers used in this study.

| Primer Name          | Sequence                                                                 |
|----------------------|--------------------------------------------------------------------------|
| HKm/fSU F1           | 5'-GGAATTTGAGCGGATAAACAATTTTCACACAGGAACAGGTTCACTCGACTCGGCGGCTCGG-3'       |
| HKm/fSU R1           | 5'-GACGAGATCATCGTGGCTGCTACGATATGATGCTGCGGAGGATCGGACGAGCGCC-3'            |
| HKm/fSD F1           | 5'-GGCGTTTGACTCGGCGGATGCTAACTAGGCCAGGCTGATGCTGCTCG-3'                   |
| HKm/fSD R1           | 5'-CCCAGGCAAATTCTGTTTTATCAGACCGCTTCTGATTACGTGTTCAGCGCGCTG-3'           |
| mifRUF1              | 5'-TTTGAATTCGCCTGGTCGAGCAGCGCA-3'                                       |
| mifRDF1              | 5'-TTTAAGCTTCTCGGCTTCGACGCCGAC-3'                                       |
| mifRDR F1            | 5'-GGCTTGCTAGCTCGGCGGACTCGGACGAGCGCTG-3'                               |
| mifRDF1              | 5'-GGCTTGCTAGCTCGGCGGACTCGGACGAGCGCTG-3'                               |
| mifSRF1              | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| mifSRDR F1           | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| GDT_mifR F1          | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| GDT_mifR R1          | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| GDT_p37_SeqF         | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| GDT_p37_SeqR         | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| mifR_seqF            | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| mifR_seqR            | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| PA5530_seqF          | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| PA5530_seqR          | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| DBS_qRT_clpXF        | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| DBS_qRT_clpXR        | 5'-GCTAGCGAGGAGAGATATCATATCGTCTCCGTCGTCGCTG-3'                         |
| qRT_rpoNF            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_rpoNR            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_idcF             | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_idcR             | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_idhF             | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_idhR             | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_sucAF            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_sucAR            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_lpd3F            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |
| qRT_lpd3R            | 5'-ATCTGGAAGCGGATCGGAAAC-3'                                             |

qRT in the primer name indicates that the primer was designed for qPCR. Broken and continuous lines below the primer sequence indicate ribosome binding and restriction sites respectively.

doi:10.1371/journal.pone.0129629.t005
The yeast colonies were checked for the presence of P3 constructs for mifS and mifSR deletions by amplification using upstream forward (mifSRUF1 and HKmifSUF, respectively) and downstream reverse (mifSRDR1 and HKmifSDR, respectively) primers. Yeast DNA was isolated from the positive colonies as described earlier [106]. E. coli was transformed with the recombinant pMQ30 plasmids containing P3s and screened for gentamycin resistance. The amplified P3s from the recombinant plasmids were sequenced to ensure fidelity. The constructs were then moved into PAO1 strain using tri-parental mating and screened for single and double crossovers using counter selection with sucrose and gentamycin as described earlier [107,108]. The presence of the gene deletions in all the mutants were confirmed using standard molecular methods (PCR and DNA sequencing of the locus). These strains are henceforth referred to as PAOΔmifS (PKM900) and PAOΔmifSR (PKM902).

Construction of complementing plasmids
DNA fragments from P. aeruginosa PAO1 with mifS (~1.77 kb) and mifR (~1.35 kb) were PCR amplified using primer pairs HK_mifSF1-HK_mifSF1R1, GDT_mifRF1-GDT_mifRR1, respectively. In order to ensure expression of the genes, the primers are designed such that the ORF will juxtapose against a strong ribosome binding site [70]. The PCR amplified products were cloned into pCR2.1 TOPO (Invitrogen, Life Technologies, Carlsbad, CA, USA) using manufacturers protocol to generate plasmids pGDT001 and pGDT002, respectively. The fidelity of the PCR amplified product was confirmed by sequencing. The fragments carrying mifS and mifR were moved into a broad host range pPSV37-Gm plasmid [103] as a Nhel-SacI fragments, downstream of an inducible P_lacUV5 promoter to generate plasmids pGDT003 and pGDT004, respectively. Henceforth, these plasmids are referred to as pMifS and pMifR.

DNA fragments from PAO1 with mifSR (~3.12 kb) and PA5530 (~1.3 kb) were PCR amplified using primer pairs HK_mifSF1-GDT_mifRR1 and GDT_PA5530F1-GDT_PA5530R1 (Table 5), respectively. The PCR amplified products were cloned directly into pPSV37-Gm plasmid as NheI-SacI fragments, downstream of an inducible P_lacUV5 promoter to generate plasmids pGDT005 and pGDT006, respectively. Sequence fidelity was confirmed by sequencing using the primers GDT_p37_SeqF-R, mifR_seqF-R, mifS_seqF-F2 and PA5530_seqF-R (Table 5). Henceforth, these plasmids are referred to as pMifSR and pPA5530.

These expression plasmids were then introduced into wild-type PAO1, PAOΔmifS, PAOΔmifR, PAOΔmifSR and PAOΔrpoN deletion mutants by electroporation [97] and gentamycin resistant colonies were selected.

Phenotypic microarray
Comparative phenotypic microarray profiles of wild-type PAO1 with PAOΔmifR and PAOΔmifS mutant were performed at BioLOG Inc. (Hayward, CA, USA). Phenotypic profiling was carried out in triplicate and data analyses was done using OmniLog PM software.

Growth curves
P. aeruginosa PAO1 and its derivatives were grown overnight at 37°C in LB broth with or without antibiotics. Overnight cultures were washed with sterile 0.85% NaCl (wt/vol) solution to remove spent and residual media. Cultures were diluted in fresh M9 minimal media to obtain equal optical densities (OD<sub>600</sub>) of 0.025. Growth of the cultures was assessed in LB broth and in M9 minimal media supplemented with glucose (30 mM), sucrose (30 mM) or TCA cycle intermediates including citrate, α-KG, succinate, fumarate, malate or oxaloacetate (at 30 mM, unless specified otherwise) as a sole carbon source in 48 and 96 well plates (Falcon). Growth was monitored by determining absorbance at 600 nm using BioTek Synergy HT (Winooski,
VT, USA) plate reader for 18–24 h at 37°C. All experiments were performed multiple times in triplicate.

Pyocyanin and pyoverdine production
Extracellular pyocyanin was quantified by extracting the pigment from culture supernatants using the chloroform-HCl method as described previously [109]. Briefly, 5 ml culture supernatants from stationary-phase cultures (~18 h) grown in King’s A medium was extracted with 3 ml chloroform. Pyocyanin was then re-extracted into 1 ml of 0.2 N HCl, resulting in a pink color, indicating the presence of pyocyanin that was read at 520 nm. The concentration is expressed as μg of pyocyanin produced per ml of culture (μg/ml), by multiplying the optical density OD520 by 17.072 [109].

To measure pyoverdine production, cells were grown overnight at 37°C in King’s B medium [98]. Pyoverdine in the supernatant was read at 405 nm and normalized to the initial cell density (OD600). Pyoverdine levels were expressed as a ratio of OD405/OD600 [110].

Minimum Inhibitory Concentration
MICs were determined using the E-test as per the manufacturers protocol (BioMerieux, USA) and/or by standard broth microdilution method [111]. The assays were performed in triplicate, each with technical triplicate, for each antibiotic in cation-adjusted Mueller Hinton broth.

RNA isolation, cDNA synthesis and qRT-PCR
RNA was isolated from P. aeruginosa wild-type PAO1, PAOΔmifR, PAOΔmifS and PAOΔmifSR strains grown in LB broth followed by 1 h treatment with 30 mM α-KG. Briefly, overnight cultures grown in LB broth at 37°C were washed with sterile 0.85% saline solution to remove spent media and were subcultured at 37°C, 200 rpm in LB media. LB broth was used as a carbon source for initial growth of cultures since PAOΔmifR, PAOΔmifS, PAOΔmifSR and PAOΔrpoN strains exhibit growth defects in the presence of α-KG alone. When the cells reached an optical density at 600 nm (OD600) of 0.6–0.7 all the cultures were treated with 30 mM α-KG for 1 h. Post treatment, RNA was stabilized by addition of phenol-ethanol mixture [112]. Stabilized RNA was then isolated using RNeasy Mini Kit (Qiagen, Inc Venio, Limburg, Netherlands) as per manufacturer’s protocol. Residual genomic DNA contamination was removed using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and RNA was repurified using Rneasy Mini Kit (Qiagen, Inc Venio, Limburg, Netherlands). Quality of purified RNA was assessed on a denaturing agarose gel (NorthernMax Gly, Ambion, Life Technologies, Carlsbad, CA, USA) and quantified at 260 nm (BioTEK, Synergy HT, Winooski, VT, USA). cDNA was then synthesized by annealing NS5 random primers to total purified RNA and subsequent extension was carried out using SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA).

qRT-PCR to study expression levels of PA5530 under α-KG induction was performed using Applied Biosystems Step One cycler and detection system with PowerSYBR Green PCR MasterMix with ROX (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). In addition RNA was isolated from PAO1, PAOΔmifR, PAOΔmifS and PAOΔmifSR strains grown in M9 Minimal media supplemented with citrate (30 mM) without α-KG treatment, as described previously. qRT-PCR to study expression levels of genes encoding sigma-54 rpoN (PA4462), isocitrate dehydrogenase (idh (PA2623) and icd (PA2624)), α-KG dehydrogenase complex (sucA (PA1585) and lpd3 (PA4829)) were done essentially as described above. The cycling conditions used were 95°C/2 minutes (holding); 40 cycles of 95°C/15 sec, 60°C/1 min (cycling); 95°C/15 sec, 60°C/1 min, 95°C/15 sec (0.6°C ramp) (melt curve). Expression was normalized to clpX.
(PA1802), whose expression was determined to remain constant between the samples and conditions tested [107].

Bioinformatic Analyses

Sequence analyses and domain organization studies were performed using the Simple Modular Architecture Research Tool (SMART) [51] and InterPro domain prediction database [52]. \textit{mifS} (P\textit{mifS}) and \textit{PA5530} (P\textit{PA5530}) promoter analyses and motif search was done using the ensemble learning method SCOPE and GLAM2 (Gapped Local Alignment of Motifs) [113,114]. Multiple sequence alignment was generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and www.pseudomonas.com [57].

Statistical Analyses

All data were analyzed for statistical significance using the Student’s \textit{t}-test on GraphPad or Analysis of Variance (ANOVA) with post-hoc testing when appropriate, on IBM SPSS Statistics 22.0 statistical analysis software. Differences were considered to be significant at \( p \)-values < 0.05.

Acknowledgments

The authors thank the following individual for their intellectual input: Kyle Martins and Jeremy Chambers (Florida International University), Deepak Balasubramanian (Harvard Medical School), Lars Dietrich (Columbia University), and Elaine Newman (Concordia University, Canada). We would like to thank Dr. D. Haas from UNIL, Switzerland for kindly providing PA0\textit{ΔrpoN} and PA0\textit{ΔrpoN::rpoN}.

Author Contributions

Conceived and designed the experiments: GT KM. Performed the experiments: GT HK EH LR. Analyzed the data: GT KM HK. Contributed reagents/materials/analysis tools: KM. Wrote the paper: GT KM.

References

1. Ramos J-L (2004) \textit{Pseudomonas}. Boston: Kluwer Academic/Plenum.
2. Ledizet M, Murray TS, Puttagunta S, Slade MD, Quagliarello VJ, Kazmierczak BI (2012) The ability of virulence factor expression by \textit{Pseudomonas aeruginosa} to predict clinical disease in hospitalized patients. PLoS One 7: e49576. doi: 10.1371/journal.pone.0049578 PMID: 23152923
3. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M (2014) Requirements for \textit{Pseudomonas aeruginosa} acute burn and chronic surgical wound infection. PLoS Genetics 10: e1004518. doi: 10.1371/journal.pgen.1004518 PMID: 25057820
4. Furukawa S, Kuchma SL, O'Toole GA (2006) Keeping their options open: acute versus persistent infections. J Bacteriol 188: 1211–1217. PMID: 16452401
5. Lyczak JB, Cannon CL, Pier GB (2000) Establishment of \textit{Pseudomonas aeruginosa} infection: lessons from a versatile opportunist. Microbes Infect 2: 1051–1060. PMID: 10967285
6. Valderrey AD, Pozuelo MJ, Jimenez PA, Macia MD, Oliver A, Rotger R (2010) Chronic colonization by \textit{Pseudomonas aeruginosa} of patients with obstructive lung diseases: cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. Diagn Microbiol Infect Dis 68: 20–27. doi: 10.1016/j.diagmicrobio.2010.04.008 PMID: 20727465
7. Manfredi R, Nanetti A, Fern M, Chiado F (2000) \textit{Pseudomonas} spp. complications in patients with HIV disease: an eight-year clinical and microbiological survey. Eur J Epidemiol 16: 111–118. PMID: 10845259
8. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL (2002) \textit{Pseudomonas aeruginosa} and other predictors of mortality and morbidity in young children with cystic fibrosis. Pediatr Pulmonol 34: 91–100. PMID: 12112774
9. Cohen-Cymberknoh M, Kerem E, Ferkol T, Elizur A (2013) Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. Thorax 68: 1157–1162. doi: 10.1136/thoraxjnl-2013-203204 PMID: 23704228

10. Lyczak JB, Cannon CL, Pier GB (2002) Lung infections associated with cystic fibrosis. Clin Microbiol Rev 15: 194–222. PMID: 11932230

11. Dart JK, Seal DV (1988) Pathogenesis and therapy of Pseudomonas aeruginosa keratitis. Eye 2 Suppl: S46–55. PMID: 3076156

12. Yeo SG, Park DC, Hong SM, Cha CJ, Kim MG (2007) Bacteriology of chronic suppurative otitis media—a multicenter study. Acta Otolaryngol 127: 1062–1067. PMID: 17851935

13. Sun Y, Karmakar M, Roy S, Ramadan RT, Williams SR, Howell S, et al. (2010) TLR4 and TLR5 on corneal macrophages regulate Pseudomonas aeruginosa keratitis by signaling through MyD88-dependent and-independent pathways. J Immunol 185: 4272–4283. doi: 10.4049/jimmunol.1000874 PMID: 20826748

14. Prevatt AR, Sedwick JD, Gajewski BJ, Antonelli PJ (2004) Hearing loss with semicircular canal transection and Pseudomonas aeruginosa otitis media. Otolaryngol Head Neck Surg 131: 248–252. PMID: 15365544

15. Balasubramanian D, Schneper L, Kumari H, Mathee K (2013) A dynamic and intricate regulatory network determines Pseudomonas aeruginosa virulence. Nucleic Acids Res 41: 1–20. doi: 10.1093/nar/gks1039 PMID: 23143271

16. Fraimow H, Nahra R (2013) Resistant Gram-negative infections. Crit Care Clin 29: 895–921. doi: 10.1016/j.ccc.2013.06.010 PMID: 24094384

17. Loomis WF Jr., Magasanik B (1967) Glucose-lactose diauxie in Escherichia coli. J Bacteriol 93: 1397–1401. PMID: 5340309

18. PII signal transduction proteins: sensors of alpha-ketoglutarate that regulate acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc Natl Acad Sci U S A 107: 50208. doi:10.1073/pnas.0910097107 PMID: 20018655

19. Collier DN, Hager PW, Phibbs PV Jr. (1996) Catabolite repression control in the Pseudomonads. Res Microbiol 147: 551–561. PMID: 9084769

20. Wolff JA, MacGregor CH, Eisenberg RC, Phibbs PV Jr. (1991) Isolation and characterization of catabolite repression control mutants of Pseudomonas aeruginosa PAO. J Bacteriol 173: 4700–4706. PMID: 1906670

21. Liu P (1952) Utilization of carbohydrates by Pseudomonas aeruginosa. J Bacteriol 64: 773–781. PMID: 13011149

22. Owen OE, Kalhan SC, Hanson RW (2002) The key role of anaplerosis and cataplerosis for citric acid cycle function. J Biol Chem 277: 30409–30412. PMID: 12087111

23. Frohman CE, Orten JM, Smith AH (1951) Chromatographic determination of the acids of the citric acid cycle in tissues. J Biol Chem 193: 277–283. PMID: 14907714

24. Krebs HA (1940) The citric acid cycle and the Szent-Gyorgyi cycle in pigeon breast muscle. Biochem J 34: 775–779. PMID: 16747218

25. Ninfa AJ, Jiang P (2005) PII signal transduction proteins: sensors of alpha-ketoglutarate that regulate nitrogen metabolism. Curr Opin Microbiol 8: 168–173. PMID: 15802248

26. He W, Miao FJ, Lin DC, Schwanert RT, Wang Z, Gao J, et al. (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. Nature 429: 188–193. PMID: 15141213

27. Feria Bourrellier AB, Valot B, Guillot A, Ambard-Bretteville F, Vidal J, Hodges M (2010) Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc Natl Acad Sci U S A 107: 502–507. doi:10.1073/pnas.0910097107 PMID: 20018655

28. Asai K, Baik SH, Kasahara Y, Moriya S, Ogasawara N (2000) Regulation of the transport system for C4-dicarboxylic acids in Bacillus subtilis. Microbiol 146:263–271.

29. Hoch JA, Silhavy TJ (1995) Two-component signal transduction. Washington, D.C.: ASM Press.

30. Stock JB, Ninfa AJ, Stock AM (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev 53: 450–490. PMID: 2556636

31. Bott M, Meyer M, Dimroth P (1995) Regulation of anaerobic citrate metabolism in Klebsiella pneumoniae. Mol Microbiol 18: 533–546. PMID: 8748036

32. Brocker M, Schaffer S, Mack C, Bott M (2009) Citrate utilization by Corynebacterium glutamicum is controlled by the CitAB two-component system through positive regulation of the citrate transport genes citH and citCBA. J Bacteriol 191: 3869–3880. doi:10.1128/JB.00113-09 PMID: 19376865

33. Yamamoto H, Murata M, Sekiguchi J (2000) The CitST two-component system regulates the expression of the Mg-citrate transporter in Bacillus subtilis. Mol Microbiol 37: 898–912. PMID: 10972810
34. Golby P, Davies S, Kelly DJ, Guest JR, Andrews SC (1999) Identification and characterization of a two-component sensor-kinase and response-regulator system (DcuS-DcuR) controlling gene expression in response to C4-dicarboxylates in *Escherichia coli*. J Bacteriol 181: 1238–1248. PMID: 9973351

35. Jiang J, Gu BH, Albright LM, Nixon BT (1989) Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum* dct genes. J Bacteriol 171: 5244–5253. PMID: 2793824

36. Reid CJ, Poole PS (1998) Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum*. J Bacteriol 180: 2660–2669. PMID: 9573150

37. Ronson CW, Astwood PM, Nixon BT, Ausubel FM (1987) Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res 15: 7921–7934. PMID: 3671068

38. Watson RJ (1990) Analysis of the C4-dicarboxylate transport genes of *Rhizobium meliloti*: nucleotide sequence and deduced products of dctA, dctB, and dctD. Mol Plant Microbe Interact 3: 174–181. PMID: 2134335

39. Valentini M, Storelli N, Lapouge K (2011) Identification of C(4)-dicarboxylate transport systems in *Pseudomonas aeruginosa* PAO1. J Bacteriol 193: 4307–4316. doi:10.1128/JB.05074-11 PMID: 21725012

40. Petrova OE, Schurr JR, Schurr MJ, Sauer K (2012) Microcolony formation by the opportunistic pathogen *Pseudomonas aeruginosa* requires pyruvate and pyruvate fermentation. Mol Microbiol 86: 819–835. doi: 10.1111/mmi.12018 PMID: 22931250

41. Kong KF, Jayawardena SR, Del Puerto A, Wiehlmann L, Tummler B, et al. (2005) Characterization of *poxB*, a chromosomal-encoded *Pseudomonas aeruginosa* oxacillinase. Gene 358: 82–92. PMID: 16120476

42. Doucette CD, Schwab DJ, Wingreen NS, Rabinowitz JD (2011) alpha-Ketoglutarate coordinates carbon and nitrogen utilization via enzyme I inhibition. Nat Chem Biol 7: 894–901. doi: 10.1038/nchembio.685 PMID: 22002719

43. Suh SJ, Runyen-Janecky L, Maleniak TC, Hager P, MacGregor CH, Zielinski-Mozny NA, et al. (2002) Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. Microbiol 148: 1561–1569. PMID: 11988531

44. Campbell JJ, Smith RA, Eagles BA (1953) A deviation from the conventional tricarboxylic acid cycle in *Pseudomonas aeruginosa*. Biochim Biophys Acta 11: 594. PMID: 13105691

45. Kornberg HL (1966) Anaplerotic sequences and their role in metabolism. Essays Biochem: 2:1–31.

46. Lundgren BR, Villegas-Penaranda LR, Harris JR, Mottem AM, Dunn DM, Boddy CN, et al. (2014) Genetic analysis of the assimilation of C5-dicarboxylic acids in *Pseudomonas aeruginosa* PAO1. J Bacteriol 196: 2543–2551. doi: 10.1128/JB.01615-14 PMID: 24794562

47. Morett E, Segovia L (1993) The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J Bacteriol 175: 6067–6074. PMID: 8407777

48. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A 95: 5857–5864. PMID: 9600884

49. Hunter S, Jones P, Mitchell A, Apweiler R, Attwood TK, Bateman A, et al. (2012) InterPro in 2011: new developments in the family and domain prediction database. Nucleic Acids Res 40: D306–312. doi: 10.1093/nar/gkr948 PMID: 22096229

50. Paget MS, Helmann JD (2003) The sigma70 family of sigma factors. Genome Biol 4: 203. PMID: 12540296
55. Gooderham WJ, Hancock RE (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev 33: 279–294. doi: 10.1111/j.1574-6976.2008.00135.x PMID: 19243444

56. Nishijyo T, Haas D, Itoh Y (2001) The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in Pseudomonas aeruginosa. Mol Microbiol 40: 917–931. PMID: 11401699

57. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, et al. (2011) Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. Nucleic Acids Res 39: D596–600. doi: 10.1093/nar/gkq869 PMID: 20929876

58. Barakat M, Ortet P, Jourlin-Castelli C, Ansaldi M, Mejean V, Whitworth DE (2009) P2CS: a two-component system resource for prokaryotic signal transduction research. BMC Genomics 10: 315. doi: 10.1186/1471-2164-10-315 PMID: 19604365

59. Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215. PMID: 10966457

60. Dandekar T, Snel B, Huynen M, Bork P (1998) Conservation of gene order: a fingerprint of proteins that physically interact. Trends Biochem Sci 23: 324–328. PMID: 9787636

61. Overbeek R, Fonstein M, D’Souza M, Pusch GD, Maltsev N (1999) The use of gene clusters to infer functional coupling. Proc Natl Acad Sci U S A 96: 2896–2901. PMID: 10077608

62. Chen YT, Chang HY, Lu CL, Peng HL (2004) Evolutionary analysis of the two-component systems in Pseudomonas aeruginosa PAO1. J Mol Evol 59: 725–737. PMID: 15599505

63. Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saqi M, Littler E, et al. (2006) Clustering of Pseudomonas aeruginosa transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics 7: 162. PMID: 16800888

64. Yeung AT, Bains M, Hancock RE (2011) The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, and antibiotic resistance in Pseudomonas aeruginosa. J Bacteriol 193: 918–931. doi: 10.1128/JB.00911-10 PMID: 21169488

65. Deretic V, Leveau JH, Mohr CD, Hibler NS (1992) In vitro phosphorylation of AlgR, a regulator of mucoidy in Pseudomonas aeruginosa, by a histidine protein kinase and effects of small phospho-donor molecules. Mol Microbiol 6: 2761–2767. PMID: 1435255

66. Lukat GS, McCleary WR, Stock AM, Stock JB (1992) Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc Natl Acad Sci U S A 89: 718–722. PMID: 1731345

67. Varughese KI (2002) Molecular recognition of bacterial phosphorelay proteins. Curr Opin Microbiol 5: 142–148. PMID: 11934609

68. Wanner BL, Wilmes-Riesenberg MR (1992) Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in Escherichia coli. J Bacteriol 174: 2124–2130. PMID: 1551836

69. Janausch IG, Zientz E, Tran QH, Kroger A, Unden G (2002) C4-dicarboxylate carriers and sensors in bacteria. Biochim Biophys Acta 1553: 39–56. PMID: 11803016

70. Lehninger AL, Nelson DL, Cox MM (2013) Lehninger principles of biochemistry. New York: W.H. Freeman.

71. Busch W, Saier MH Jr. (2004) The IUBMB-endorsed transporter classification system. Mol Biotechnol 27: 253–262. PMID: 15247499

72. Busch W, Saier MH Jr. (2002) The transporter classification (TC) system, 2002. Crit Rev Biochem Mol Biol 37: 287–337. PMID: 12449427

73. Saier MH Jr., Tran CV, Barabote RD (2006) TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic Acids Res 34: D181–186. PMID: 16381841

74. Davies SJ, Golby P, Omrani D, Broad SA, Harrington VL, Guest JR, et al. (1999) Inactivation and regulation of the aerobic C(4)-dicarboxylate transport (dctA) gene of Escherichia coli. J Bacteriol 181: 5624–5635. PMID: 10482502

75. Yarosh OK, Charles TC, Finan TM (1989) Analysis of C4-dicarboxylate transport genes in Rhizobium meliloti. Mol Microbiol 3: 813–823. PMID: 2546011

76. Finan TM, Wood JM, Jordan DC (1981) Succinate transport in Rhizobium leguminosarum. J Bacteriol 148: 193–202. PMID: 7287623

77. Youn JW, Jokivuori E, Kramer R, Marin K, Wendisch VF (2009) Characterization of the dicarboxylic acid transporter DctA in Corynebacterium glutamicum. J Bacteriol 191: 5480–5488. doi: 10.1128/JB.00640-09 PMID: 19581365
78. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. (2015) CDD: NCBI's conserved domain database. Nucleic Acids Res 43: D222–226. doi: 10.1093/nar/gku1221 PMID: 25414356

79. Seol W, Shatkin AJ (1991) *Escherichia coli* kgtP encodes an alpha-ketoglutarate transporter. Proc Natl Acad Sci U S A 88: 3802–3806. PMID: 2053984

80. Potvin E, Sanschagrin F, Levesque RC (2008) Sigma factors in *Pseudomonas aeruginosa*. FEMS Microbiol Rev 32: 38–55. PMID: 18070067

81. Li W, Lu CD (2007) Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa*. J Bacteriol 189: 5413–5420. PMID: 17545289

82. Zhang XX, Rainey PB (2008) Dual involvement of CbrAB and NtrBC in the regulation of histidine utilization in *Pseudomonas fluorescens* SBW25. Genetics 178: 185–195. doi: 10.1534/genetics.107.081984 PMID: 18202367

83. Cases I, Ussery DW, de Lorenzo V (2003) The sigma54 regulon (sigmulon) of *Pseudomonas putida*. Environ Microbiol 5: 1281–1293. PMID: 14641574

84. Kohler T, Cayrol JM, Ramos JL, Harayama S (1989) Nucleotide and deduced amino acid sequence of the RpoN sigma-factor of *Pseudomonas putida*. Nucleic Acids Res 17: 10125. PMID: 2602128

85. Behrends V, Bell TJ, Liebeke M, Cordes-Blauert A, Ashraf SN, Nair C, et al. (2013) Metabolite profiling to characterize disease-related bacteria: gluconate excretion by *Pseudomonas aeruginosa* mutants and clinical isolates from cystic fibrosis patients. J Biol Chem 288: 15098–15109. doi: 10.1074/jbc.M112.442814 PMID: 23572517

86. Ronson CW, Nixon BT, Albright LM, Ausubel FM (1987) *Rhizobium meliloti* ntrA (rpoN) gene is required for diverse metabolic functions. J Bacteriol 169: 2424–2431. PMID: 3034866

87. Merrick MJ (1993) In a class of its own—the RNA polymerase sigma factor sigma 54 (sigma N). Mol Microbiol 10: 903–909. PMID: 7934866

88. Browne P, Barret M, O’Gara F, Morrissey JP (2010) Computational prediction of the Crc regulon identifies genus-wide and species-specific targets of catabolite repression control in *Pseudomonas* bacteria. BMC Microbiol 10: 300. doi: 10.1186/1471-2180-10-300 PMID: 21108798

89. Moreno R, Ruiz-Manzano A, Yuste L, Rojo F (2007) The *Pseudomonas putida* Crc global regulator is an RNA binding protein that inhibits translation of the AlkS transcriptional regulator. Mol Microbiol 64: 665–675. PMID: 17462015

90. Sonnleitner E, Abdou L, Haas D (2009) Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 106: 21866–21871. doi: 10.1073/pnas.pnas.0910308106 PMID: 20080802

91. Wurtzel O, Yoder-Himes DR, Han K, Dandekar AA, Edelheit S, Greenberg EP, et al. (2012) The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. PLoS Pathog 8: e1002945. doi: 10.1371/journal.ppat.1002945 PMID: 23028334

92. Hebert SC (2004) Physiology: orphan detectors of metabolism. Nature 429: 143–145. PMID: 15141197

93. Long LH, Halliwell B (2011) Artefacts in cell culture: alpha-ketoglutarate can scavenge hydrogen peroxide generated by ascorbate and epigallocatechin gallate in cell culture media. Biochem Biophys Res Commun 406: 20–24. doi: 10.1016/j.bbr.2011.01.091 PMID: 21281600

94. Bascom-Slack CA, Dawson D (1998) A physical assay for detection of early meiotic recombination intermediates in Saccharomyces cerevisiae. Mol Gen Genet 258: 512–520. PMID: 9699333

95. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

96. Shanks RM, Caiazzo NC, Hinsa SM, Toutain CM, O’Toole GA (2006) Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from Gram-negative bacteria. Appl Environ Microbiol 72: 5027–5036. PMID: 16820502

97. Choi KH, Kumar A, Schweizer HP (2006) A 10-min method for preparation of highly electrophoretic *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64: 391–397. PMID: 15987659

98. King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med 44: 301–307. PMID: 13184240

99. Heurlier K, Denervaud V, Pessi G, Reimann C, Haas D (2003) Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. J Bacteriol 185: 2227–2235. PMID: 12644493
100. Kessler B, de Lorenzo V, Timmis KN (1992) A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol Gen Genet 233: 293–301. PMID: 1318499

101. Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76: 1648–1652. PMID: 377280

102. Rietisch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 102: 8006–8011. PMID: 15911752

103. Lee PC, Stopford CM, Svenson AG, Rietisch A (2010) Control of effector export by the Pseudomonas aeruginosa type III secretion proteins PcrG and PcrV. Mol Microbiol 75: 924–941. doi: 10.1111/j.1365-2958.2009.07027.x PMID: 20487288

104. Horton RM, Cai ZL, Ho SN, Pease LR (1990) Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 8: 528–535. PMID: 2357375

105. Schweizer HP, Hoang TT (1995) An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa. Gene 158: 15–22. PMID: 7789804

106. Shanks RM, Kadouri DE, MacEachran DP, O’Toole GA (2009) New yeast recombineering tools for bacteria. Plasmid 62: 88–97. doi: 10.1016/j.plasmid.2009.05.002 PMID: 19477196

107. Balasubramanian D, Schnepfer L, Merighi M, Smith R, Narasimhan G, Lory S, et al. (2012) The regulatory repertoire of Pseudomonas aeruginosa AmpC ss-lactamase regulator AmpR includes virulence genes. PloS one 7: e34067. doi: 10.1371/journal.pone.0034067 PMID: 22479525

108. Kumari H, Balasubramanian D, Zincke D, Mathee K (2014) Role of Pseudomonas aeruginosa AmpR on beta-lactam and non-beta-lactam transient cross-resistance upon pre-exposure to subinhibitory concentrations of antibiotics. J Med Microbiol 63: 544–555. doi: 10.1099/jmm.0.070185-0 PMID: 24464693

109. Essar DW, Eberly L, Hadero A, Crawford IP (1990) Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 172: 884–900. PMID: 2153661

110. Shen J, Meldrum A, Poole K (2002) FpvA receptor involvement in pyoverdine biosynthesis in Pseudomonas aeruginosa. J Bacteriol 184: 3268–3275. PMID: 12029043

111. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standards (2012). Wayne (Pennsylvania). Clinical and Laboratory Standards Institute 32: M07-A09.

112. Brenchik A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, et al. (2009) The GacS/GacA signal transduction system of Pseudomonas aeruginosa acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol Microbiol 73: 434–445. doi: 10.1111/j.1365-2958.2009.06782.x PMID: 19602144

113. Chakravarty A, Carlson JM, Khetani RS, Gross RH (2007) A novel ensemble learning method for de novo computational identification of DNA binding sites. BMC Bioinform 8: 248.

114. Frith MC, Saunders NF, Kobe B, Bailey TL (2008) Discovering sequence motifs with arbitrary insertions and deletions. PLoS Comput Biol 4: e1000071. doi: 10.1371/journal.pcbi.1000071 PMID: 18437229