Genetic Analysis of the Assimilation of C$_5$-Dicarboxylic Acids in *Pseudomonas aeruginosa* PAO1

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There is a wealth of information on the genetic regulation and biochemical properties of bacterial C$_4$-dicarboxylate transport systems. In sharp contrast, there are far fewer studies describing the transport and assimilation of C$_5$-dicarboxylates among bacteria. In an effort to better our understanding on this subject, we identified the structural and regulatory genes necessary for the utilization of α-ketoglutarate (α-KG) in *Pseudomonas aeruginosa* PAO1. The PA5530 gene, encoding a putative dicarboxylate transporter, was found to be essential for the growth of *P. aeruginosa* PAO1 on both α-KG and glutarate (another C$_5$-dicarboxylate). Metabolite analysis confirmed that the PA5530 gene was necessary for the uptake of extracellular α-KG. Like other substrate-inducible transporter genes, expression of the PA5530 gene was induced by extracellular C$_5$-dicarboxylates. It was later found that the expression of the PA5530 gene was driven solely by a −24/−12 promoter recognized by the alternative sigma factor RpoN. Surprisingly, the enhancer binding protein MifR, which is known to have an essential role in biofilm development, was required for the expression of the PA5530 gene. The MifR protein is homologous to other transcriptional regulators involved in dicarboxylate assimilation, suggesting that MifR might interact with RpoN to activate the expression of the PA5530 gene in response to extracellular C$_5$-dicarboxylates, especially α-KG. The results of this study provide a framework for exploring the assimilation of α-KG in other pseudomonads.

The C$_5$-dicarboxylate α-ketoglutarate (α-KG) is widely recognized for being an intermediate of both carbon and nitrogen metabolism. The other C$_5$-dicarboxylate commonly encountered is glutarate, which is an intermediate of lysine and tryptophan metabolism. It has become increasingly apparent that α-KG plays a larger and more profound role in bacterial physiology than originally believed. For example, it is now known that α-KG directly regulates nitrogen metabolism via binding to and controlling the activity of the nitrogen regulatory protein PII (1–3). There is also compelling evidence that endogenous α-KG is a natural protectant against both oxidative damage (4, 5) and cyanide poisoning (6). α-KG-dependent hydroxylases are ubiquitous among bacteria and participate in various cellular functions (7), including the assimilation of key nutrients, e.g., sulfur and phosphate, and the biosynthesis of complex molecules such as antibiotics and lipid A. Interestingly, there have been a few recently published papers reporting that α-KG might be a preferred nutrient for some bacteria during the course of an infectious state (8–10). α-KG is a suitable growth substrate for most bacteria, but the genes involved in the transport and assimilation of α-KG have been investigated for only a small number of bacterial species, including *Escherichia coli* (10–12), *Staphylococcus aureus* (13), *Bacillus licheniformis* (14), *Lactococcus lactis* (15), *Xanthomonas oryzae* (9), and *Rhizobium tropici* (8). Missing from the literature are studies describing the genetic regulation of α-KG assimilation in *Pseudomonas*, which is surprising, because these bacteria are well known for their versatile metabolism, enabling them to consume a wide range of substrates.

The assimilation of α-KG has been documented in previous studies involving *Pseudomonas* (16–20). Campbell and Stokes observed that acetate-grown *P. aeruginosa* displayed a significant lag time when transferred into α-KG medium (19), hinting at the probable existence of an inducible α-KG transporter. *Pseudomonas* cultures grown under conditions of nitrogen limitation are well known to accumulate extracellular α-KG, which is readily depleted with the addition of exogenous ammonium. Probably the most complete study on α-KG transport in *Pseudomonas* was that by Edwards et al., which biochemically characterized C$_5$-dicarboxylate transport in *P. putida* (21). Those authors observed that transport of α-KG in *P. putida* was induced by extracellular C$_5$-dicarboxylates such as α-KG and glutarate (21). Despite these observations, the genes encoding the transport and regulatory proteins involved in α-KG uptake had not been identified for any given *Pseudomonas* species.

Because of the limited information available on α-KG assimilation in *Pseudomonas*, we decided to identify the genetic network surrounding α-KG transport in *Pseudomonas aeruginosa* PAO1. An initial survey of the sequenced genome of *P. aeruginosa* PAO1 revealed that the predicted PA5530 protein is homologous to the well-characterized α-KG transporter, KgtP, of *E. coli* K-12 (11). Subsequent analysis confirmed that the PA5530 gene was required for the uptake of extracellular α-KG for *P. aeruginosa* PAO1. Expression of the PA5530 gene was induced by extracellular C$_5$-dicarboxylates, and this expression was dependent on the alternative sigma factor σ^{54} or RpoN. Unexpectedly, the expression of the PA5530 gene also required the transcriptional regulator MifR, which was found previously to be involved in biofilm formation (22). MifR shares significant homology (>60%) with other regu-

TABLE 1 Strains, plasmids, and oligonucleotides used in the current study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Reference or source
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type	23
PW9687	PA5166-F04::ISphoA/hah derivative of PAO1	24
PW10371	PA5530-C01::ISphoA/hah derivative of PAO1	24
PAO6359	*rpoN*::Ω-Km derivative of PAO1	25
Δ*mifR* PAO1	Δ*mifR* derivative of PAO1	This study
Δ*mifR* PW9687	Δ*mifR* derivative of PW9687	This study
PA14	Wild type	26
ΔPA5530 PA14	ΔPA5530 derivative of PA14	This study
PAK	Wild type	27
ΔPA5530 PAK	ΔPA5530 derivative of PAK	This study
Escherichia coli Top10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL* (Str^r) *endA1* λ⁻	Invitrogen
Plasmids		
pCR-Blunt	Cloning plasmid; Km^r	Invitrogen
pTrc99a	Expression plasmid; Cb^r	Pharmacia
pBBR1MCS-5	Broad-host-range plasmid; Gm^r	28
pEX18ApGW	Plasmid for gene deletions in *P. aeruingosa*; Cb^r Gm^r	29
ΔP*lac*-pBBR1MCS-5	pBBR1MCS-5 minus *lac* promoter; Gm^r	This study
pBRL474	PA5530 gene in pCR-Blunt; Km^r	This study
pBRL479	PA5530 gene in pBBR1MCS-5; Gm^r	This study
pBRL476	PA5530-*lacZ* in pCR-Blunt; Km^r	This study
pBRL485	PA5530-*lacZ* in ΔP*lac*-pBBR1MCS-5; Gm^r	This study
pBRL487	PA5530-*lacZ* with mutated RpoN promoter; Gm^r	This study
pBRL511	*mifR*::Gm^r in pEX18ApGW; Cb^r Gm^r	This study
pBRL538	PA5530::Gm^r in pEXT18ApGW; Cb^r Gm^r	This study
pBRL549	*mifSR* operon in pCR-Blunt; Km^r	This study
pBRL561	*mifS*-Δ*mifR* operon in pCR-Blunt; Km^r	This study
pBRL566	*mifSR* operon (forward orientation) in pTrc99a; Cb^r	This study
pBRL567	*mifSR* operon (backward orientation) in pTrc99a; Cb^r	This study
pBRL570	*mifS*-Δ*mifR* operon (forward orientation) in pTrc99a;Cb^r	This study
pJRH07	*mifR* gene in pCR-Blunt; Km^r	This study
pJRH08	*mifR* gene in pBBR1MCS-5; Gm^r	This study
Oligonucleotides		
BL342.f	ATGACCATGATTACGGATTCACTG	
BL342.r	GCAGTTATTTTTGACACCAGACCAACTGGTA	
BL434.f	GCATCTAGACTGATCAGCACATCCAAGACAAC	
BL434.r	GCAGAGCTCTCAATCGGTCGTGATCTTCGA	
BL435.f	GCAGATCGGCGAGTTCTCC	
BL435.r	GAATCCGTAATCATGGTCATCGTGTTTCCTCTTTTCGTTGTTG	
BL438.f	GCTTTCCCGCGTTAACACGGCACCTGCTATC	
BL438.r	GATAGCAGGTGCCGTGTTAACGCGGGAAAGC	
BL466.f	TACAAAAAAGCAGGCTGACCAGGTGATCTTCGTCGAC	
BL466.r	TCAGAGCGCTTTTGAAGCTAATTCGGAAGGGTTTCTCGATGAAGTCG	
BL467.f	AGGAACTTCAAGATCCCCAATTCGCGAACATTTCGCCAGCGTTG	
BL467.r	TACAAGAAAGCTGGGTCTCTTCGCCGACGAAATCGC	
BL552.f	TACAAAAAAGCAGGCTGAAAGCGCCAACGCCATTTC	
BL552.r	TCAGAGCGCTTTTGAAGCTAATTCGGATGATCAGCGAGCCAAAGC	
BL553.f	AGGAACTTCAAGATCCCCAATTCGCTTCTACACCTACACCACCTAC	
BL553.r	TACAAGAAAGCTGGGTCAATCGGTCGTGATCTTCGAG	
BL554.f	GCATCTAGACGTTTCGATCCGCCGATGTC	
BL554.r	GCAGAGCTCAGTTGGCGAAGGATCTCTGAC	
JRH04.f	GACTCTAGATAAGAAGGAGATATACCATGAGCGACCAGGTGATCTTC	
JRH04.r	GACCTCGAGAGTTGGCGAAGGATCTCTGAC	

lators involved in α-KG utilization, including KgtR of *R. tropici* CIAT899 (8) and KguR of *E. coli* CTF073 (10). The results presented here indicate that MifR is a homolog of the KgtR/KguR proteins and participates with RpoN to activate the expression of the putative α-KG transporter (PA5530) in response to extracellular α-KG.

MATERIALS AND METHODS

Bacteria, plasmids, and media. Bacterial strains and plasmids used in the current study are given in Table 1. Bacteria were grown in Lennox broth (BD Difco), nutrient broth no. 2 (Oxoid), or modified M9 minimal medium (30), containing 5 μM FeSO$_4$. Solid bacteriological medium was prepared with the addition of BD Bacto agar at 15 g liter^{-1}. The following

antibiotics were used for plasmid selection: kanamycin (Km) (50 μg ml^{-1} for *E. coli*), carbenicillin (Cb) (100 μg ml^{-1} for *E. coli* or 200 μg ml^{-1} for *P. aeruginosa*), and gentamicin (Gm) (10 μg ml^{-1} for *E. coli* or 30 μg ml^{-1} for *P. aeruginosa*).

Molecular biology methods. DNA was purified by using Promega nucleic acid purification kits. Restriction enzymes and ligases were products of New England BioLabs. PrimeStar polymerase (TaKaRa Biosciences) was used for all PCRs, which were done according to the recommended protocols for PrimeStar polymerase. Oligonucleotides used for PCR are provided in Table 1. Genomic DNA from *P. aeruginosa* PAO1 was used for all PCR applications. PCR products were cloned into pCR-Blunt by using the Zero Blunt PCR cloning kit (Invitrogen). Cloned DNA was verified by sequencing (Genewiz).

Construction of ΔP*lac*-pBBR1MCS-5. A promoterless plasmid suitable for the housing and analysis of reporter genes in *P. aeruginosa* was constructed. The *lac* promoter positioned upstream of the multiple-cloning site of plasmid pBBR1MCS-5 was removed via SphI-KpnI double digestion. The SphI-KpnI-digested pBBR1MCS-5 fragment was blunted by using *Pfu* DNA polymerase (Agilent Technologies) and subsequently religated to yield ΔP*lac*-pBBR1MCS-5.

Cloning of the PA5530, *mifR* (PA5511), and *mifSR* (PA5512-PA5511) genes. The PA5530, *mifR*, and *mifSR* genes were PCR amplified with primer pairs BL434.f/BL434.r, JRH04.f/JRH04.r, and BL554.f/BL554.r, respectively. The PA5530, *mifR*, and *mifSR* PCR products were cloned into pCR-Blunt to generate plasmids pBRL474, pJRH07, and pBRL549, respectively. The PA5530 and *mifR* genes were individually subcloned into the XbaI/SacI sites of pBBR1MCS-5 to yield pBRL479 and pJRH08, respectively.

The XbaI/SpeI *mifSR* fragment from pBRL549 was cloned into the XbaI site of pTrc99a with either a forward or backward orientation to give plasmid pBRL566 or pBRL567, respectively. Plasmid pBRL549 was digested with SalI to excise an internal portion (nucleotides 27 to 830) of the *mifR* gene, thereby yielding plasmid pBR561. The resulting XbaI/SpeI *mifS*-Δ*mifR* fragment was cloned from pBRL561 into the XbaI site of pTrc99a with a forward orientation, generating pBRL570.

Cloning of the PA5530-*lacZ* fusion. The 1,069-bp 5′ region upstream of the predicted PA5530 open reading frame (ORF) was PCR amplified with primer pair BL435.f/BL435.r, and the β-galactosidase (*lacZ*) ORF of *E. coli* K-12 MG1655 was PCR amplified with primer pair BL342.f/BL342.r. The PA5530 promoter and *lacZ* PCR products were assembled into a single fragment via fusion PCR (31), using primer pair BL435.f/BL342.r. The PA5530-*lacZ* fusion was cloned into pCR-Blunt to give pBRL476. The XbaI/SpeI PA5530-*lacZ* fragment of pBRL482 was subcloned into the XbaI site of ΔP*lac*-pBBR1MCS-5 to yield pBRL485. The "GG" dinucleotide positioned 105 bp upstream of the *lacZ* ORF in the PA5530-*lacZ* reporter (in pBRL485) was changed to "AA" by using the QuikChange kit (Agilent Technologies) and primer pair BL438.f/BL438.r. The resulting plasmid, pBRL488, was sequenced to verify that the desired substitution was present.

Deletion of the PA5530 and *mifR* (PA5511) genes in *P. aeruginosa*. The PA5530 and *mifR* genes were deleted in *P. aeruginosa* according to the method of Choi and Schweizer (29). Procedures for plasmid construction, electroporation, screening, selection, and marker removal were conducted exactly as described previously (29). Briefly, the PA5530::Gm and *mifR*::Gm cassettes were cloned into plasmid pEX18ApGW to give pBRL511 and pBRL538, respectively. The PA5530 gene was deleted in *P. aeruginosa* PA14 and PAK by using pBRL511, whereas the *mifR* gene was deleted in *P. aeruginosa* PAO1 and PW9687 by using pBRL538. The Gm markers were removed by using plasmid pFLP2. The ΔPA5530 and Δ*mifR* mutations were verified by PCR.

Growth experiments on C$_5$-dicarboxylates. All *P. aeruginosa* strains were grown in triplicate. For each replicate, 50 ml of M9 minimal medium supplemented with 5 μM FeSO$_4$ and 20 mM C$_5$-dicarboxylate (α-KG or glutarate) (in a 500-ml baffle shake flask) was inoculated to an initial optical density at 600 nm (OD$_{600}$) of ∼0.1. Cultures were grown at 37°C at

200 rpm, and the absorbance at 600 nm (OD$_{600}$) was measured at 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 24 h postinoculation. Note that M9 minimal medium did not have to be supplemented with gentamicin to select for recombinant *P. aeruginosa* strains harboring plasmid pBRL479 or pJRH08; the presence of C$_5$-dicarboxylate as a sole carbon source was sufficient for selection.

Measurement of extracellular α-KG from *P. aeruginosa* cultures. Experiments were done in triplicate. *P. aeruginosa* PAO1 and the PA5530 transposon mutant (strain PW10317) were grown in 2.0 ml of nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm for 18 h. Afterwards, 50 ml of nutrient broth no. 2 (Oxoid) (in a 500-ml baffle shake flask) was inoculated with 0.5% of a seed culture, and the culture was grown at 37°C at 200 rpm. At OD$_{600}$ values of 0.2, 0.4, 0.8, and 1.6, an aliquot of culture (1.0 ml) was passed through a 0.22-μm membrane (Millipore) via vacuum filtration. The filtrate (0.5 ml) was added to an equal volume (0.5 ml) of 0.8 M perchloric acid, and the solution was vortexed vigorously for 1 min.

Each prepared sample (20 μl) was combined with an equal volume (20 μl) of 1,2-diamino-4,5-methylenedioxybenzene (DMB) derivatization reagent (32), and the mixture was incubated in the dark at 50°C for 2.5 h. If needed, samples were diluted prior to the DMB derivatization so that the measured concentration of the DMB–α-KG derivative was between 20 and 200 μM (dynamic range of the method). The DMB-derivatized α-KG was analyzed in each sample by electrospray ionization-liquid chromatography mass spectrometry (ESI-LC-MS), which consisted of an API2000 LC tandem mass spectrometry (LC/MS/MS) system equipped with a turbo-ion spray source interfaced with a Prominence UFLC. The column used for separation was a C$_{18}$ reversed-phase Hypersil (100-mm- by 2.1-mm internal diameter [ID], 3-μm particle size, and 120-Å pore size). Mobile phase A (H$_2$O with 0.05% formic acid) and mobile phase B (95% acetonitrile–5% H$_2$O with 0.05% formic acid) were used for all separations. The elution procedure consisted of an isocratic profile of 15 min of 20% mobile phase B in mobile phase A and a linear gradient from 20 to 100% mobile phase B in mobile phase A over 1 min, followed by an isocratic profile of 100% mobile phase B for 4 min with a flow rate of 0.30 ml min^{-1}. Electrospray ionization in the positive mode was performed by using the turbo-ion spray source with an ion spray voltage of 4,500 V, a desolvation temperature of 300°C, and gas 1 and gas 2 set at 20 and 30, respectively. Mass spectra were collected over the range 200 to 300 *m/z* with a declustering potential of 22 V, a focusing potential of 400 V, and an entrance potential of 5 V. The DMB–α-KG derivative was detected by ion extraction of the [M + H]$^+$ *m/z* of 262.9 to 263.5. All traces were smoothed by using a Savitzky-Golay filter, and the area under the peak was determined by using Analyst classic parameters from the Applied Biosystems software (version 1.5). Concentrations reported for α-KG represent mean values (± standard deviations [SD]).

PA5530-*lacZ* expression experiments. Each treatment was tested in triplicate, and galactosidase (LacZ) activity was measured by using the Miller assay as described previously (23). Recombinant *P. aeruginosa* and *E. coli* strains harboring pBRL485 were grown in nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm. When the cells reached an OD$_{600}$ of ∼0.3, exogenous substrates were added to a final concentration of 0.02 to 20 mM, and LacZ activity was assayed at either 30 or 90 min postinduction.

Microarray analysis. Microarray experiments were done in triplicate. *P. aeruginosa* PAO1 was grown in nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm to an OD$_{600}$ of 0.3. α-KG was then added to the cultures at a final concentration of either 0 or 20 mM. At 30 min postinduction, an aliquot of culture (1.0 ml) was mixed with 0.5 ml RNAprotect Bacteria reagent (Qiagen). The bacteria in the treated samples were lysed by using an enzymatic proteinase K digestion approach, as detailed in the RNAprotect Bacteria reagent handbook. The RNA was purified by using the RNeasy minikit (Qiagen) with an on-column DNase digestion step. PCR and a Bioanalyzer (Agilent Technologies) were used to check the purified RNA samples for DNA contamination and overall quality. Microarray studies were performed by the Microarray Core Facility (Upstate Medical University, Syracuse, NY) by using *P. aeruginosa* PAO1 Affymetrix GeneChip
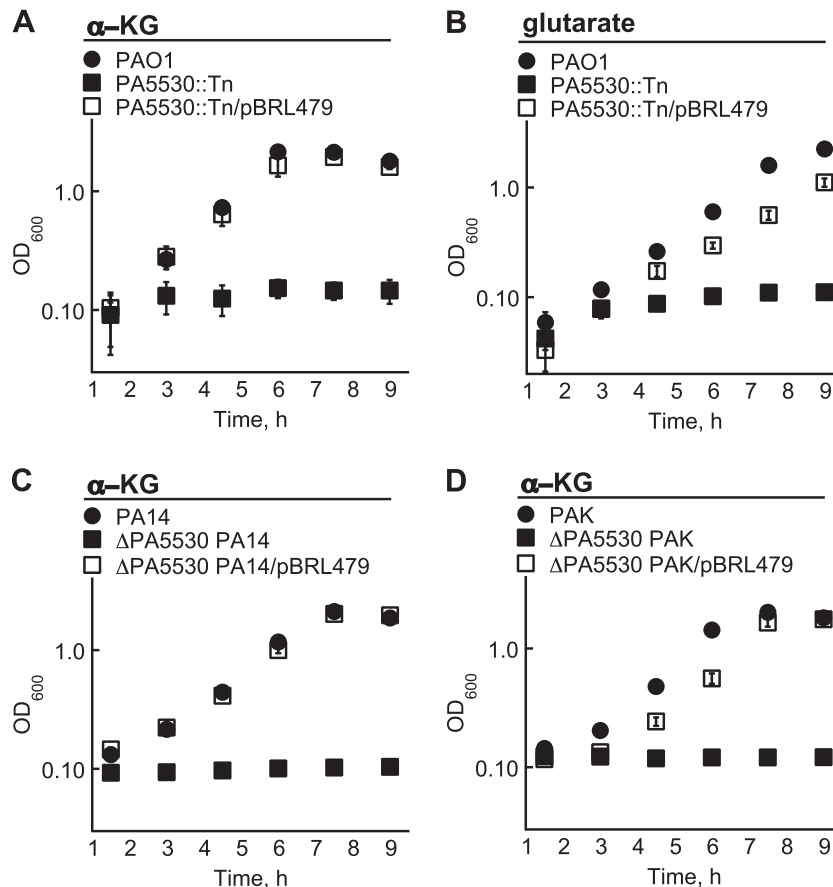
FIG 1 The PA5530 gene is required for the growth of *P. aeruginosa* on C_5-dicarboxylates. (A and B) A PA5530 transposon mutant (PA5530::Tn) of *P. aeruginosa* PAO1 did not grow in M9 minimal medium when the sole carbon source was 20 mM α-KG (A) or glutarate (B). (C and D) α-KG was also not a viable carbon source for ΔPA5530 derivatives of *P. aeruginosa* PA14 (C) and PAK (D). Growth on C_5-dicarboxylates was recovered for all strains when the PA5530 gene was expressed from the *lac* promoter of pBBR1MCS-5 (pBRL479). Data points represent mean values ± SD (*n* = 3).

arrays. Methods for conducting the microarray experiments, including data processing and statistical analysis, were done exactly as previously described (23).

Microarray data accession number. Data from the microarray experiment were deposited in the Gene Expression Omnibus (GEO) database (33) under accession no. GSE54032.

RESULTS

The PA5530 gene is required for growth of *P. aeruginosa* on C_5-dicarboxylates. The protein responsible for α-KG transport in *P. aeruginosa* PAO1 was expected to possess significant homology to the α-KG:H^+ symporter (KgtP) of *E. coli* K-12 MG1655. After performing a BLASTP search against the protein database of *P. aeruginosa* PAO1, the putative PA5530 protein was found to have 70% homology (55% identity) to KgtP of *E. coli* K-12. Consistent with the idea that the PA5530 protein functions as an α-KG transporter, a *P. aeruginosa* PAO1 strain harboring a transposon insertion within the PA5530 gene could not utilize α-KG as a carbon source (Fig. 1A). Additionally, the PA5530 mutant did not grow on glutarate-minimal medium (Fig. 1B), showing that the assimilation of C_5-dicarboxylates converges on the PA5530 gene. Growth on C_5-dicarboxylates was restored in the PA5530 mutant when the PA5530 gene was expressed from the *lac* promoter on the broad-host-range plasmid pBBR1MCS-5.

The PA5530 gene was also required for the growth of *P. aerugi-*

nosa strains PA14 and PAK on α-KG. The PA5530 gene was deleted in *P. aeruginosa* strains PA14 and PAK, and the resulting ΔPA5530 *P. aeruginosa* mutants were assayed for growth on α-KG. As expected, neither ΔPA5530 mutant grew on α-KG unless the PA5530 gene was heterologously expressed from pBBR1MCS-5 (Fig. 1). The necessity of the PA5530 gene for α-KG assimilation appears to be a shared trait of *Pseudomonas aeruginosa* strains PAO1, PA14, and PAK.

We next confirmed that the PA5530 gene is required for α-KG uptake in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 and the PA5530 mutant were grown in nutrient broth no. 2 (Oxoid), and the extracellular α-KG concentrations were measured at cell densities (OD_{600}) of 0.2, 0.4, 0.8, and 1.6. At an OD_{600} of 0.2, *P. aeruginosa* PAO1 and the PA5530 mutant had comparable extracellular α-KG concentrations of 240 (±38) μM and 384 (±32) μM, respectively. By the next doubling (OD_{600} of 0.4), extracellular α-KG concentrations decreased to <20 μM (lower detection limit) for *P. aeruginosa* PAO1 but increased to 898 (±122) μM for the PA5530 mutant. Interestingly, *P. aeruginosa* PAO1 at an OD_{600} of 0.8 accumulated extracellular α-KG to a concentration of 88 (±24) μM, which decreased to <20 μM at an OD_{600} of 1.6. In contrast, the PA5530 mutant exhibited extracellular α-KG concentrations of 868 (±72) μM and 1,134 (±48) μM at OD_{600} values of 0.8 and 1.6, respectively. The elevated extracellular α-KG
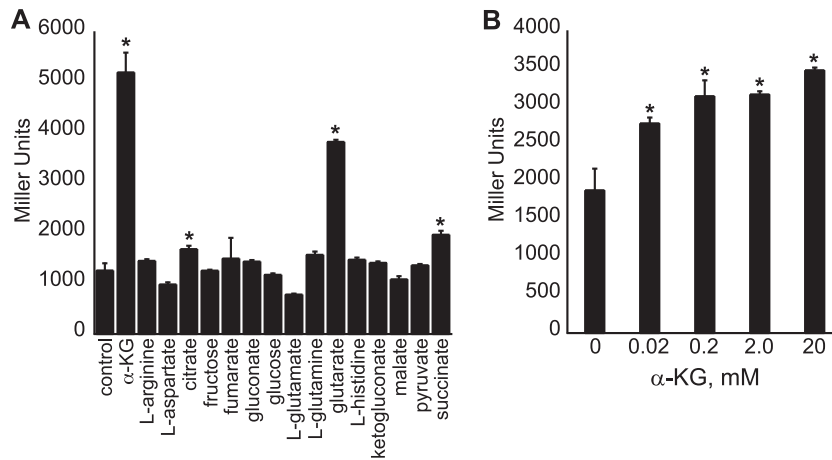
FIG 2 Expression of a PA5530-*lacZ* reporter is induced by extracellular C$_5$-dicarboxylates. The 5′ 1,069-bp regulatory region upstream of the PA5530 ORF was fused to *lacZ*, and the resulting PA5530-*lacZ* reporter was introduced into *P. aeruginosa* PAO1. Recombinant cells were grown in nutrient broth no. 2 (Oxoid) to an OD$_{600}$ of 0.3 and subsequently induced with various organic compounds provided at a final concentration of 20 mM. (A) At 90 min postinduction, LacZ activity was highest for cells treated with α-KG or glutarate. (B) The expression level of PA5530-*lacZ* increased within 30 min after the addition of α-KG to a final concentration of 0.02 to 2 mM. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

concentration observed for the PA5530 mutant is a characteristic also found in a Δ*kgtP E. coli* strain (12) and shows that the disruption of the PA5530 gene hinders the uptake of α-KG in *P. aeruginosa* PAO1. It should be noted that the α-KG content of nutrient broth no. 2 (Oxoid) was found to be <20 μM. Therefore, the measured extracellular α-KG concentration was a product of *Pseudomonas* metabolism and not an artifact of nutrient broth no. 2 (Oxoid).

Expression of the PA5530 gene is regulated by extracellular α-KG. Previous biochemical studies have shown the existence of an inducible α-KG transporter in *Pseudomonas* (21). To determine if the expression of the PA5530 gene is induced by the presence of extracellular C$_5$-dicarboxylates, the 1,069-bp 5′-regulatory region immediately upstream of the PA5530 start codon was fused to a DNA segment encoding the β-galactosidase (*lacZ*) ORF of *E. coli* K-12 MG1655. The resulting PA5530-*lacZ* fusion was cloned into a promoterless plasmid, ΔP*lac*-pBBR1MCS-5, and subsequently electroporated into *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 harboring the PA5530-*lacZ* reporter was grown in nutrient broth no. 2 (Oxoid) to an OD$_{600}$ of 0.3, at which time various organic acids were added to a final concentration of 20 mM. As shown in Fig. 2A, expression of PA5530-*lacZ* increased by 330% (±28%) within 90 min of the addition of α-KG. Glutarate also induced the expression of PA5530-*lacZ* by 224% (±8.0%). Of the remaining carbon sources tested, only succinate and citrate were found to have an inducing effect, increasing PA5530-*lacZ* expression levels by ∼50%. These data confirm that expression of the PA5530 gene is strongly induced in the presence of extracellular C$_5$-dicarboxylates, with preference toward α-KG. For example, the PA5530-*lacZ* expression level increased by 48% (±22%) within 30 min after the addition of α-KG to a final concentration of 20 μM (Fig. 2B).

The data from the PA5530-*lacZ* reporter experiments showed that the expression of the PA5530 gene is induced by extracellular α-KG; however, the number and range of genes whose expression is regulated by α-KG were not known. We therefore determined the transcriptional response of *P. aeruginosa* PAO1 to extracellular

α-KG using Affymetrix GeneChips. In triplicate, *P. aeruginosa* PAO1 cells were grown in nutrient broth no. 2 (Oxoid) to an OD$_{600}$ of 0.3 and then treated with either 0 or 20 mM α-KG. At 30 min postinduction, cells were harvested, and total RNA was isolated. Following microarray and statistical analyses, a total of four genes displayed changes in transcript levels by >2-fold with the addition of exogenous α-KG.

The changes in transcript levels for three of the four genes were near the 2-fold cutoff: PA0865 (−2.8 ± 0.7), PA4131 (−2.5 ± 0.3), and PA5170 (3.7 ± 1.6). The PA4131 gene encodes a putative iron-sulfur protein and has no known function. The physiological roles of the PA0865 (*hpd*) and PA5170 (*arcD*) genes were previously analyzed (34, 35), and their known functions cannot be readily extrapolated to α-KG metabolism. Because of this uncertainty and the slight changes observed in their transcript levels, these genes are not considered to directly participate in α-KG assimilation. Expectedly, the transcript level of the PA5530 gene increased by 17-fold (±5.0-fold) with the addition of exogenous α-KG. The microarray results reinforce the findings of the PA5530-*lacZ* experiments, and collectively, they show that α-KG induces the expression of the PA5530 gene. The inducible expression of the PA5530 gene is a behavior reminiscent of other bacterial transporter genes and argues that the PA5530 gene most likely encodes the α-KG transporter in *P. aeruginosa* PAO1.

Expression of the PA5530 gene is dependent on RpoN. The sigma factor RpoN recognizes a −24/−12 promoter with the consensus sequence 5′-TGGCACG-N4-TTGCW-3′ (W is A/T) (36). Located 92 bp upstream of the predicted start codon of the PA5530 ORF is a putative RpoN promoter, 5′-TGGCACG-N4-CTGCT-3′. To determine if this predicted RpoN promoter is functional, the highly conserved "GG" motif of the −24 element was changed to "AA" in the PA5530-*lacZ* reporter. This mutation in the RpoN promoter of the PA5530-*lacZ* reporter reduced its expression by >95% in *P. aeruginosa* PAO1 (Fig. 3); this finding is consistent with the "GG" motif being a key determinant of RpoN-mediated transcription. Furthermore, there was a >95% decrease in the expression level of the PA5530-*lacZ* reporter in an *rpoN*
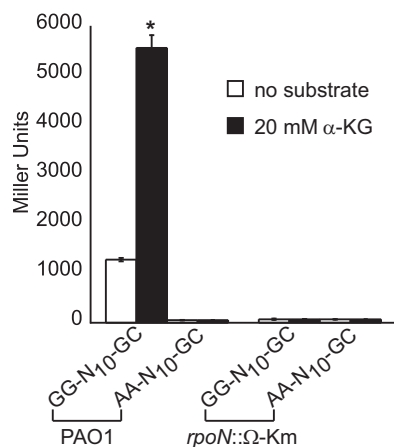
FIG 3 RpoN is essential for the expression of the PA5530-*lacZ* reporter. Positioned 93 bp upstream of the PA5530 ORF is a sequence resembling a −24/−12 promoter (GG-N$_{10}$-GC). Replacement of the conserved "GG" dinucleotide of the −24 element with "AA" in the PA5530-*lacZ* reporter (denoted AA-N$_{10}$-GC) reduced its expression by >95% in *P. aeruginosa* PAO1. Additionally, expression of the PA5530-*lacZ* reporter was null in an *rpoN*::Ω-Km derivative of *P. aeruginosa* PAO1. Note that all strains were grown in nutrient broth no. 2 (Oxoid) to an OD$_{600}$ of 0.3 and then induced with 20 mM α-KG. LacZ activity was measured at 90 min postinduction. Data points represent mean values ± SD (*n* = 3). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes (*P* < 0.0001), which are marked (asterisk).

mutant compared to wild-type *P. aeruginosa* PAO1 (Fig. 3). The addition of exogenous α-KG did not change the expression of PA5530-*lacZ* in the *rpoN* mutant. These results support a model in which RpoN and its cognate −24/−12 promoter direct the transcription of the PA5530 gene in *P. aeruginosa* PAO1.

The *mifR* gene is required for expression of the PA5530 gene in *P. aeruginosa* PAO1. RpoN requires additional regulators, known as enhancer binding proteins (EBPs), to activate the transcription of targeted genes (37). Having established that RpoN is completely necessary for the expression of the PA5530 gene, we

next sought to determine the EBP that participates with RpoN in this process. To this end, we scanned the chromosomal region surrounding the PA5530 locus to identify genes encoding known or hypothetical EBPs. In the vicinity of the PA5530 locus is the *mifR* (PA5511) gene. The EBP MifR has been observed to be required for microcolony formation (22, 38), but it also possesses significant homology (>60%) to the DctD family of response regulators. DctD response regulators are EBPs that activate the transcription of genes whose products participate in the transport of dicarboxylates from the environment (39, 40). For *P. aeruginosa* PAO1, DctD (PA5166) regulates the transport of C$_4$-dicarboxylates such as fumarate, malate, and succinate (41). We therefore wondered if MifR might be a DctD homolog that regulates the transport of C$_5$-dicarboxylates in *P. aeruginosa* PAO1.

In support of MifR having a role in the utilization of C$_5$-dicarboxylates, a Δ*mifR* mutant of *P. aeruginosa* PAO1 could not use α-KG or glutarate as a growth substrate (Fig. 4). Expression of the PA5530 gene from plasmid pBBR1MCS-5 rescued the growth of the Δ*mifR* mutant on both of these C$_5$-dicarboxylates. This finding suggested that the diminished capacity of the Δ*mifR* mutant to grow on C$_5$-dicarboxylates might be a result of insufficient expression of the PA5530 gene. This suspicion was confirmed when it was observed that the expression of the PA5530-*lacZ* reporter was reduced by >95% in the Δ*mifR* mutant compared to wild-type *P. aeruginosa* PAO1 (Fig. 5). We also found that the *dctD* gene had no impact on the expression of PA5530-*lacZ*; α-KG induced the expression of PA5530-*lacZ* in a *dctD* mutant to levels observed in wild-type cells (Fig. 5). As expected, deletion of the *mifR* gene in the *dctD* mutant abolished the expression of PA5530-*lacZ*. The inability of the Δ*mifR* mutant to grow on C$_5$-dicarboxylates or express the PA5530-*lacZ* reporter gives credibility to the idea that the primary function of MifR is the regulation of C$_5$-dicarboxylate transport in *P. aeruginosa* PAO1.

The response regulator gene *mifR* is in a putative operon with its predicted sensor kinase partner gene *mifS*. Sensing and responding to extracellular α-KG are most likely mediated by the MifSR two-component signal transduction system (TCS) in *P. aeruginosa* PAO1. To test this hypothesis, the *mifSR* genes were
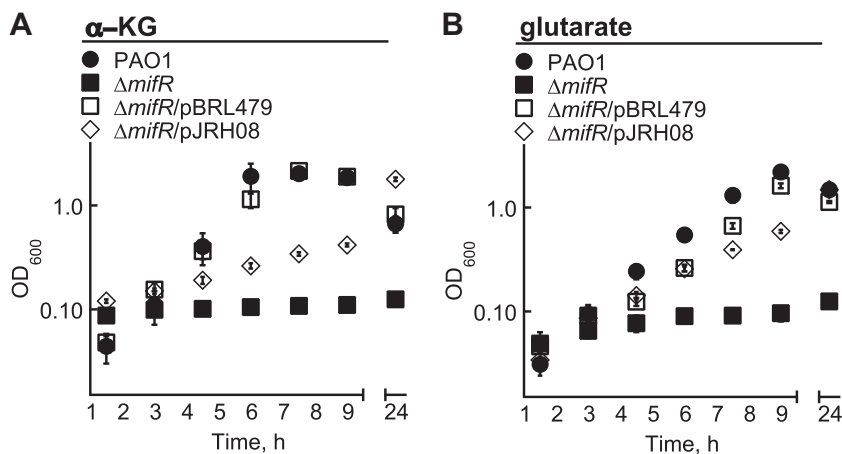

FIG 4 Heterologous expression of the PA5530 gene rescues the growth of a Δ*mifR* mutant of *P. aeruginosa* PAO1 on C$_5$-dicarboxylates. A Δ*mifR* mutant of *P. aeruginosa* PAO1 could not grow in M9 minimal medium with either 20 mM α-KG (A) or glutarate (B) as the sole carbon source. Expression of the PA5530 gene from the *lac* promoter of pBBR1MCS-5 (pBRL479) restored the growth of the Δ*mifR* mutant on these C$_5$-dicarboxylates. There was partial complementation of the Δ*mifR* mutant with pJRH08 (*mifR* gene in pBBR1MCS-5). Full complementation most likely requires optimizing the expression of the *mifR* gene in plasmid pJRH08 (for an example, see reference 34). Data points represent mean values ± SD (*n* = 3).
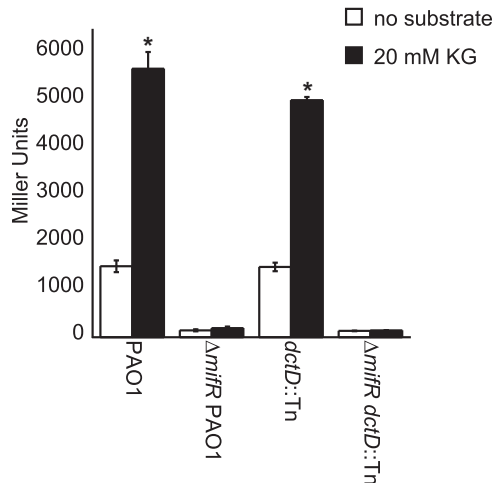
FIG 5 Expression of the PA5530-*lacZ* reporter is dependent on the *mifR* gene. Expression of the PA5530-*lacZ* reporter was reduced by >95% in a Δ*mifR* mutant of *P. aeruginosa* PAO1 compared to that observed in wild-type cells. Cells harboring a *dctD* transposon insertion (*dctD*::Tn) displayed wild-type levels of LacZ activity. However, deletion of the *mifR* gene in *dctD*::Tn cells eliminated the expression of PA5530-*lacZ*. These findings suggest that MifR regulates C₅-dicarboxylate transport, whereas DctD is involved in the transport of C₄-dicarboxylates. Note that all strains were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then induced with 20 mM α-KG. LacZ activity was measured at 90 min postinduction. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

heterologously expressed in *E. coli* Top10 cells that also harbored the PA5530-*lacZ* reporter. As shown in Fig. 6, the basal expression of the *mifSR* operon from the *trc* promoter of plasmid pTrc99a allowed *E. coli* Top10 to express the PA5530-*lacZ* reporter when challenged with external α-KG. Specifically, LacZ activity increased by >1,000% within 120 min of the addition of 20 mM α-KG. This induction was dependent on the *mifR* gene, because *E. coli* Top10 harboring (i) a *mifS*-Δ*mifR* operon or (ii) a *mifSR* operon in the backward orientation of the *trc* promoter of pTrc99a failed to express the PA5530-*lacZ* reporter in the presence of extracellular α-KG. Glutarate induced the expression of the PA5530-*lacZ* reporter by 125% (±10%), whereas C₄-dicarboxylates had no effect on PA5530-*lacZ* expression. These findings show that the *mifSR* genes are sufficient for activating the expression of the PA5530-*lacZ* reporter in nonnative *E. coli* and thus suggest that MifR is a direct regulator of the PA5530 gene.

DISCUSSION

The main objective of this study was to determine the gene encoding the α-KG transporter in *P. aeruginosa* PAO1. Based on homology to the prototypic α-KG transporter KgtP, we identified two genes, PA0229 (*pcaT*) and PA5530, which might encode the α-KG transporter. PA0229 or PcaT is annotated as a putative α-KG transporter in the Pseudomonas Genome Database (42), but PcaT proteins function in the transport of β-ketoadipate (43), which is a C₆-dicarboxylate. In *P. aeruginosa* PAO1, the *pcaT* gene is part of the predicted *pcaTBCD* operon, whose products participate in the breakdown of aromatic compounds, i.e., the protocatechuate
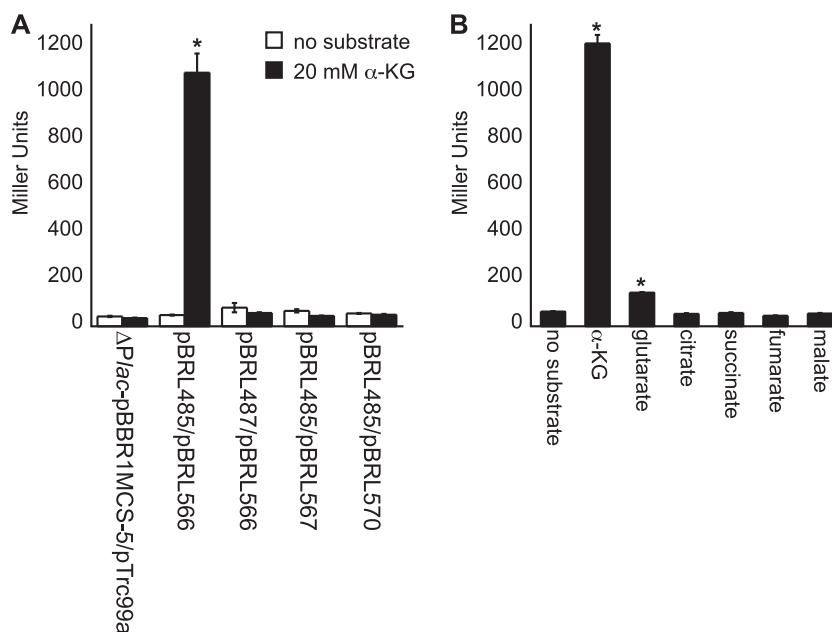

FIG 6 α-KG induces expression of the PA5530-*lacZ* reporter in nonnative *E. coli* only when the *mifSR* genes are heterologously expressed. (A) *E. coli* Top10 cells that basally expressed the *mifSR* operon from the *trc* promoter of pTrc99a (pBRL566) responded to extracellular α-KG via increasing the expression level of the PA5530-*lacZ* reporter by >1,000% compared to the level in noninduced cells. *E. coli* Top10 cells expressing nonfunctional *mifSR* alternatives, including a *mifSR* operon oriented in the opposite direction of the *trc* promoter (pBRL567) or a *mifS*-Δ*mifR* operon (pBRL570), did not express the PA5530-*lacZ* reporter. Furthermore, the *mifSR* operon did not cause a change in the expression of the PA5530-*lacZ* reporter in which the "GG" dinucleotide of the −24 element of the putative RpoN promoter was replaced with "AA" (pBRL487). (B) Lastly, expression of PA5530-*lacZ* in *E. coli*/pBRL566 was induced by extracellular C₅-dicarboxylates. These results show that the *mifSR* genes can directly regulate the expression of the PA5530 gene in response to extracellular α-KG. For all experiments, cells were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then induced with 20 mM substrate. LacZ activity was measured at 120 min postinduction. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

branch of the β-ketoadipate pathway (44). Consequently, PcaT was considered to be a transporter for β-ketoadipate and not α-KG. This made the PA5530 gene a favorable candidate for encoding the α-KG transporter in *P. aeruginosa* PAO1.

A transposon insertion within the PA5530 gene in *P. aeruginosa* PAO1 prevented this bacterium from using C₅-dicarboxylates as growth substrates. Follow-up experiments showed that the disruption of the PA5530 gene caused a significant accumulation of extracellular α-KG. For example, both wild-type *P. aeruginosa* PAO1 and a PA5530 mutant produced ∼200 μM extracellular α-KG within 90 min of being inoculated into a rich medium. Whereas wild-type cells were able to assimilate this extracellular α-KG, lowering its levels below 20 μM, the PA5530 mutant continued to accumulate extracellular α-KG, which eventually reached concentrations of >1.0 mM. This result is consistent with the PA5530 gene functioning in α-KG transport, but we did not anticipate that the levels of extracellular α-KG would be in the millimolar range. The generation and subsequent uptake of extracellular α-KG are well-known characteristics of *P. aeruginosa* cultures. However, the magnitude or significance of this activity when *P. aeruginosa* is grown in a rich medium has not been reported previously. The metabolic process responsible for the continuous production of extracellular α-KG is a topic worth exploring.

α-KG did induce the expression of the PA5530 gene, as determined by both microarray and *lacZ* reporter experiments. In fact, the exogenous addition of 20 μM α-KG was sufficient to stimulate the expression of the PA5530 gene. Expression of the PA5530 gene was also induced by extracellular glutarate, reaffirming that the PA5530 gene has all the elements associated with an inducible C₅-dicarboxylate transporter. Namely, the PA5530 gene (i) encodes a putative KgtP homolog, (ii) was essential for the utilization of C₅-dicarboxylates, (iii) was required for the uptake of extracellular α-KG, and (iv) was genetically regulated by extracellular C₅-dicarboxylates.

Upstream of the PA5530 ORF is a −24/−12 promoter recognized by the sigma factor RpoN. We did not find any sequence resembling a σ⁷⁰-type promoter within the vicinity of the predicted start codon of the PA5530 ORF. The changing of the highly conserved "GG" dinucleotide of the −24 element to "AA" completely eliminated the expression of the PA5530 gene. Additionally, the PA5530 gene was not expressed in an *rpoN*-deficient derivative of *P. aeruginosa* PAO1. Both of these results suggest that RpoN is the sigma factor responsible for the expression of the PA5530 gene. The genetic regulation of C₅-dicarboxylate transport by RpoN might be a common mechanism employed by *Pseudomonas*, because the *rpoN* gene was also required for the growth of *P. putida* on α-KG (45).

One of the most interesting findings of this study was the requirement of the *mifR* gene for the assimilation of C₅-dicarboxylates in *P. aeruginosa* PAO1. The response regulator MifR (PA5511) and its partner sensor kinase MifS (PA5512) comprise a TCS that was initially found to be essential for the development of mature biofilms (22). Later studies showed that in the absence of the *mifR* gene, pyruvate fermentation was suboptimal and thus unable to support the formation of microcolonies (38). In our study, a ΔmifR mutant could not utilize C₅-dicarboxylates due to a significant reduction (>95%) in the expression of the PA5530 gene.

A reasonable explanation as to why the *mifR* gene was required for the expression of the PA5530 gene was found when we closely examined the amino acid sequence of the MifR protein. Like all other enhancer binding proteins, MifR has the conserved RpoN interaction domain, but more importantly, MifR is 70% homologous (54% identical) to the response regulator DctD (PA5166) of *P. aeruginosa* PAO1. The presence of dual DctD regulators (commonly annotated DctD1 and DctD2) has been reported for the sequenced genomes of several *Pseudomonas*-related bacteria, including *Ralstonia solanacearum*, *Azotobacter vinelandii*, *Pseudomonas protegens*, and *Burkholderia mallei* (NCBI Genome Database). The DctBD TCS of *P. aeruginosa* PAO1 is involved in the assimilation of C₄-dicarboxylates (41), and we found that the *dctD* gene had no effect on the expression of the PA5530 gene. It is plausible that MifS and MifR function as a DctBD-type TCS that responds to extracellular C₅-dicarboxylates via activating the expression of the PA5530 gene. Some bacteria are known to use a TCS, e.g., KguSR (10) and KgtSR (8), to regulate α-KG assimilation, and we found that the heterologous expression of the *mifSR* genes in *E. coli* was sufficient to activate the expression of the PA5530-*lacZ* reporter in response to extracellular α-KG. Biochemical evidence is needed to verify that MifR binds to and regulates the expression of the PA5530 gene.

Lastly, it will be valuable to determine if the PA5530 gene or α-KG has any role in biofilm development in *P. aeruginosa* PAO1. Cells within the interior of matured biofilms are considered to be in an anaerobic environment (46, 47), and recently, pathogenic *E. coli* strain CTF073 was reported to preferentially assimilate α-KG as a carbon source under anaerobic growth conditions (10). It is possible that MifR is required for the anaerobic metabolism of α-KG in *P. aeruginosa* PAO1. A recent transcriptomic study found that the transcript levels of several genes whose products are involved in anaerobic metabolism were deregulated in a ΔmifR mutant of *P. aeruginosa* PAO1 (38). We have now just begun to analyze α-KG utilization for anaerobically grown *P. aeruginosa* PAO1, including its ΔPA5530 and ΔmifR derivatives.

ACKNOWLEDGMENTS

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