Transcriptional Regulation of Carnitine Catabolism in Pseudomonas aeruginosa by CdhR

Jamie A. Meadows,a Matthew J. Wargoa,b

aDepartment of Microbiology and Molecular Genetics, University of Vermont Larner College of Medicine, Burlington, Vermont, USA
bThe Vermont Lung Center, University of Vermont Larner College of Medicine, Burlington, Vermont, USA

ABSTRACT The common environmental bacterium and opportunistic pathogen Pseudomonas aeruginosa encodes diverse metabolic pathways and associated regulatory networks allowing it to thrive in these different environments. In an effort to understand P. aeruginosa metabolism and detection of host-derived compounds, we previously identified CdhR and GbdR as members of the AraC transcription factor family that regulate catabolism of the quaternary amine compounds carnitine and glycine betaine, respectively. In this study, our goal was to further characterize regulation of carnitine catabolism by the transcription factor CdhR. CdhR binds in a concentration-dependent manner upstream of the carnitine catabolism operon promoter (PcaiXcdhCABhocS). We identified the CdhR binding site and determined that it overlaps with the GbdR binding site in the caiX-cdhR intergenic region. Carnitine catabolism is repressed by glucose and glycine betaine, and here we show this happens at the transcriptional level. Furthermore, we show that CdhR enhances its own expression and that GbdR contributes to cdhR expression by enhancing the level of basal expression. The intertwined regulation of caiX and cdhR transcription by GbdR and CdhR suggests that carnitine catabolism is under tight but tunable control.

IMPORTANCE Pathogens must metabolize host-derived compounds during infection and properly regulate the responsible pathways. Carnitine is a common eukaryotic-associated quaternary amine compound that can be catabolized by Pseudomonas aeruginosa. Here we expand on our understanding of how this metabolic pathway is regulated and provide details on how carnitine catabolism is intertwined with glycine betaine catabolism at the level of transcriptional control.

KEYWORDS metabolism, osmoprotectant, quaternary amine, transcriptional regulation

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen found in a wide variety of environments, often enriched in the drinking water distribution system, from which it can readily contaminate surfaces and medical devices in hospitals (1–4). The ability to transition from the preinfection niche to the host is likely a key to its success as a pathogen. P. aeruginosa encodes diverse pathways for metabolism of host-derived and host-independent carbon and nitrogen sources, allowing it to survive and thrive while undergoing these environmental transitions.

L-Carnitine (here referred to as “carnitine”) and O-acylcarnitines are quaternary amine compounds abundant in host tissues, one function of which is to shuttle fatty acids in and out of the mitochondria for β-oxidation in animals (5). There are no animal enzymes that can degrade carnitine (6); consequently any degradation in the host is due to bacteria, either by an aerobic pathway like P. aeruginosa (7) or an anaerobic pathway like many bacteria in the mammalian intestine (8). P. aeruginosa can acquire carnitine from the environment by import through the somewhat promiscuous ABC

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Address correspondence to Matthew J. Wargo, mwargo@uvm.edu.
transporter CbcWV using the CaiX periplasmic substrate binding protein (9). The enzymes required for carnitine or short-chain acylcarnitine catabolism in P. aeruginosa are encoded in the carnitine catabolism operon, caiX-cdhCAB-hocS (Fig. 1A), enabling metabolism for osmoprotection, virulence factor induction, and nutrition (10–13). Medium- and long-chain acylcarnitines, with the exception of octanoylcarnitine, can be used as sole carbon sources as well (10), but the enzymes required for the hydrolysis of these compounds have not been identified.

Part of the metabolic flexibility of P. aeruginosa can be attributed to its large repertoire of regulatory proteins—more than 9% of the genome is dedicated to transcriptional regulation (14). One such regulator is CdhR, which is divergently transcribed from the carnitine catabolism operon and is required for growth on carnitine and induction of the carnitine operon (11). Aerobic carnitine degradation (Fig. 1B) leads to the formation of glycine betaine (GB), the catabolic genes for which are transcriptionally regulated by GbdR (15). GbdR and CdhR not only regulate catabolism of related quaternary amine compounds, but both are AraC family transcription factors that belong to the same glutamine amidotransferase-1-like (GATase-1) transcriptional regulator subfamily (GATRs) and are similar in sequence (62% positive and 44% identical) (16, 17).

In this study, we expand our understanding of the regulation of carnitine catabolism by identifying the CdhR binding site, determining essential binding site residues, and demonstrating that catabolite repression of carnitine catabolism by glucose and glycine betaine functions at the level of transcription of the carnitine operon. We show that GbdR can bind the intergenic region of caiX-cdhR in an orientation supporting

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**FIG 1** Diagram of the P. aeruginosa PAO1 carnitine catabolism operon and the catabolic pathway. (A) Arrows represent the individual open reading frames of the carnitine catabolism operon and the regulator chdR. Below the arrow is the designated gene name. (B) Diagram of the cdhR-caiX intragenic region organized such that caiX transcription occurs left to right. The orange box denotes the cdhR 5’ UTR, the dark green box marks the position of the CdhR and GbdR binding sites (CdhR binding sequence listed below), and the light green box denotes the caiX 5’ UTR. (C) Diagram of the converging carnitine and choline catabolism pathways. Black arrows represent an enzymatic step in the catabolic pathway, and the gene names are italicized below. The blue arrows represent positive regulation by either CdhR or GbdR, and the T-bar represents repression by BetI.
regulation of cdhR. Finally, CdhR positively regulates its own expression in the presence of carnitine but represses basal expression in the absence of ligand, a repression that is alleviated when GbdR is present, suggesting a potential hierarchy of CdhR and GbdR binding at their overlapping binding sites.

RESULTS

Mapping the caiX promoter region. The carnitine catabolism operon, caiX-cdhCAB-hocS (Fig. 1A), is driven from a promoter located between caiX and cdhR (Fig. 1B) and encodes proteins that are responsible for the hydrolysis of short-chain acylcarnitines (10) and degradation of carnitine to glycine betaine (GB) (11, 18) (Fig. 1C). cdhR is divergently transcribed from the carnitine catabolism operon (Fig. 1A) and was previously shown to be required for induction of caiX and growth on carnitine (11). Primer extension was used to define the transcriptional start site of caiX and cdhR. Plasmids containing the target regions were used to increase RNA copy number, particularly for cdhR, as native transcripts are at low abundance (19). The length of the caiX primer extension product placed the transcriptional start site 25 bases upstream of the translational start site at a thymine residue. The length of the cdhR primer extension product placed the transcriptional start site 63 bases upstream of the translational start site at a cytosine. The relative sizes of the two untranscribed regions (UTRs) and their spacing compared to the CdhR binding site are shown in Fig. 1B.

To expand upon our understanding of CdhR’s role in carnitine catabolism, we narrowed down the binding site of CdhR by promoter mapping of PcaiX using four sequentially shorter lacZYA transcriptional reporters. The constructs end at bp +3 from the caiX transcriptional start site and begin at bp −399 (pJAM22), bp −302 (pJAM23), bp −216 (pJAM24), and bp −112 (pJAM25) (Fig. 2A). In the presence of carnitine, all constructs except pJAM25 were induced, indicating that the binding site of CdhR is between bp −216 and −112 or overlaps −112 (Fig. 2A) in relation to the transcriptional start site.

Based upon CdhR-dependent transcriptional induction of caiX (11) (Fig. 2A), we assayed the capability of purified maltose binding protein (MBP)-CdhR to bind to the upstream activation sequence (UAS) of caiX. Using biotin-labeled caiX UAS as the DNA probe and purified MBP-CdhR, electrophoretic mobility shift assays (EMSAs) revealed that MBP-CdhR binds the caiX UAS in a concentration-dependent manner (Fig. 2B, lanes 1 to 3). The binding interaction between MBP-CdhR and caiX probe can be competed with unlabeled caiX probe (lane 4), but not an unlabeled dhcA probe (lane 5), and...
MBP-CdhR does not shift the nonspecific UAS, dhcA (lanes 6 to 8). These data demonstrate that MBP-CdhR binds specifically to the caiX UAS.

Identification of the CdhR binding site sequence and bases required for induction of caiX. Promoter mapping and EMSAs determined the region of DNA necessary for carnitine-dependent caiX transcriptional induction and CdhR binding (Fig. 2). To further characterize the CdhR DNA contact site within the caiX UAS, we employed DNase I footprinting. There was a characteristic and site-specific protection of caiX UAS DNA as the MBP-CdhR concentration increased (Fig. 3A). Comparing the zone of protection to the A+G ladder, we were able to identify the CdhR contact region, stretching 34 bp (Fig. 3A). The CdhR binding site reveals that each half-site contains the sequence GGTCGC with a 15-bp spacer, which is very similar to the binding sites of three other related P. aeruginosa transcription factors, GbdR (17), SouR (20), and ArgR (21). To determine the importance of the half-site residues, we made mutations in the distal half-site by systematically changing partially overlapping dinucleotides to adenosines (pJAM122 to pJAM127). Induction from these mutated cdhR binding sites demonstrated that the second guanine residue in the half-site is important for caiX expression, but complete abolishment of carnitine-dependent induction requires an additional mutation of the adjacent thymine residue (Fig. 3B).

Glucose and glycine betaine repress the transcription of caiX. In P. aeruginosa, the enzymatic activity of carnitine dehydrogenase (CDH) declines until no longer detectable if the cells are switched from carnitine to glucose as the sole carbon source (22). CDH activity oscillates when carnitine is the sole carbon source, predicted to occur when the catabolic product glycine betaine is produced, resulting in induction of a negative-feedback oscillation loop (22). We used the caiX-lacZYA transcriptional reporter (pJAM22) to determine if catabolite repression is transcriptionally regulated. Maximal catabolite repression is seen at 4 mM glucose, while GB represses transcription to a lesser extent and at a higher concentration (Fig. 4).

FIG 3  The CdhR binding site and key residues for CdhR-dependent induction. (A) A DNase I footprinting assay was performed by taking the caiX UAS end labeled with ³²P and adding increasing concentrations of MBP-CdhR, followed by DNase I treatment and non-denaturing 5% polyacrylamide TBE gel. The first lane of the gel is the A+G sequencing ladder, and the nanomolar concentration of MBP-CdhR is marked. (B) The caiX enhancer site was mutated by changing two bases at a time (in red and underlined) in the caiX distal binding site to adenosines and fused to lacZ. The P. aeruginosa PA14 wild type carrying each of the plasmids was grown in MOPS with 20 mM pyruvate at 20 µg·ml⁻¹, with or without 1 mM carnitine for 4 h, and then β-galactosidase activity was reported as Miller units. Error bars represent standard deviations from three biological replicates, and results are representative of three independent experiments. Data were analyzed using a two-way analysis of variance (ANOVA) with a Sidak’s multiple-comparison posttest comparing each mutant’s pyruvate to carnitine. Abbreviations: P, pyruvate; C, carnitine; n.s., not significant; *, P < 0.05; ***, P < 0.001.
CdhR can bind, but does not regulate, the ABC transporter cbcXWV. After establishing the CdhR binding sequence, GGTCGC-[N15]-GGTCGC, we searched for this sequence in the *P. aeruginosa* PAO1 genome using the DNA motif search tool from the *Pseudomonas* Genome Database website (23). Only two identical sites were identified within intergenic regions: caiX-cdhR and cbcX-sdaB, both of which are involved in carnitine metabolism (9, 11). CbcXWV is an ABC transporter, and the core transporter proteins CbcWV are required for growth on carnitine along with the substrate binding component CaiX (9). An MBP-CdhR EMSA with the cbcXWV UAS probe showed MBP-CdhR binding in a concentration-dependent manner (Fig. 5A), but quantitative reverse...
transcription-PCR (qRT-PCR) revealed that carnitine cannot support induction of cbcX (Fig. 5B). Deletion of cdhCA eliminates production of GB from carnitine (Fig. 1B) (11); therefore, for carnitine to lead to cbcXWV induction, GB must be produced to enable cbcXWV induction via GbdR (17).

Roles of GbdR and CdhR at the caiX-cdhR intergenic region. The ability of CdhR to bind a known member of the GbdR regulon (cbcX) (17, 24), the detection of the GbdR binding consensus in the caiX-cdhR intergenic region (17), and the overlapping positions of the CdhR and GbdR consensus sites in the caiX UAS led us to investigate the role of GbdR in carnitine regulation. We predicted that GbdR would be able to bind the cdhR UAS, and an EMSA with purified MBP-GbdR demonstrated that MBP-GbdR binds the caiX-cdhR intergenic probe in a concentration-dependent manner (Fig. 6A). When the conserved CG residues for the GbdR-binding distal half-site were mutated to AA (GCCGC to GCAAC), binding was lost (Fig. 6A), as previously seen for similar mutations in the plcH and choE distal half-sites (17). Since GbdR binds the caiX-cdhR intergenic region, induction of caiX (pJAM22) was assessed in the wild type (WT) and a gbdR deletion mutant in both PA14 and PAO1 backgrounds. The gbdR deletion mutant has less induction of PcaiX-lacZ (Fig. 6C), but this defect is likely due to a defect in carnitine import (9) (Fig. 4).

After establishing that CdhR and GbdR bind the intergenic region of caiX-cdhR (Fig. 2B, 3A, and 6), we wanted to determine how these two transcription factors (TFs) impacted cdhR expression. Using two different translational reporter fusions—one carried on a plasmid in both PA14 and PAO1 backgrounds and one integrated into the chromosome at the attTn7 site—it became apparent that CdhR has a role in its own
expression. In the wild type, carnitine increased expression of cdhR compared to the
basal expression level (pyruvate) (Fig. 7A). In the absence of gbdR, carnitine still induces
cdhR, but basal expression of cdhR is decreased compared to that of the wild type and
a cdhR deletion mutant, suggesting that GbdR functions to relieve repression at this
locus (Fig. 7A). The similarity of the activity in the double deletion mutant with the gbdR
single deletion in the presence of carnitine suggests that carnitine detection by CdhR
allows relief of the baseline CdhR-dependent repression. The effects of CdhR and GbdR

FIG 7 CdhR promotes cdhR expression, and GbdR dampens basal repression. (A) WT, ΔcdhR ΔgbdR, and ΔcdhR ΔgbdR strains in both PA14 (14)
and PA01 (1) backgrounds carrying a cdhR-lacZ translational plasmid reporter (pJAM135) were grown in MOPS with 20 mM pyruvate and
20 µg·ml⁻¹ gentamicin, with or without 1 mM carnitine, and β-galactosidase activity was reported as fold change over WT pyruvate. Data were
analyzed using a two-way ANOVA with a Sidak’s multiple-comparison posttest comparing to the WT pyruvate condition within each strain. (PA14
was not compared to PA01.) *, P < 0.05. (B) PA01 WT, ΔcdhR, and ΔgbdR strains, all with the translational fusion cdhR-yfp integrated at the attTn7
site, were grown on MOPS agar pads with 20 mM pyruvate and with or without 1 mM carnitine. Cells were imaged under phase-contrast and
YFP fluorescence every 10 min at 32°C. Data were analyzed using a one-way ANOVA with a Dunnnett’s multiple-comparison posttest comparing
each time point within a strain to pyruvate at time zero (t = 0). ***, P < 0.001.
on cdhR expression are also seen with single-cell expression levels (Fig. 7B), showing both the general effects seen with the population assessment (Fig. 7A), as well as the heterogeneity in individual cell expression. These findings are summarized in a genetic model (Fig. 8).

DISCUSSION

The metabolic diversity of *P. aeruginosa* is controlled by a vast set of regulators, including one-component transcription factors that are often critical for regulating catabolism of alternate nutrient sources (25). One such transcription factor family that can control carbon metabolism and virulence is the AraC family (26, 27). In this study, we expand on our understanding of carnitine catabolism and show how two AraC family transcription factors, CdhR and GbdR, whose metabolic pathways converge at glycine betaine (GB) (Fig. 1C), are intertwined (Fig. 8). We identified the CdhR binding site in the *caiX-cdhR* intergenic region and through reporter and DNA binding assays reveal this site as an additional GbdR binding site. Through reporter fusions, we were able to show that CdhR regulates its own expression and is responsive to carnitine. We also show that GbdR binding to the *caiX-cdhR* region assists in regulating carnitine catabolism by inhibiting repression of *cdhR* transcription, thus maintaining the basal *cdhR* expression level.

The first question we addressed was how CdhR binds and regulates the carnitine operon. CaiF in *Escherichia coli* is the only other carnitine regulator that has been characterized to date, and it is a degenerate AraC TF that lacks the traditional N-terminal domain but maintains the helix-turn-helix (HTH) DNA binding domains (28). The CaiF amino acid sequence is 13% identical and 23% similar with 44% gaps compared to CdhR, with most of the similar regions within the HTH domains, therefore likely functioning in a manner much different than CdhR. caiF is transcribed in the opposite direction from the *E. coli* carnitine metabolism operon (*caiTABCDE*), which codes for utilization of carnitine as a terminal electron acceptor generating γ-butyrobetaine and binds to inverted repeats in the *caiF* UAS (28, 29). *P. aeruginosa* and other Gram-negative bacteria, capable of utilizing carnitine as a carbon and/or nitrogen source, contain a carnitine catabolism operon capable of generating glycine betaine and are organized in similar fashion to one another with an AraC family transcription factor divergently transcribed from the catabolic operon (Fig. 1A) (30). CdhR binds to direct repeats, and the binding site is upstream of the *caiX* promoter (Fig. 1B and 3A), categorizing CdhR as a class I activator (binds upstream and recruits RNA polymerase via the C-terminal domain of the alpha subunit [31]) that requires both half-sites for induction of *caiX* (Fig. 3B).

Kleber and Aurich analyzed the activity of carnitine dehydrogenase (CDH) with respect to glucose and glycine betaine (GB) and showed that glucose, as a preferred carbon source, is catabolite repressive (22), whereas glycine betaine leads to repression of CDH activity, resulting in oscillations of activity as carnitine is catabolized to glycine betaine (22). Our data demonstrate that repression of carnitine catabolism by glucose and glycine betaine can be controlled at the level of transcription (Fig. 4). Negative
feedback by glycine betaine is likely GbdR dependent based on GbdR’s capability to bind the caiX-cdhR intergenic region (Fig. 6B) and GbdR’s responsiveness to GB (32). P. aeruginosa maintains intracellular glycine betaine pools, and GbdR fine-tuning of carnitine catabolism may be directly related to sustaining the homeostatic levels of glycine betaine, as the GB pool has a physiological impact on nutrients, osmoprotection, and virulence (33).

We performed an alignment using the Pseudomonas.com DNA motif search tool of the newly identified CdhR binding sequence to the PAO1 genome and identified two intergenic regions: caiX-cdhR and cbcX-sdaB (23). This led us to investigate if CdhR contributes to the regulation of carnitine import by the ABC transporter CbcWV in association with CaiX, which is required for growth on carnitine (9). Even though CdhR binds to the cbcXWV UAS in vitro, it does not contribute to cbcXWV expression (Fig. 5). We propose that an unknown transporter imports carnitine, which is metabolized to GB, and this GB drives expression of cbcXWV in a GbdR-dependent manner, which coupled with CdhR-dependent expression of caiX allows for a larger flux of carnitine needed to support growth (9). This is similar to the mechanism of choline import for cbcXWV induction, which is termed priming (24).

We previously characterized the GbdR regulon and identified the intergenic region of caiX-cdhR to have a GbdR binding site (17). Here we report that GbdR binds the caiX-cdhR intergenic region in vitro and the conserved CG residues are necessary for GbdR binding, as mutation of these residues to AA results in loss of binding (Fig. 6A). Based upon the conserved residues being located in the GbdR distal half-site (17), the orientation of GbdR binding is likely toward cdhR activation and not caiX. Interestingly, the GbdR and CdhR binding sites overlap in the caiX-cdhR intergenic region, which led to the hypothesis that GbdR has a role in regulating carnitine catabolism and, in particular, cdhR.

We propose a model (Fig. 8) showing the genetic network containing CdhR and GbdR. In the absence of carnitine, CdhR binds to its caiX-cdhR intergenic target sequence site in a manner that inhibits cdhR expression. GbdR competes for this binding site and limits CdhR-dependent inhibition of cdhR. Analysis of the DNA sequence up- and downstream of the CdhR binding site reveals multiple CdhR half-sites. These half-sites could participate in inhibition of CdhR basal expression by looping. CdhR inhibition of its own expression may be similar to the AraC “light-switch” mechanism, in which the regulator binds upstream sites to loop DNA and restrict polymerase access to the promoter (34). Another possibility is that CdhR oligomerizes along the DNA, nucleated at these half-sites, to dampen cdhR basal expression. In this model, GbdR would compete for binding, relieving CdhR-dependent cdhR repression. Upon CdhR detection of carnitine, a change occurs allowing increased expression of cdhR and caiX (Fig. 7). As the catabolic product GB builds up, the cell controls the flux of carnitine catabolism by GB-dependent transcriptional repression (Fig. 4), which is likely regulated by GbdR (Fig. 8).

In conclusion, we show that CdhR regulates the carnitine catabolic operon by directly binding the caiX UAS, and this is likely the only site in the genome where its potential binding is effective in altering gene transcription. We also show that catabolic repression of carnitine catabolism can function through direct repression of carnitine catabolic operon transcription. Finally, we show the role of GbdR in regulation at this site. These data suggest a system for fine-tuning carnitine catabolism in relation to other carbon sources and that both GbdR and CdhR alter transcription from the caiX-cdhR intergenic regulatory region.

**MATERIALS AND METHODS**

**Strains and growth conditions.** P. aeruginosa wild-type strains PAO1 and PA14 and their derivatives (Table 1) were maintained on Pseudomonas isolation agar (PIA [Difco]) plates or Lennox broth (LB) liquid, and when necessary 50 or 40 μg·ml⁻¹ gentamicin was added to the media, respectively. Escherichia coli NEB5α or T7 Express E. coli (NEB C3016) cells were maintained on LB plates with 10 μg·ml⁻¹ gentamicin, LB liquid with 7 μg·ml⁻¹ gentamicin, LB plates or liquid with 125 μg·ml⁻¹ carbenicillin, or LB plates or liquid with 100 μg·ml⁻¹ kanamycin.

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Deletion constructs. A deletion of PA5389 (cdhR) in PAO1 was made in the wild-type background (MJ79). The upstream and downstream regions of PA5389 were PCR amplified from PAO1 genomic DNA with the primers 5389GOIF1KpnI, 5389SOEGOIR1, 5389SOEGOIF1, and 5389GOIR1BamHI (Table 2). The splice overlap extension PCR product was ligated into the Zero Blunt plasmid pCR-Blunt (Invitrogen), excised with EcoRI, and ligated into similarly cut pMQ30 to generate pJAM90. Conjugation of donor E. coli S17/pir carrying pJAM90 with PAO1 and subsequent screening were done as previously described (10), generating strain JM236. The deletion strain, JM179, was verified by lack of growth on carnitine and by PCR.

A double mutant of cdhR and gbdR in PA14 was made using the PA14 ΔgbdR strain MJ26 as the recipient strain for mating with E. coli S17/pir carrying pJAM90 as described above. The double mutant strain was verified by PCR. The PAO1 cdhR gbdR double mutant was made using the PAO1 ΔcdhR strain JM236 as the recipient strain for mating with E. coli S17/pir carrying pPA5280KO (15). The double mutant was verified by PCR and its inability to grow on glycine betaine.

Construction of transcriptional reporter constructs. Promoter mapping of PA5388 (caiX) was done using four different truncations of the caiX upstream region cloned as transcriptional reporter constructs. pMW79 was used as the PCR template for amplification of the upstream region of caiX, using

### TABLE 1 Strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference or source |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| P. aeruginosa PAO1|                         |                     |
| MJ79              | Wild type               | 14                  |
| MJ80              | ΔgbdR                   | 15                  |
| JM236             | ΔcdhR                   | This study          |
| JM253             | Wild type attTn7:88-89intYFPCFP-2 | This study       |
| JM339             | ΔcdhR attTn7:88-89intYFPCFP-2 | This study       |
| JM340             | ΔgbdR attTn7:88-89intYFPCFP-2 | This study       |
| MJ784             | ΔgbdR ΔcdhR             | This study          |
| **P. aeruginosa PA14** |                       |                     |
| MJ101             | Wild type               | 42                  |
| MJ11              | ΔcdhR                   | 11                  |
| MJ26              | ΔgbdR                   | 15                  |
| MJ262             | ΔcdhCA                  | 11                  |
| JM179             | ΔcdhR ΔgbdR             | This study          |
| E. coli MJ340     | Wild-type S17/pir       |                     |
| DH5α              | NEB C2987               | NEB                 |
| T7Express         | NEB C3016               | NEB                 |
| **Plasmids**      |                         |                     |
| pMQ30             | Suicide vector, Gm'     | 36                  |
| pMQ80             | High-copy-no. *Pseudomonas* vector, Gm' | 36 |
| pMal-C2X          | T7-expressing vector, MBP N-terminal tag, Amp' | NEB |
| pTNS2             | Plasmid carrying attTn7 transposase | 43 |
| pUC18-mini-Tn7T-Gm| Gm' on mini-Tn7T        | 35                  |
| pUC18-mini-Tn7T-Gm-eyfp| Gm' on mini-Tn7T with YFP | 35 |
| pUCP22            | High-copy-no. *Pseudomonas* stabilization vector, Gm' | 44 |
| pMW5              | lacZYA in pUCP22        | 32                  |
| pMW79             | PA14 genomic clone of PAS380-PAS389 in pMQ80 | 11 |
| pPA5380KO         | gbdR deletion construct in pEX18-Gm | 15 |
| pJMAM22           | Promoter caiX-lacZYA transcriptional fusion A | This study |
| pJMAM23           | Promoter caiX-lacZYA transcriptional fusion B | This study |
| pJMAM24           | Promoter caiX-lacZYA transcriptional fusion C | This study |
| pJMAM25           | Promoter caiX-lacZYA transcriptional fusion D | This study |
| pJMAM50           | PAS389 in pMal-C2X      | This study          |
| pJMAM76           | YFP-CFP in pMQ80       | This study          |
| pJMAM86           | CFP PAS388-PAS389 intergenic region YFP in pUC18mini, DR2 | This study |
| pJMAM90           | PAS389 deletion construct in pMQ30 | This study |
| pJMAM122          | Promoter caiX-lacZYA transcriptional fusion | This study |
| pJMAM123          | Promoter caiX-lacZYA mut 1 transcriptional fusion | This study |
| pJMAM124          | Promoter caiX-lacZYA mut 2 transcriptional fusion | This study |
| pJMAM125          | Promoter caiX-lacZYA mut 3 transcriptional fusion | This study |
| pJMAM126          | Promoter caiX-lacZYA mut 4 transcriptional fusion | This study |
| pJMAM127          | Promoter caiX-lacZYA mut 5 transcriptional fusion | This study |
| pJMAM130          | Promoter caiX-lacZYA mut 6 transcriptional fusion | This study |
| pJMAM131          | C terminus of lacZ in pUCP22 | This study |
| **P. aeruginosa** |                         |                     |
| pJMAM135          | Promoter caiX-lacZYA in pUCP22 | This study |

Deletion constructs. A deletion of PAS389 (cdhR) in PAO1 was made in the wild-type background (MJ79). The upstream and downstream regions of PAS389 were PCR amplified from PAO1 genomic DNA with the primers 5389GOIF1KpnI, 5389SOEGOIR1, 5389SOEGOIF1, and 5389GOIR1BamHI (Table 2). The splice overlap extension PCR product was ligated into the Zero Blunt plasmid pCR-Blunt (Invitrogen), excised with EcoRI, and ligated into similarly cut pMQ30 to generate pJMAM90. Conjugation of donor E. coli S17/pir carrying pJMAM90 with PAO1 and subsequent screening were done as previously described (10), generating strain JM236. The deletion strain, JM179, was verified by lack of growth on carnitine and by PCR.

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**TABLE 2** Primers used in this study

| Primer          | Sequence (5’ to 3’)* |
|-----------------|----------------------|
| **Deletion constructs** |                      |
| 5389G0F1KpnI    | ATAGGGTACCCAGAAGACCCACCCACTGCT |
| 5389G0EG0R1     | AAGTACGACGCGACTGACCAGGGGAGACCCGAGAACG |
| 5389G0EG0IF1    | GCTTCTCGGTAATGGGCGCTTCAGTGGTGAGTCGCTTCTGATT |
| 5389G0IR1BamHI  | ATCGTCTCGGTAATGGGCGCTTCAGTGGTGAGTCGCTTCTGATT |
| **Protein expression construct** |                   |
| 5389Mal-c2xF    | GCATCAAGATTTTCCAGGACCTTCAGTGGTGAGTCGCTTCTGATT |
| 5389Mal-c2xR    | GCATCAAGATTTTCCAGGACCTTCAGTGGTGAGTCGCTTCTGATT |
| **EMSA probes** |                        |
| PAS388promF3    | AAGCTTGTGCGCGGAGGTAGAGTGC |
| PAS388promR     | TGGAGTGCTTGGATTCGCCAGGAGAGG |
| PAS388promRbiot | Biotin-TGGATTGTTTCTGCGAGGT |
| 5389EMS-F       | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5389EMS-R-biot  | Biotin-TGGATTGTTTCTGCGAGGT |
| 5389EMS-Mut3F   | GAGCCGCCGCGCAGGACGCGCACTCGGAGGACG |
| cbxXprom-F      | CCCGCCAAGACCCACACTATGAT |
| cbxXprom-R-biot | Biotin-GAATCTCCTGGCAGGGTTAAG |
| dhcprom-F-biot  | Biotin-GAGGCTTCTCCAGGCTCT |
| dhcprom-R       | GGAATGGTACCCCTCCCGGCTTCTGTTGATT |
| dhcprom-F       | GAACTCCTCCTGCGAGGTAAGG |
| **Transcriptional reporters** |                       |
| PAS388promR     | TGGAGTGCTTGGATTCGCCAGGAGAGG |
| PAS388promF1    | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| PAS388promF3    | GCTTCTCGGTAATGGGCGCTTCAGTGGTGAGTCGCTTCTGATT |
| PAS388promF2    | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| PAS388promF4    | GCTTCTCGGTAATGGGCGCTTCAGTGGTGAGTCGCTTCTGATT |
| 5388pos         | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut1        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut2        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut3        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut4        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut5        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| 5388mut6        | ATGAAAGCTTCCAGCAGGAGGAAACCCAGAG |
| **Translational reporters** |                       |
| 2-lacZCtermFhindcln | GCAACGCTTTTCTGCAAGGCCATGAGGTAAGTGTTATGC |
| 2-lacZCtermRsmakpn | CGGTACCCCGGAGATCCATTATTTTTTCCAGGCCACCCAGGCC |
| YFP R HindIII   | GCATCAAGCTTCCAGCAGGAGGAAACCCAGAG |
| YFP F Kpn Sal   | GACGCGCTTACCATGCTCGACCATATGCTGAGGAGAGGAAAGG |
| YFP R HindIII   | GACGCGCTTACCATGCTCGACCATATGCTGAGGAGAGGAAAGG |
| YFP R HindIII   | GACGCGCTTACCATGCTCGACCATATGCTGAGGAGAGGAAAGG |
| YFP R HindIII   | GACGCGCTTACCATGCTCGACCATATGCTGAGGAGAGGAAAGG |
| 88-89YC-DR#2ycF | GGGACACCACCCCGTGAAAGAACGCTTCCAGCAGGTAAGTGCTGAGGAGGAAAGG |
| 88-89YC-DR#2ycR | CGGCACGGCCGCGTGAAGCAGTCCAGCAGGTAAGTGCTGAGGAGGAAAGG |

* 6-FAM, 6-carboxyfluorescein.

5389G0promR as the reverse primer for all products with the forward primers PAS388promF1, PAS388promF2, PAS388promF3, and PAS388promF4. These four PCR products were ligated into the pCR-Blunt, digested with KpnI and HindIII, and ligated into similarly cut pMW5 to yield four different 5388lacZYA transcriptional fusions (pJAM22 to pJAM25). Each was transformed by electroporation into PA14 wild type and selected on PIA-gentamicin plates.

To assess which residues were essential for induction of *caix*, seven different transcriptional reporters were constructed by amplifying the *caix* binding site from pMW79 using the PAS388promR primer and seven different forward primers: P5388pos, P5388mut1, P5388mut2, P5388mut3, P5388mut4, P5388mut5, and P5388mut6. PCR products were digested with HindIII and KpnI and ligated into similarly cut pMW5. The resulting plasmids, pJAM122 to pJAM127 and pJAM130, were transformed into PA14 wild type by electroporation and selected on PIA-gentamicin.

**Construction of cdhR translational reporter constructs.** To determine control of *cdhR*, a divergent fluorescent translational reporter with yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) on either side of the intergenic region of PAS388 and PAS389 was constructed. YFP was amplified using primers YFP F Kpn Sal and YFP R HindIII from the template pUC18miniTn7-Gm-eyfp (35). The YFP fragment was ligated into pCR Zero Blunt plasmid, digested with HindIII and KpnI, and subsequently ligated into pMW5 to yield the resulting plasmids, pJAM128 to pJAM131, which were transformed into PA14 wild type by electroporation and selected on PIA-gentamicin.

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PA5388promR as the reverse primer for all products with the forward primers PAS388promF1, PAS388promF2, PAS388promF3, and PAS388promF4. These four PCR products were ligated into the pCR-Blunt, digested with KpnI and HindIII, and ligated into similarly cut pMW5 to yield four different 5388lacZYA transcriptional fusions (pJAM22 to pJAM25). Each was transformed by electroporation into PA14 wild type and selected on PIA-gentamicin plates.

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**Construction of cdhR translational reporter constructs.** To determine control of *cdhR*, a divergent fluorescent translational reporter with yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) on either side of the intergenic region of PAS388 and PAS389 was constructed. YFP was amplified using primers YFP F Kpn Sal and YFP R HindIII from the template pUC18miniTn7-Gm-eyfp (35). The YFP fragment was ligated into pCR Zero Blunt plasmid, digested with HindIII and KpnI, and subsequently ligated into pMW5 to yield the resulting plasmids, pJAM128 to pJAM131, which were transformed into PA14 wild type by electroporation and selected on PIA-gentamicin.
ligated into similarly cut pMIQ80. The resulting plasmid was digested with EcoRI and SacI and ligated with an EcoRI- and SacI-cut P. aeruginosa-codon-biased CFP, to generate pJAM76. The PAS388-PAS389 intergenic region was amplified using primers 88-89YCDR#2ycF and 88-89YCDR#2ycR. pJAM76 was digested with Sall to linearize the plasmid for recombination of the PAS388-PAS389 intergenic regions using yeast cloning in Saccharomyces cerevisiae via the method of Shanks et al. (36). The resultant plasmid was digested with HindIII, and the insert was ligated into similarly cut pUC18-mini-Tn7-Gm, yielding pJAM86, which was coelectroporated with pTNS2 to insert CFP-PAS388-PAS389-YFP onto the chromosome at the arfT7 site (35) of the PA01 wild-type, PA01 ΔcdhR, and PA01 ΔgbdR strains.

A PAS389 translational lacZ fusion was made by inserting a gblock (IDT) that has 320 bp of the upstream region of PAS389 into the translational start site of PAS389 and then from the translational start site of lacZ to +850 bases in the lacZ gene into pJAM131. pJAM131 was built by PCR amplification of the C-terminal end of lacZ with primers 2-lacZCtermFhindcla and 2-lacZCtermRsmakpn, digested with HindIII and KpnI, and ligated into a similarly cut pUCP22. The gblock was ligated into HindIII and Clal and ligated into similarly cut pJAM131 to make pJAM135.

Cloning and expression of MBP-CdhR. The plasmid pJAM50, expressing a maltose binding protein (MBP) fusion to the amino terminus of CdhR, was made by amplifying cdhR with primers 5389Mal-C2XF and 5389Mal-C2XR and ligating the product into pCR-Blunt. The cdhR coding segment was excised with EcoRI and HindIII and ligated into the similarly cut pMal-C2X (NEB), transformed by electroporation into T7 Express E. coli (NEB C2566), and selected on LB-carbenicillin to generate strain JM153.

To express MBP-CdhR, 1 liter of JM153 was grown at 37°C in LB with 75 μg/ml-1 carbencillin to an optical density at 600 nm (OD600) of 0.4. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for an additional 3 h. Cells were collected by centrifugation and resuspended in 20 mM Tris HCl–150 mM NaCl (pH 7.4) with 1× Halt protease inhibitor cocktail (Thermo) and washed with cold phosphate buffered saline (PBS). The cells were lysed by French pressure using yeast cloning in Saccharomyces cerevisiae and then resuspended in MOPS, and then inoculated into MOPS with 20 mM pyruvate and 20 mM glucose at 2, 4, or 10 mM or had glycine betaine at 20 or 40 mM. Cultures were induced for 4 h at 37°C, and β-galactosidase activities were performed according to Miller (37).

EMSAs. To determine binding of MBP-CdhR or MBP-GbdR to promoters, EMSAs were performed as previously described (17). Briefly, caix, dhc, cbcx, cdxR, or cdhR mutant promoter DNA fragments were made by PCR amplification from PA14 genomic DNA template with primers listed in the EMSA primer section in Table 2 and verified by sequencing. The DNA probes were dialyzed in 1/4 Tris-EDTA (TE) on a 0.025-μm-pore filter for 20 min. EMSAs were conducted following the LightShift chemiluminescent EMSA kit instructions (Pierce) as modified by Hampel et al. (17) with labeled probes used at 1 fmol·µl−1 and unlabeled competitor at 600 fmol·µl−1. Samples were electrophoresed on 5% Tris-borate-EDTA (TBE) nondenaturing gels, transferred to BioDyne-B nylon membrane (Thermo), and detected using the Thermo chemiluminescent nucleic acid detection module per the manufacturer's instructions.

DNase I footprinting. DNase I footprinting was performed as described previously (17). Briefly, the target DNA was made by labeling the S′ end of either PAS388promR or PAS388promF2 primer with [32P]dCTP using T4 polynucleotide kinase and [γ-32P]ATP and amplifying the PA14 caix upstream region. The radiolabeled PCR product was purified from a 5% polyacrylamide Tris-borate-EDTA gel. The MBP-CdhR footprinting assay was conducted as in Breznowitz et al. (38), as modified in the study by Hampel et al. (17).
Fluorescence microscopy. *P. aeruginosa* PAO1 WT, ΔcdtR, and ΔgbdR strains with the divergent fluorescent reporter PA3288-PA5389 intergenic region CPYFP-DsRed (pJAM86) were grown overnight in MOPS with 20 mM pyruvate and 5 mM glucose at 37°C. Cells were collected by centrifugation, washed, and resuspended in MOPS to an OD_{600} of 1.0. A 1/20 dilution was made, and 1 μl was placed on the center of an agar pad (1.5% low-melting-point agarose, MOPS medium with 20 mM pyruvate, and with or without 1 mM carnitine). The agar pad was placed cell side down on a 50-mm glass bottom cell culture dish (Warner Instruments). Samples were imaged on a Nikon Ti-E every 10 min for 6 h at 32°C. Image stacks were imported into Fiji 2.0.0-rc-29/1.49 s (39) using the Bio-Formats importer 5.1.1 (40). A rolling variance filter on the contrast channel was used to define background areas, and after an additional threshold was applied, the “find maxima” macro was used to define cell areas. Using masks from the contrast channel, mean pixel intensity measurements were taken from the YFP channel. A framewise background intensity correction was performed for each cell using R 3.2.0 (41).

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