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RESEARCH ARTICLE

DdaR (PA1196) Regulates Expression of Dimethylarginine Dimethylaminohydrolase for the Metabolism of Methylarginines in *Pseudomonas aeruginosa* PAO1

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ABSTRACT Dimethylarginine dimethylaminohydrolases (DDAHs) catalyze the hydrolysis of methylarginines to yield L-citrulline and methylamines as products. DDAHs and their central roles in methylarginine metabolism have been characterized for eukaryotic cells. While DDAHs are known to exist in some bacteria, including *Streptomyces coelicolor* and *Pseudomonas aeruginosa*, the physiological importance and genetic regulation of bacterial DDAHs remain poorly understood. To provide some insight into bacterial methylarginine metabolism, this study focused on identifying the key elements or factors regulating DDAH expression in *P. aeruginosa* PAO1. First, results revealed that *P. aeruginosa* can utilize N^G,N^G-dimethyl-L-arginine (ADMA) as a sole source of nitrogen but not carbon. Second, expression of the *ddaH* gene was observed to be induced in the presence of methylarginines, including N^G-monomethyl-L-arginine (L-NMMA) and ADMA. Third, induction of the *ddaH* gene was shown to be achieved through a mechanism consisting of the putative enhancer-binding protein PA1196 and the alternative sigma factor RpoN. Both PA1196 and RpoN were essential for the expression of the *ddaH* gene in response to methylarginines. On the basis of the results of this study, PA1196 was given the name DdaR, for dimethylarginine dimethylaminohydrolase regulator. Interestingly, DdaR and its target *ddaH* gene are conserved only among *P. aeruginosa* strains, suggesting that this particular *Pseudomonas* species has evolved to utilize methylarginines from its environment.

IMPORTANCE Methylated arginine residues are common constituents of eukaryotic proteins. During proteolysis, methylarginines are released in their free forms and become accessible nutrients for bacteria to utilize as growth substrates. In order to have a clearer and better understanding of this process, we explored methylarginine utilization in the metabolically versatile bacterium *Pseudomonas aeruginosa* PAO1. Our results show that the transcriptional regulator DdaR (PA1196) and the sigma factor RpoN positively regulate expression of dimethylarginine dimethylaminohydrolases (DDAHs) in response to exogenous methylarginines. DDAH is the central enzyme of methylarginine degradation, and its transcriptional regulation by DdaR-RpoN is expected to be conserved among *P. aeruginosa* strains.

KEYWORDS PA1196, DdaR, enhancer-binding protein, RpoN, dimethylarginine dimethylaminohydrolase, methylarginine, ADMA, *Pseudomonas aeruginosa*

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Many eukaryotic proteins are posttranslationally modified through arginine methylation. At the center of this posttranslational modification are enzymes known as protein arginine methyltransferases (PRMTs) that methylate the guanidino nitrogen atoms of arginine residues in *S*-adenosylmethionine-dependent reactions (1, 2). Because PRMTs have differences in specificity and prevalence, the methylation patterns vary among arginine residues. Consequently, a few methylarginines are known to exist, including N^G-monomethyl-L-arginine (L-NMMA), asymmetric N^G,N^G-dimethyl-L-arginine (ADMA), and symmetric N^G,$N^{G'}$-dimethyl-L-arginine (SDMA). During protein turnover or proteolysis, ADMA and L-NMMA are released within the cell in their free forms (3, 4), which allows them to act as competitive inhibitors in arginine utilization pathways (5–7). To circumvent this problem, cells rely on an enzyme called dimethylarginine dimethylaminohydrolase (DDAH) that hydrolyzes L-NMMA and ADMA to generate L-citrulline and either mono- or dimethylamine as end products (3, 8). In contrast, SDMA is not an inhibitor of the arginine utilization pathways, nor is it a substrate for DDAH (8, 9). SDMA is removed from the body through excretion in the urine (4, 10).

The metabolism of methylarginines, especially ADMA, has been extensively studied as it pertains to eukaryotes. Interestingly, genes encoding DDAHs are found in some bacteria, including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Sinorhizobium meliloti*, and *Streptomyces coelicolor* (11). The DDAHs of *M. tuberculosis*, *P. aeruginosa*, and *S. coelicolor* have been biochemically investigated and were shown to catalyze the hydrolysis of both ADMA and L-NMMA (11). The presence of DDAH suggests that these bacteria might be able to utilize methylarginines as a source of carbon and/or nitrogen. As a first step in understanding such metabolism, we sought to identify the genes and regulatory mechanisms necessary for methylarginine utilization in *P. aeruginosa* PAO1.

DDAH of *P. aeruginosa* PAO1 has been biochemically characterized (12–15) and is encoded by the *PA1195* (*ddaH*) gene. The *ddaH* gene has distinguishable features suggesting that it is under the regulation of the alternative sigma factor σ^{54} or RpoN (16, 17). First, located 50 bp upstream of the start codon of *ddaH* is a putative −24/−12 promoter (Sigma 54 Promoter Database [http://www.sigma54.ca]). The −24/−12 promoter is a highly conserved nucleotide sequence that is recognized by RpoN for transcriptional activation (17, 18). Second, the adjacent *PA1196* gene encodes a putative enhancer-binding protein (EBP) with an unknown function (19, 20). EBPs are transcriptional regulators that interact specifically with RpoN to activate transcription from −24/−12 promoters (19, 21, 22). Upon stimulation, EBPs become active and hydrolyze ATP. This nucleotide hydrolysis provides the energy needed for the RpoN-RNA polymerase holoenzyme to transition from a closed to an open complex (21).

In the current study, PA1196 was hypothesized to be a key regulator of *ddaH* expression in *P. aeruginosa* PAO1. Specifically, it was proposed that PA1196 in concert with RpoN activates the transcription of *ddaH* in response to methylarginines, such as ADMA and L-NMMA. The *PA1196* gene is in a predicted operon with *PA1197*, a gene encoding a putative Sir2 protein. Sir2 proteins often function as deacetylases and have regulatory roles in cellular processes, including transcriptional repression or posttranslational modifications (23, 24). It was therefore considered that PA1197 might also have some regulatory role in methylarginine utilization in *P. aeruginosa* PAO1.

RESULTS

PA1196 is required for utilization of ADMA as a nitrogen source for *P. aeruginosa* PAO1. The *ddaH*, *PA1196*, and *PA1197* genes were deleted from *P. aeruginosa* PAO1 to create a series of unmarked deletion mutants: the Δ*ddaH*, Δ*PA1196*, and Δ*PA1197* mutants. The mutants and wild-type PAO1 strain were grown in minimal medium supplemented with ADMA as a sole source of either carbon or nitrogen (Fig. 1). As a sole carbon source in the presence of NH_4Cl, ADMA did not support the growth of any of the mutants or PAO1 (Fig. 1A). Cell densities reached a maximum optical density at 600 nm (OD_{600}) of ~0.1 at 48 h postinoculation on ADMA as a carbon source. However, when ADMA served as the sole nitrogen source in the presence of gluconate, slow growth was observed for the Δ*PA1197* mutant and PAO1 (Fig. 1B). For these two

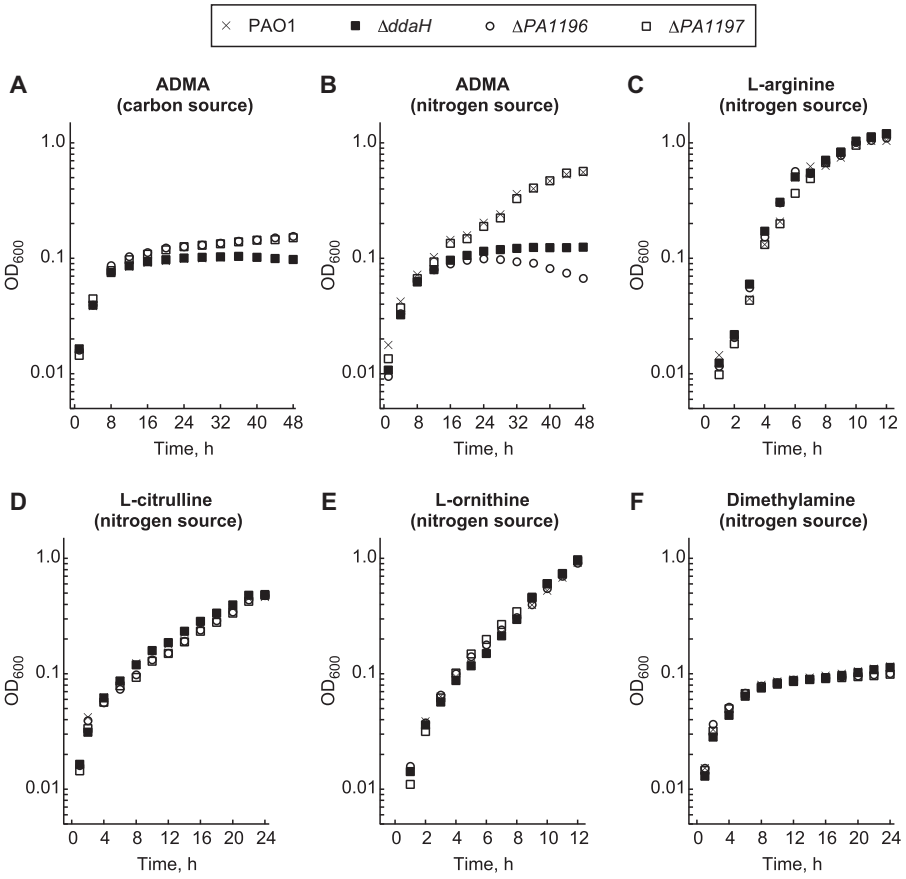

FIG 1 PA1196 is required for *P. aeruginosa* PAO1 to grow on ADMA as a nitrogen source. The growth of *P. aeruginosa* PAO1 and the Δ*ddaH*, Δ*PA1196*, and Δ*PA1197* mutants in minimal medium supplemented with the following nitrogen (10 mM) and carbon (15 mM) sources was measured: NH_4Cl and ADMA (A), ADMA and gluconate (B), L-arginine and gluconate (C), L-citrulline and gluconate (D), L-ornithine and gluconate (E), and dimethylamine and gluconate (F). The strains were grown in 96-well polystyrene plates at 37°C for 24 to 48 h. Data points represent mean values ($n = 4$), with SDs being <5.0%. Error bars were omitted for clarity.

strains, cell densities reached a maximum OD_{600} of ~0.6 at 48 h postinoculation on ADMA as a nitrogen source. The Δ*ddaH* and Δ*PA1196* mutants exhibited no growth on ADMA as a nitrogen source; cell densities reached a maximum OD_{600} of ~0.1 (Fig. 1B). Identical growth curves were observed for all strains on L-arginine, L-citrulline, and L-ornithine as nitrogen sources (Fig. 1C to E), suggesting that deletion of *ddaH*, *PA1196*, and *PA1197* has no effect on the utilization of these compounds. Dimethylamine did not serve as a nitrogen source for any of the mutants or PAO1 (Fig. 1F).

Expression of either the *PA1196* or the *ddaH* gene from the *lac* promoter on plasmid pBBR1MCS-5 restored the growth of the Δ*PA1196* mutant on ADMA as a nitrogen source (Fig. 2). The finding that plasmid-derived expression of *ddaH* compensated for the ADMA-related growth deficiency of the Δ*PA1196* mutant was an indicator that expression of the *ddaH* gene is possibly deregulated or insufficient in the absence of the *PA1196* gene. Collectively, the results of these growth assays demonstrate that *ddaH* (as expected) and *PA1196* are essential for ADMA utilization in *P. aeruginosa* PAO1.

Expression of *ddaH* is induced by ADMA. To explore the expression of *ddaH*, a LacZ reporter was constructed by fusing the ~500-bp 5′ regulatory region of *ddaH* with the *lacZ* open reading frame (ORF) of *Escherichia coli*. The *ddaH*::*lacZ* fusion was cloned into the promoter-less, low-copy-number plasmid ΔP*lac*-pBBR1MCS-5. Afterwards, *P. aeruginosa* PAO1 harboring *ddaH*::*lacZ* was grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with various substrates, each at a final concentration

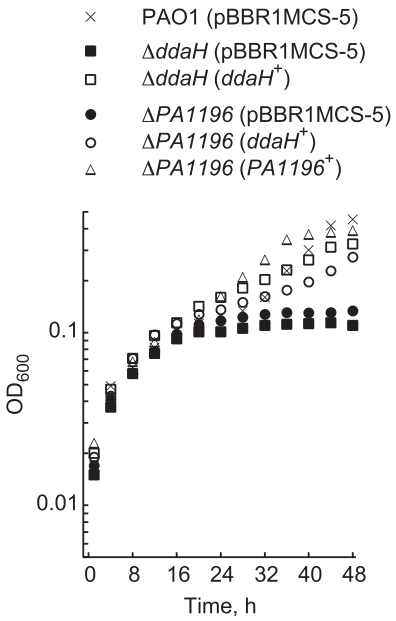

FIG 2 Plasmid-based expression of *PA1196* or *ddaH* rescues the growth of the Δ*PA1196* mutant on ADMA. Growth in minimal medium supplemented with 15 mM gluconate, 10 mM ADMA, and 30 μg ml^{-1} gentamicin was measured for *P. aeruginosa* PAO1 harboring an empty plasmid (pBBR1MCS-5), the Δ*ddaH* mutant harboring either an empty plasmid (pBBR1MCS-5) or pBRL685 (*ddaH*$^+$), and the Δ*PA1196* mutant harboring either an empty plasmid (pBBR1MCS-5), pBRL685 (*ddaH*$^+$), or pBRL686 (*PA1196*$^+$). Note that the *ddaH* and *PA1196* genes were cloned under the control of the *lac* promoter of pBBR1MCS-5 to generate pBRL685 and pBRL686, respectively. Recombinant strains were grown in 96-well polystyrene plates at 37°C for 48 h. Data points represent mean values ($n = 4$), with SDs being <5.0%. Error bars were omitted for clarity.

of 0.1 mM. As shown in Fig. 3A, LacZ activities increased >2-fold at 1.0 h after the addition of ADMA. LacZ activities also increased >2-fold with the addition of L-NMMA, a monomethylated derivative of L-arginine. In contrast, LacZ activities did not change with the addition of L-arginine, L-citrulline, or no substrate.

The effects of various concentrations of exogenous ADMA on the expression of *ddaH*::*lacZ* were measured. Final ADMA concentrations of 0.01, 0.1, and 1.0 mM caused LacZ activities to increase >2-fold when *ddaH*::*lacZ* was expressed in *P. aeruginosa* PAO1 (Fig. 3B). The addition of ADMA to 0.001 mM did not significantly affect *ddaH*::*lacZ* expression. These results indicate that expression of *ddaH* in *P. aeruginosa* PAO1 is inducible by ADMA at relatively low concentrations.

DDAH is regulated by PA1196. It was next determined what role PA1196 and PA1197 might have on expression of the *ddaH* gene. Mutants (the Δ*ddaH*, Δ*PA1196*, and Δ*PA1197* mutants) and PAO1 harboring *ddaH*::*lacZ* were grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either no substrate or 0.1 mM ADMA. LacZ activities were subsequently measured at 1.0 h after the addition of the substrate. It was found that the addition of ADMA induced the expression of *ddaH*::*lacZ* for both the Δ*PA1197* mutant and PAO1; i.e., LacZ activities increased >2-fold for both strains (Fig. 4). For the Δ*ddaH* mutant, LacZ activities increased >7-fold with the addition of ADMA. This elevated fold change compared to the activity observed in PAO1 was expected, because the Δ*ddaH* mutant is unable to catabolize or enzymatically break down ADMA. For the Δ*PA1196* mutant, there was no change in LacZ activity with the addition of ADMA. This result is consistent with PA1196 being a positive regulator of the *ddaH* gene.

Deletion of the *PA1197* gene did not affect expression of *ddaH*::*lacZ*, indicating that PA1197 is not necessary for the transcription of *ddaH* in response to ADMA. Therefore, it was considered possible that PA1197 regulates the actual function or catalytic activity of DDAH. To address this possibility, cell extracts from mutant and PAO1 strains grown

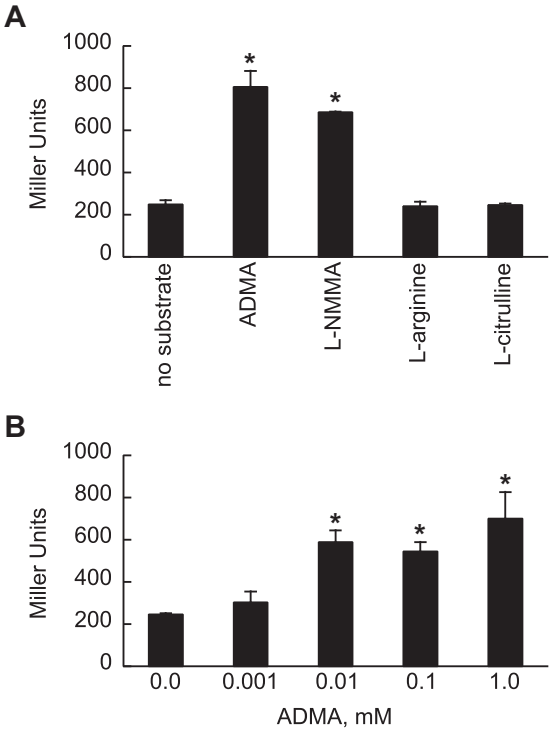

FIG 3 Methylarginines induce expression of *ddaH*::*lacZ* in *P. aeruginosa* PAO1. The ~500-bp 5′ regulatory region of *ddaH* was fused to *E. coli lacZ*, and the resulting construct was cloned into a promoter-less plasmid to give *ddaH*::*lacZ*. *P. aeruginosa* PAO1 harboring *ddaH*::*lacZ* was grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either 0.1 mM ADMA, L-NMMA, L-arginine, or L-citrulline (A) or ADMA at various concentrations, 0.001, 0.01, 0.1, or 1.0 mM (B). LacZ activities were measured 1.0 h after the addition of the substrate. Data points represent mean values ($n = 3$) ± SDs. ANOVA with a Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

in gluconate-minimal medium were prepared in either the absence or the presence of ADMA. DDAH activity, i.e., the formation of L-citrulline from ADMA, was measured for each cell extract. For all mutant and PAO1 strains grown in the absence of ADMA, the resulting cell extracts yielded DDAH activities of ~20 U (Fig. 5). However, when the bacteria were grown in the presence of ADMA, DDAH activities were ~100 U in cell extracts prepared from the Δ*PA1197* mutant and PAO1 (Fig. 5). The addition of ADMA caused an ~5-fold increase in DDAH activity. In contrast, cell extracts prepared from the Δ*ddaH* and Δ*PA1196* mutants grown in the presence of ADMA generated DDAH

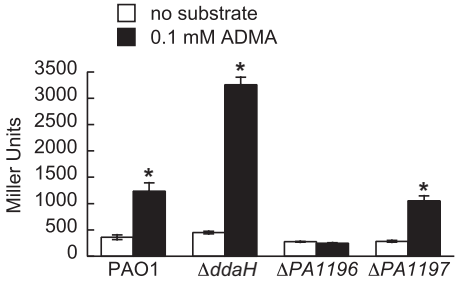

FIG 4 PA1196 is essential for induction of *ddaH*::*lacZ* in response to ADMA. Expression of *ddaH*::*lacZ* was determined in *P. aeruginosa* PAO1 and the Δ*ddaH*, Δ*PA1196*, and Δ*PA1197* mutants. Strains harboring *ddaH*::*lacZ* were grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either no substrate or 0.1 mM ADMA. LacZ activities were measured at 1.0 h after the addition of the substrate. Data points represent mean values ($n = 3$) ± SDs. ANOVA with a Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

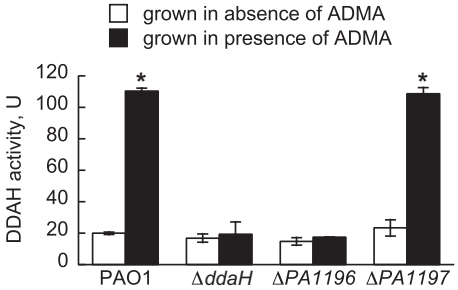

FIG 5 DDAH activity is dependent on PA1196. *P. aeruginosa* PAO1 and the Δ*ddaH*, Δ*PA1196*, and Δ*PA1197* mutants were grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either no substrate or 1.0 mM ADMA. At 2.0 h after the addition of the substrate, cells were collected by centrifugation, washed, and subsequently lysed via sonication. The lysates were cleared of cellular debris, concentrated, and then assayed for DDAH activity, which is reported in units (U), defined as the formation of L-citrulline (nanomoles $hour^{-1}$ $milligram^{-1}$ total protein). Data points represent mean values ($n = 2$) ± SDs. ANOVA with a Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

activities of ~20 U, which was similar to the value measured in the absence of ADMA. These findings confirm that PA1196 is essential for DDAH activity in *P. aeruginosa* PAO1.

ADMA induces expression of *ddaH*::*lacZ* in *Pseudomonas putida* cells that express *PA1196*. Because *P. putida* KT2440 does not possess any homologs of DDAH or PA1196, it was considered a suitable host to validate the central role of PA1196 in the expression of the *ddaH* gene. To this end, the *PA1196* gene was cloned under the control of the *lac* promoter on plasmid pBBR1MCS-3, and the resulting plasmid-derived *PA1196* was cotransformed with *ddaH*::*lacZ* into *P. putida* KT2440. Recombinant cells were grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either no substrate or 0.1 mM ADMA. LacZ activities were measured 2.0 h after the addition of the substrate. As shown in Fig. 6, the addition of ADMA induced LacZ activity >20-fold for *P. putida* KT2440 cells harboring plasmid-derived *PA1196* and *ddaH*::*lacZ*. PA1196 was essential for this induction, because cells that harbored *ddaH*::*lacZ* in combination with either an empty plasmid (pBBR1MCS-3) or a plasmid having *PA1196* cloned backwards relative to the orientation of the *lac* promoter on pBBR1MCS-3 yielded only background levels of LacZ activity with the addition of ADMA.

RpoN is required for induction of *ddaH*::*lacZ*. A putative RpoN promoter with the sequence T**GG**CGCGTGGCTT**GC**A (Sigma 54 Promoter Database [http://www.sigma54.ca]) is located 50 bp upstream of the *ddaH* ORF. The GG and GC nucleotides (which appear in bold type in the promoter sequence) of the −24 and −12 elements, respectively, are crucial for promoter activity. In order to evaluate the role of this promoter in *ddaH* expression, the GG nucleotides of the −24 element were replaced with AA in the *ddaH*::*lacZ* reporter. As expected, this mutation ($-P_{RpoN}$) rendered *ddaH*::*lacZ* unresponsive to ADMA in *P. aeruginosa* PAO1 (Fig. 7). Furthermore, the addition of ADMA did not induce expression of *ddaH*::*lacZ* in an *rpoN* mutant (the *rpoN*::Ω-Km mutant) of *P. aeruginosa* PAO1. These findings are in agreement and indicate that expression of *ddaH* is regulated through RpoN.

ADMA utilization in other strains of *P. aeruginosa*. DDAHs and homologs of PA1196 are found in other *P. aeruginosa* strains (20), which suggests that methylarginine utilization is conserved among them. Indeed, growth on ADMA as a nitrogen source was observed for *P. aeruginosa* strains PA14 and PAK (Fig. 8A). In comparison, DDAHs are not predicted to occur in other *Pseudomonas* spp., and consistent with their absence, neither *P. putida* nor *Pseudomonas fluorescens* grew on ADMA as a nitrogen source (Fig. 8A). Lastly, we decided to evaluate the growth of *P. aeruginosa* PAO1 in gluconate-minimal medium supplemented with various concentrations of ADMA. As shown in Fig. 8B, identical growth curves were observed for *P. aeruginosa* PAO1 on 2.0, 5.0, and 10 mM ADMA. Supplementation with ADMA at 0.2 mM also resulted in

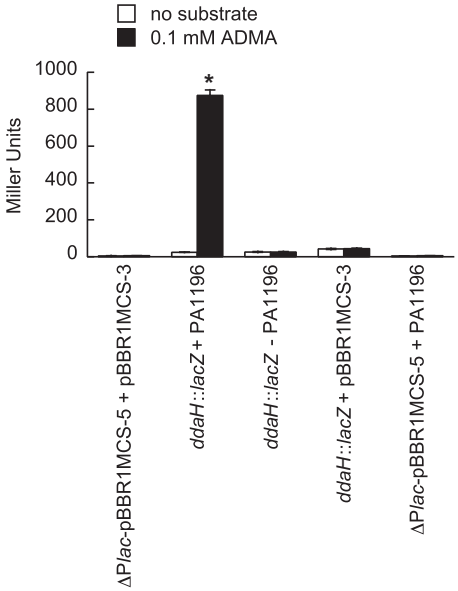

FIG 6 PA1196 is essential for induction of *ddaH::lacZ* in *P. putida* KT2440. *P. putida* KT2440 was cotransformed with several plasmid combinations, including (i) *ddaH::lacZ* and pBRL720 (+*PA1196*), (ii) *ddaH::lacZ* and an empty plasmid (pBBR1MCS-3), (iii) *ddaH::lacZ* and pBRL719 (−*PA1196*), (iv) Δ*Plac*-pBBR1MCS-5 and pBRL720 (+*PA1196*), and (v) empty plasmids (Δ*Plac*-pBBR1MCS-5 and pBBR1MCS-3). Recombinant strains were grown in gluconate-minimal medium to an OD_{600} of 0.3 and then challenged with either no substrate or 0.1 mM ADMA. LacZ activities were measured at 2.0 h after the addition of the substrate. Note that the *PA1196* gene was cloned with either a forward (pBRL720) or a backward (pBRL719) orientation relative to the orientation of the *lac* promoter of pBBR1MCS-3. Data points represent mean values ($n = 3$) ± SDs. ANOVA with a Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with an asterisk.

detectable growth but at a reduced level compared to that observed for the higher ADMA concentrations.

DISCUSSION

Methylarginine degradation in *P. aeruginosa* PAO1. A proposed pathway for the breakdown of methylarginines in *P. aeruginosa* PAO1 is given in Fig. 9. Extracellular methylarginines are believed to be transported through PA1194 and/or ArcD (PA5170). The *PA1194* gene encodes a transport protein exhibiting 60% similarity (49% identity) to ArcD, an L-arginine/L-ornithine antiporter (25, 26). Similar to DDAH and PA1196, homologs of PA1194 exist in other strains of *P. aeruginosa*. This suggests that PA1194 may have evolved to function as a methylarginine/L-ornithine antiporter. Interestingly, results from the LacZ assays involving *P. putida* KT2440 (Fig. 6) indicate that this bacterium is capable of the uptake of extracellular ADMA, even though it does not possess a homolog of PA1194. *P. putida* KT2440 does, however, harbor an L-arginine/L-ornithine antiporter, ArcD (PP_1002), which could be responsible for methylarginine transport in this bacterium. Although PA1194 and ArcD are potentially involved in the transport of methylarginines, further studies are needed to elucidate the actual mechanisms underlying the uptake of methylarginines in *P. aeruginosa*.

The central catabolic step in methylarginine utilization is catalyzed by DDAH, which hydrolyzes intracellular methylarginines to generate mono- or dimethylamine and L-citrulline as products. Mono- and dimethylamines have not been reported to be viable nutrients for *P. aeruginosa*, and in this study, dimethylamine did not serve as a nitrogen source for *P. aeruginosa* PAO1 (Fig. 1). However, L-citrulline is an intermediate of arginine degradation (the arginine deiminase or ArcABC pathway) and has been shown to be a poor nitrogen source for *P. aeruginosa* (27–29). On the basis of this information, it is predicted that the L-citrulline product of DDAH catalysis is converted into L-ornithine and carbamoyl phosphate via catabolic ornithine carbamoyltransferase (ArcB) (Fig. 9). Carbamate kinase (ArcC) then transfers the phosphorous group from

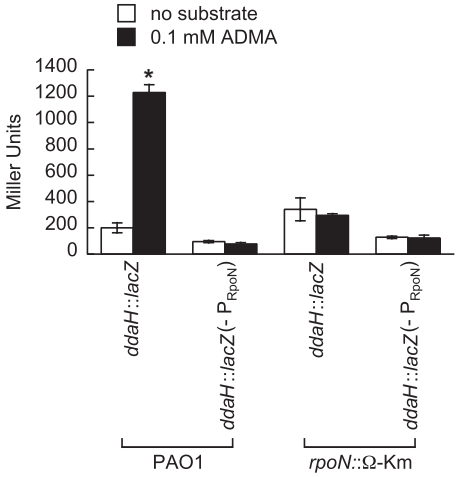

FIG 7 RpoN and its cognate promoter are required for induction of *ddaH*::*lacZ* in response to ADMA. Expression of *ddaH*::*lacZ* was measured in *P. aeruginosa* PAO1 and an *rpoN* mutant (the *rpoN*::Ω-Km mutant). In addition, expression was measured for a *ddaH*::*lacZ* construct that had a mutated RpoN promoter ($-P_{RpoN}$); i.e., the conserved GG dinucleotide of the −24 element of the RpoN promoter was changed to AA. Strains harboring the *ddaH*::*lacZ* constructs were grown in gluconate-minimal medium supplemented with 1.0 mM L-glutamine to an OD_{600} of 0.3 and then challenged with either no substrate or 0.1 mM ADMA. LacZ activities were measured 1.0 h after the addition of the substrate. Data points represent mean values ($n = 3$) ± SDs. ANOVA with a Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with an asterisk.

carbamoyl phosphate onto ADP, thus yielding ATP, CO_2, and NH_3 as products. This series of reactions would account for the ability of *P. aeruginosa* PAO1 to use ADMA as a nitrogen source, albeit poorly (Fig. 1).

The significance or importance of methylarginine utilization by *P. aeruginosa* has yet to be established. Data from the literature indicate that there is ~0.2 to 20 μmol of ADMA per kg of plant tissue (30) and ~0.01 to 1.0 μmol of ADMA per g of animal protein (31), and plasma concentrations of ADMA are on the order of 0.3 to 0.7 μM in

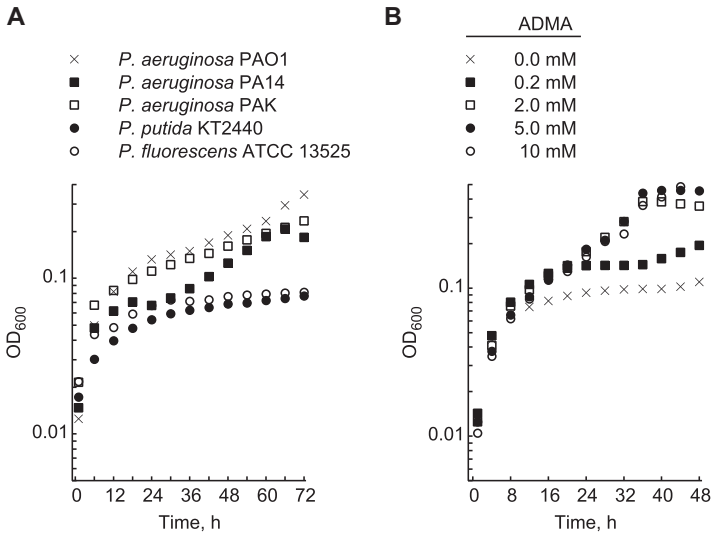

FIG 8 ADMA serves as a nitrogen source for other strains of *P. aeruginosa*. (A) Growth in minimal medium supplemented with 15 mM gluconate and 10 mM ADMA was measured for various *P. aeruginosa* strains (PAO1, PA14, and PAK), *P. putida* KT2440, and *P. fluorescens* ATCC 13525. Strains were grown in 96-well polystyrene plates at 30°C for 72 h. (B) Growth in minimal medium supplemented with 15 mM gluconate and various ADMA concentrations (0.0, 0.2, 2.0, 5.0, and 10 mM) was measured for *P. aeruginosa* PAO1 in 96-well polystyrene plates at 37°C for 48 h. Data points represent mean values ($n = 4$), with SDs being <5.0%. Error bars were omitted for clarity.

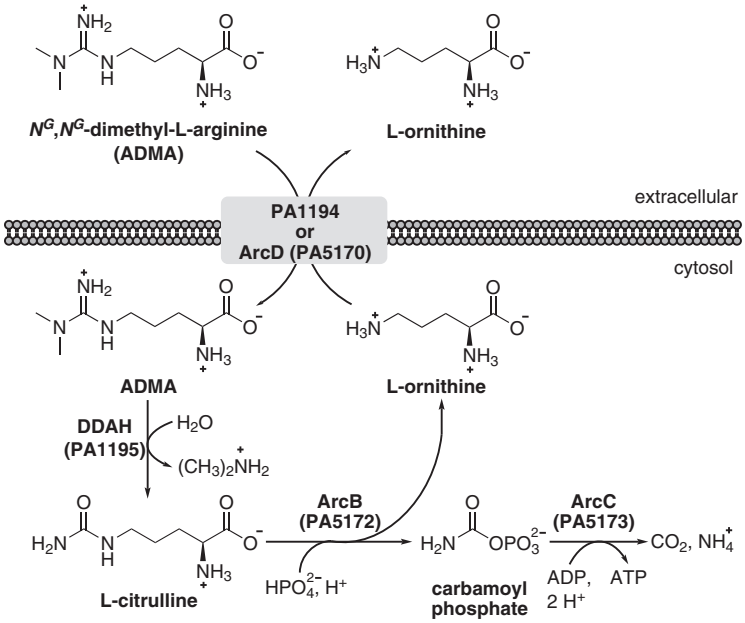

FIG 9 Proposed pathway for methylarginine degradation in *P. aeruginosa* PAO1. It is hypothesized that extracellular methylarginines are transported into the bacterium through PA1194 and/or ArcD (PA5170). PA1194 shares 60% similarity (49% identity) with ArcD (PA5170), an L-arginine/L-ornithine antiporter. This suggests that PA1194 might function as a dedicated methylarginine/L-ornithine antiporter. Upon entry, intracellular methylarginines are hydrolyzed into L-citrulline and either mono- or dimethylamine via DDAH (PA1195). Using phosphate as a substrate, ornithine carbamoyltransferase, ArcB (PA5172), catalyzes the conversion of L-citrulline into L-ornithine and carbamoyl phosphate. While the L-ornithine product can be used for the uptake of methylarginines, carbamoyl phosphate is broken down into ATP, CO_2, and NH_3 in a reaction catalyzed by carbamate kinase, ArcC (PA5173).

healthy humans (32, 33). These values in combination with the results from this study suggest that *P. aeruginosa* assimilates exogenous methylarginines to supplement its growth rather than using these compounds as a main source of nitrogen and/or energy. Additionally, L-NMMA and ADMA are inhibitors of nitric oxide (NO) production in eukaryotes (7), so it is possible that methylarginine degradation serves as a protective measure for NO signaling in *P. aeruginosa*.

DdaR (PA1196) as a regulator of DDAH in *P. aeruginosa* PAO1. One of the more significant findings of this study was the identification of the putative EBP PA1196 as an essential regulator of the expression of the *ddaH* gene in response to methylarginines. Consequently, PA1196 was named DdaR, for dimethylarginine dimethylaminohydrolase regulator. DdaR has a predicted modular structure comprised of an N-terminal sensor domain, a central sigma 54 interaction domain, and a C-terminal DNA-binding domain that possesses a FIS-type helix-turn-helix motif (19, 20). DdaR is not a homolog of any previously characterized EBP and therefore represents a class of EBPs whose regulatory activities are mediated through methylarginines. Homologs of DdaR are found only in the genomes of other strains of *P. aeruginosa*. DdaR homologs do not occur in other *Pseudomonas* spp., nor are they found in other bacteria which possess DDAHs. This suggests that the DdaR-RpoN mechanism for regulating expression of *ddaH* is unique to *P. aeruginosa*. Despite DDAH and PA1196 being conserved among *P. aeruginosa* strains, the ability to grow on methylarginines as a sole source of nitrogen varies among them (Fig. 8). The reasons behind this growth variation is unclear but might be related to factors such as the experimental growth temperature, RpoN activity, L-citrulline degradation, and tolerance to dimethylamine.

Apart from *ddaH*, it is not clear what other genes are regulated by DdaR. The *arcBC* genes, which are central in L-citrulline degradation, do not possess any putative −24/−12 promoters (Sigma 54 Promoter Database [http://www.sigma54.ca]). Both PA1194 and ArcD (PA5170) are hypothesized to be involved in methylarginine trans-

TABLE 1 Bacteria used in the current study

Strain	Relevant characteristic(s)	Reference or source
Pseudomonas aeruginosa		
PAO1	Wild type	46
PAO1 Δ*ddaH*	Δ*ddaH* (Δ*PA1195*) mutant derivative of PAO1	This study
PAO1 Δ*PA1196*	Δ*PA1196* mutant derivative of PAO1	This study
PAO1 Δ*PA1197*	Δ*PA1197* mutant derivative of PAO1	This study
PAO6359	*rpoN*::Ω-Km mutant derivative of PAO1	46
PA14	Wild type	47
PAK	Wild type	48
Pseudomonas putida KT2440	Wild type	ATCC
Pseudomonas fluorescens ATCC 13525	Wild type	ATCC
Escherichia coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^r) *endA1* λ$^-$	Invitrogen

port, but neither corresponding gene possesses a −24/−12 promoter. The absence of −24/−12 promoters among these genes is a clear indicator that they are not direct targets of transcriptional activation by RpoN. More extensive analyses, e.g., transcriptomic studies, are needed to determine the scope and magnitude of genes whose expression is affected by DdaR.

The function of PA1197 remains unclear. The *ddaR* gene is in a putative operon with *PA1197*, which encodes a Sir2-type deacetylase. While this operon structure would suggest that PA1197 has some role in methylarginine utilization, we found that a Δ*PA1197* mutant exhibited wild-type growth on ADMA under the conditions tested in this study (Fig. 1). In addition, DDAH activity and expression of a *ddaH*::*lacZ* reporter were not affected by a Δ*PA1197* mutation (Fig. 4 and 5). These findings argue that PA1197 is not essential for DDAH activity and methylarginine utilization in *P. aeruginosa* PAO1. It is possible that PA1197 affects methylarginine utilization under conditions not tested in this study, including under conditions of oxygen limitation and in biofilms. For example, in the presence of NO, the catalytic cysteine residue of DDAH undergoes *S*-nitrosylation (34, 35), a modification that inhibits enzymatic activity. PA1197 might participate in the alleviation of this inhibition through some unknown mechanism.

MATERIALS AND METHODS

Bacteria and media. The bacteria used in the current study are listed in Table 1. Bacteria were grown in either Lennox broth (LB) or minimal medium (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 8.6 mM NaCl, 1.0 mM $MgSO_4$, 5.0 μM $FeSO_4$, pH 7.0). Minimal medium was supplemented with 15 mM carbon source and 10 mM nitrogen source as specified for each set of experiments (see below). Solid bacteriological media were prepared with the addition of Difco Bacto agar at 15 g liter^{-1}. For plasmid selection in *E. coli*, the media were supplemented with carbenicillin (100 μg ml^{-1}), gentamicin (20 μg ml^{-1}), or kanamycin (50 μg ml^{-1}). For plasmid and marker selection in *Pseudomonas* spp., the media were supplemented with carbenicillin (200 μg ml^{-1}), gentamicin (30 μg ml^{-1}), or tetracycline (25 μg ml^{-1}).

General molecular biology procedures. The plasmids and oligonucleotides (primers) used in the current study are listed in Table 2. The restriction endonucleases, T4 ligase, and Phusion DNA polymerase used for cloning purposes were purchased from New England BioLabs. Promega nucleic acid purification kits were used for DNA isolation. Genomic DNA from *P. aeruginosa* PAO1 served as the template in all PCRs.

Deletion of the *PA1195* (*ddaH*), *PA1196*, and *PA1197* genes in *P. aeruginosa* PAO1. The *PA1195* (*ddaH*), *PA1196*, and *PA1197* genes were deleted from *P. aeruginosa* PAO1 using well-established procedures (36–38). The primers and plasmids pertaining to the gene deletions are given in Table 2.

Cloning of the *PA1195* (*ddaH*) and *PA1196* genes. The *ddaH* and *PA1196* ORFs were PCR amplified from the genomic DNA of *P. aeruginosa* PAO1 using primer pairs BL614.f/BL614.r and BL615.f/BL615.r, respectively. The desired *ddaH* and *PA1196* PCR products were gel purified and cloned into pCR-Blunt (Invitrogen) to generate the plasmids pBRL671 and pBRL672, respectively. To install a 5′ ribosome-binding site (RBS), the *ddaH* and *PA1196* ORFs were subcloned into the NdeI/BamHI sites of pET15b (EMD Millipore) to generate the plasmids pBRL674 and pBRL675, respectively. The RBS-*ddaH* and RBS-*PA1196* fragments were then each subcloned into the XbaI/SacI sites of the expression plasmid pBBR1MCS-5 (39)

TABLE 2 Plasmids and oligonucleotides used in the current study[a]

Plasmid or oligonucleotide	Relevant characteristics or sequence	Reference/source or purpose
Plasmids		
pCR-Blunt	Cloning plasmid; Km^r	Invitrogen
pDONR221	Gateway cloning plasmid; Km^r	Invitrogen
pET15b	Expression plasmid; Cb^r	EMD Millipore
pBBR1MCS-3	Broad-host-strain plasmid; Tc^r	39
pBBR1MCS-5	Broad-host-strain plasmid; Gm^r	39
pEX18ApGW	Gene deletion plasmid for *P. aeruginosa*; Cb^r	36
pFLP2	Carries FLP recombinase; Cb^r	49
pPS856	Carries FRT-*accC1*-FRT marker; Gm^r	49
Δ*Plac*-pBBR1MCS-5	pBBR1MCS-5 minus the *lac* promoter; Gm^r	37
pBRL631	*ddaH*::FRT-*aacC1*-FRT in pDONR221; Km^r Gm^r	This study
pBRL633	*PA1197*::FRT-*aacC1*-FRT in pDONR221; Km^r Gm^r	This study
pBRL635	*PA1196*::FRT-*aacC1*-FRT in pDONR221; Km^r Gm^r	This study
pBRL637	*ddaH*::FRT-*aacC1*-FRT in pEX18ApGW; Cb^r Gm^r	This study
pBRL638	*PA1197*::FRT-*aacC1*-FRT in pEX18ApGW; Cb^r Gm^r	This study
pBRL639	*PA1196*::FRT-*aacC1*-FRT in pEX18ApGW; Cb^r Gm^r	This study
pBRL645	*ddaH*::*lacZ* in pCR-Blunt; Km^r	This study
pBRL649	*ddaH*::*lacZ* in Δ*Plac*-pBBR1MCS-5; Gm^r	This study
pBRL671	*ddaH* in pCR-Blunt; Km^r	This study
pBRL672	*PA1196* in pCR-Blunt; Km^r	This study
pBRL674	*ddaH* in pET15b; Cb^r	This study
pBRL675	*PA1196* in pET15b; Cb^r	This study
pBRL685	*ddaH* in pBBR1MCS-5; Gm^r	This study
pBRL686	*PA1196* in pBBR1MCS-5; Gm^r	This study
pBRL688	*ddaH*::*lacZ* with mutated RpoN promoter; Gm^r	This study
Oligonucleotides		
BL342.f	ATGACCATGATTACGGATTCACT	*E. coli lacZ*
BL342.r	TTATTTTTGACACCAGACCAACTGG	*E. coli lacZ*
BL579.f	TACAAAAAAGCAGGCTCGAACGCATCCTGGTCATCAC	*PA1197* UpF-GWL
BL579.r	TCAGAGCGCTTTTGAAGCTAATTCGTGCTTCTGCAACTCGGCGATG	*PA1197* UpR-Gm
BL580.f	AGGAACTTCAAGATCCCCAATTCGGTACCGCGAATTGCGCAAAG	*PA1197* DnF-Gm
BL580.r	TACAAGAAAGCTGGGTCGATAAATGTGACTTACCACCTG	*PA1197* DnR-GWR
BL581.f	GGCCTGCAGCGTATCGATC	*ddaH*::*lacZ*
BL581.r	GAATCCGTAATCATGGTCATGACGATTTTCCTCGTGTGG	*ddaH*::*lacZ*
BL587.f	TACAAAAAAGCAGGCTCGCTTCGCGTGGAAATCGTC	*ddaH* UpF-GWL
BL587.r	TCAGAGCGCTTTTGAAGCTAATTCGCGAGCGATGATGTGCTTGAAC	*ddaH* UpR-Gm
BL588.f	AGGAACTTCAAGATCCCCAATTCGGTCAACGAAAGGGTGATCATGC	*ddaH* DnF-Gm
BL588.r	TACAAGAAAGCTGGGTCAGTTTTCCCTTGTCCGAATCG	*ddaH* DnR-GWR
BL614.f	GCCATATGTTCAAGCACATCATCGCTC	*ddaH* ORF
BL614.r	GCGGATCCGAGCTCTCAGAAGCGCAGCGACATGC	*ddaH* ORF
BL615.f	GCCATATGGCGCTTGTCGAGGAACC	*PA1196* ORF
BL615.r	GCGGATCCGAGCTCTCAGGCTGGCGGATCGAG	*PA1196* ORF
BL618.f	GGCGTTGGCTAACGCGTGGCTT	Mutagenesis of pBRL649
BL618.r	CGGGAATTGCCTTGGAAATCC	Mutagenesis of pBRL649
ZS412.f	TACAAAAAAGCAGGCTATGGCGCTTGTCGAGGAAC	*PA1196* UpF-GWL
ZS412.r	TCAGAGCGCTTTTGAAGCTAATTCGGGCCCCCACGTAGGCCTG	*PA1196* UpR-Gm
ZS413.f	AGGAACTTCAAGATCCCCAATTCGGTGATCCGGCGCAGCCTGTTGCTGC	*PA1196* DnF-Gm
ZS413.r	TACAAGAAAGCTGGGTTCAGGCTGGCGGATCGAG	*PA1196* DnR-GWR
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA	FRT-*aacC1*-FRT marker
Gm-R	CGAATTGGGGATCTTGAAGTTCCT	FRT-*aacC1*-FRT marker
GW-*attB1*	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Gateway cloning primer
GW-*attB2*	GGGGACCACTTTGTACAAGAAAGCTGGGT	Gateway cloning primer

[a]Plasmids carry the *bla*, *aacC1*, *aph(3′)-II*, and/or *tetC* gene encoding resistance to carbenicillin (Cb^r), gentamicin (Gm^r), kanamycin (Km^r), and/or tetracycline (Tc^r), respectively. FRT, FLP recombination target. The following abbreviations referring to the target and purpose of gene deletion primers correspond to the gene deletion strategy for *P. aeruginosa* (49): UpF, up forward; UpR, up reverse; DnF, down forward; DnR, down reverse; GWL, Gateway left; GWR, Gateway right; and Gm, gentamicin marker.

to give plasmids pBRL685 and pBRL686, respectively. Lastly, the *PA1196* ORF from pBRL672 was subcloned into the XbaI site of pBBR1MCS-3 (39) with either a backward (pBRL719) or a forward (pBRL720) orientation relative to the orientation of the *lac* promoter.

Cloning of the *ddaH*::*lacZ* reporter. The *ddaH*::*lacZ* reporter was constructed through fusion PCR (40). Briefly, the primers BL581.f and BL581.r were used to PCR amplify the 5′ regulatory region (~500 bp) of the *ddaH* gene. The primers BL342.f and BL342.r were used to PCR amplify the *E. coli lacZ* ORF (~3000 bp). The *ddaH* and *lacZ* amplicons were next fused together in a PCR with the

primers BL581.f and BL342.r. The desired *ddaH*::*lacZ* fusion was gel purified and cloned into pCR-Blunt (Invitrogen) to generate pBRL645. The *ddaH*::*lacZ* fusion was subcloned into the XbaI site of Δ*Plac*-pBBR1MCS-5 (37) to give pBRL649. In addition, Q5 site-directed mutagenesis (NEB) was performed using primers BL618.f and BL618.r to change the GG nucleotides of the −24 element of the RpoN promoter to AA in *ddaH*::*lacZ* of pBRL649. The resulting plasmid, pBRL688, was sequenced to verify the presence of the desired mutation.

Growth assays. ADMA hydrochloride and L-NMMA acetate were purchased from Sigma-Aldrich. However, due to the presence of acetate in the L-NMMA preparation, this methylarginine was excluded from the growth assays. Furthermore, for the experiments to be cost-effective, growth was assayed using a microplate reader. Growth was measured in quadruplicate for each *P. aeruginosa* strain. Bacteria were initially grown on LB agar plates at 37°C for 24 h. Single colonies were inoculated into 2 ml of LB (in a 16- by 100-mm culture tube), and the inoculated or seed cultures were grown at 37°C at 200 rpm for 24 h. The wells of 96-well polystyrene plates were filled with 0.2 ml of minimal medium supplemented with 15 mM gluconate and 10 mM nitrogen source (ADMA, L-arginine, L-citrulline, dimethylamine, L-ornithine, or NH_4Cl). The minimal medium-filled wells were inoculated with 1% (vol/vol) LB-grown seed culture. The inoculated plates were incubated at 37°C with continuous slow shaking in a Synergy HT BioTek microplate reader. The absorbance at 600 nm (OD_{600}) of each well was measured at 1.0-h intervals for a total duration of 24 to 48 h.

Exceptions to this procedure included the following: (i) when ADMA was tested as a carbon source, the minimal media were supplemented with 15 mM ADMA and 10 mM NH_4Cl, (ii) for complementation experiments, the media were supplemented 30 μg ml^{-1} gentamicin for plasmid selection, and (iii) a temperature of 30°C (instead of 37°C) was used when the growth of various *Pseudomonas* spp. on ADMA as a nitrogen source was tested.

***β*-Galactosidase (LacZ) assays.** LacZ activities were measured using the Miller assay (40, 41), and experiments were conducted in triplicate for each strain and/or condition. The *ddaH*::*lacZ* reporter (pBRL649) was electroporated into *P. aeruginosa*, and colonies were selected on LB agar plates supplemented with 30 μg ml^{-1} gentamicin. Individual colonies were inoculated into 2 ml of LB (in a 16- by 100-mm culture tube) supplemented with 30 μg ml^{-1} gentamicin, and the inoculated or seed cultures were grown at 37°C at 200 rpm for 24 h. Minimal medium (1 ml in a 16- by 100-mm culture tube) supplemented with 15 mM gluconate, 10 mM NH_4Cl, and 30 μg ml^{-1} gentamicin was inoculated with 1% (vol/vol) LB-grown seed culture, and the inoculated cultures were grown at 37°C at 200 rpm. At an OD_{600} of 0.3, cultures were treated with a final concentration of 0.1 mM substrate, which included ADMA, L-NMMA, L-citrulline, or L-arginine. LacZ activities were measured at 1.0 h afer the addition of the substrate. Analysis of variance (ANOVA) was done using Dunnett's *post hoc* test (α value, 0.05) to identify significant differences ($P < 0.0001$) in LacZ activities.

P. putida KT2440 was cotransformed with the *ddaH*::*lacZ* reporter (pBRL649) and plasmid-derived *PA1196* (pBRL720) via electroporation (42). Recombinant colonies were selected on LB supplemented with 30 μg ml^{-1} gentamicin and 25 μg ml^{-1} tetracycline. Individual colonies were inoculated into 2 ml of LB (in a 16- by 100-mm culture tube) supplemented with 30 μg ml^{-1} gentamicin and 25 μg ml^{-1} tetracycline, and the inoculated or seed cultures were grown at 30°C at 200 rpm for 24 h. Minimal medium (1 ml in a 16- by 100-mm culture tube) supplemented with 15 mM gluconate, 10 mM NH_4Cl, 30 μg ml^{-1} gentamicin, and 25 μg ml^{-1} tetracycline was inoculated with 2% (vol/vol) LB-grown seed culture, and the inoculated cultures were grown at 30°C at 200 rpm. At an OD_{600} of 0.3, the cultures were treated with either no substrate or 0.1 mM ADMA. LacZ activities were measured at 2.0 h after the addition of the substrate. LacZ assays (controls) were also performed with *P. putida* KT2440 cotransformed with empty plasmids pBBR1MCS-3/Δ*Plac*-pBBR1MCS-5, pBBR1MCS-3/pBRL649, pBRL720/Δ*Plac*-pBBR1MCS-5, and pBRL719/pBRL649. ANOVA was done using Dunnett's *post hoc* test (α value, 0.05) to identify significant differences ($P < 0.0001$) in LacZ activities.

Measurement of DDAH activity. DDAH activity was measured in duplicate for each *P. aeruginosa* strain per tested condition. Bacteria were initially grown on LB agar plates at 37°C for 24 h. Single colonies were used to inoculate 2 ml of LB (in a 16- by 100-mm culture tube), and the inoculated or seed cultures were grown at 37°C at 200 rpm for 24 h. Minimal medium (5 ml in a 50-ml conical centrifuge tube) supplemented with 15 mM gluconate and 10 NH_4Cl was inoculated with 1% (vol/vol) LB-grown seed culture. The inoculated cultures were grown at 37°C at 200 rpm to an OD_{600} of 0.3 and subsequently treated with either no substrate or 1.0 mM ADMA. At 2.0 h after the addition of the substrate, bacteria were harvested via centrifugation. Extracts were prepared from the cells as previously described (43). Briefly, cells were lysed by sonication on ice, debris and unlysed cells were removed by centrifugation, and the cell-free supernatants were washed and concentrated in a Microcon centrifugal filter (Millipore). The protein content of cell extracts was quantified using the Bradford assay (Pierce). The DDAH activity in the cell extracts was measured using established procedures (44, 45) and is reported in units (U), defined as the amount of L-citrulline (nanomoles hour^{-1}) formed per milligram of total protein. ANOVA was done using Dunnett's *post hoc* test (α value, 0.05) to identify significant differences ($P < 0.0001$) in DDAH activities.

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