

# Arginine racemization by coupled catabolic and anabolic dehydrogenases

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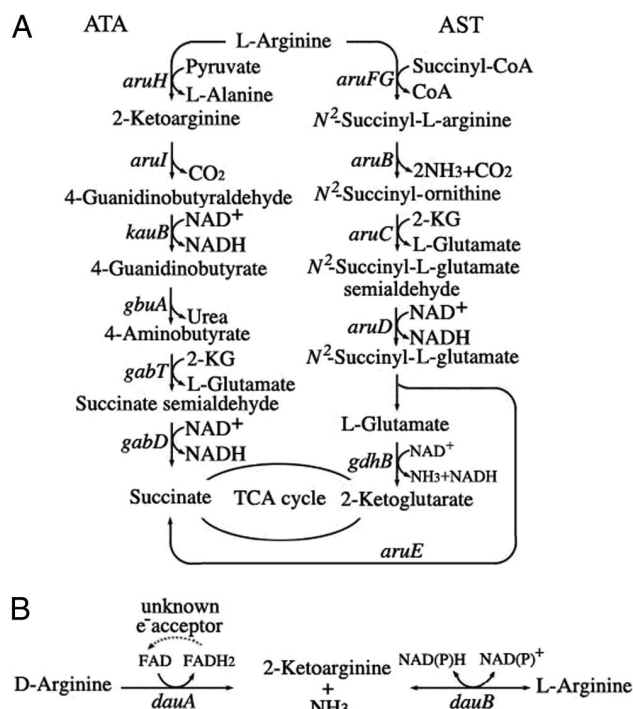
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D-Amino acids exist in living organisms as specialized components of many different machineries. Biosynthesis of D-amino acids from racemization of predominant L-enantiomers is catalyzed by a single enzyme. Here, we report the finding of a novel 2-component amino acid racemase for D-to-L inversion in D-arginine metabolism of *Pseudomonas aeruginosa*. From DNA microarray analysis, the putative *dauBAR* operon (for D-arginine utilization) of unknown functions was found to be highly induced by D-arginine. The importance of the *dau* operon in D-arginine metabolism was demonstrated by the findings that strains with a lesion at *dauA* or *dauB* failed to use D-arginine as sole carbon source. Two lines of evidence suggest that DauA and DauB are required for D-to-L racemization of arginine. First, growth complementation of an L-arginine auxotroph by D-arginine was abolished by a lesion at *dauA* or *dauB*. Second, D-arginine induced L-arginine-specific genes in the parental strain PAO1 but not in its *dauA* or *dauB* mutants. This hypothesis was further supported by activity measurements of the purified enzymes: DauA catalyzes oxidative deamination of D-arginine into 2-ketoarginine and ammonia, and DauB is able to use 2-ketoarginine and ammonia as substrates and convert them into L-arginine in the presence of NADPH or NADH. Thus, we propose that DauA and DauB are coupled catabolic and anabolic dehydrogenases to perform D-to-L racemization of arginine, which serves as prerequisite of D-arginine utilization through L-arginine catabolic pathways.

amino acid | arginine dehydrogenase | racemase

Although L-amino acids are the predominant amino acids in protein synthesis, D-amino acids serve as specialized components of many types of machineries in living organisms. In mammals, D-serine and D-aspartate are associated with cell aging and neural signaling (1, 2). In bacteria, some D-amino acids are essential ingredients of cell wall synthesis (3). Endogenous D-amino acids are produced by racemization from the prevalent L-amino acids through the action of racemases. Amino acid racemases are classified into 2 groups: pyridoxal 5' phosphate-dependent and phosphate-independent enzymes (4). Completely different reaction mechanisms have been proposed for these 2 groups of enzymes for the spatial rearrangement of  $\alpha$ -hydrogen in the corresponding amino acids. Nevertheless, racemization of amino acids reported so far is catalyzed by a single enzyme.

When provided in excess, some D-amino acids can be used as nutrients to support growth by bacteria. In most cases, D-amino acid oxidase or dehydrogenase catalyzes the oxidative deamination as the first step in catabolism. *Pseudomonas aeruginosa*, an opportunistic human pathogen with an enormous catabolic capacity, is capable of growing on D-arginine as the sole source of carbon and nitrogen (5). The presence of an inducible D-arginine dehydrogenase activity in this organism was initially reported by Haas and coworkers (6), and 2-ketoarginine derived from this reaction could be converged into the arginine transaminase (ATA) pathway (7, 8), 1 of the 4 pathways for L-arginine catabolism in pseudomonads (Fig. 1). In fact, it has been proposed that L-arginine might be converted into D-arginine via racemization (6), reminiscent of L-alanine utilization through a catabolic alanine racemase and D-alanine



**Fig. 1.** Arginine catabolic pathways in *P. aeruginosa* PAO1. (A) Relevant intermediates and genes of the ATA and AST pathways were shown. (B) Proposed functions of *dauA* and *dauB* in conversion of D-arginine to L-arginine.

dehydrogenase in *Escherichia coli* and many bacteria (9, 10). Existence of an arginine racemase in *P. aeruginosa* was supported by growth complementation of arginine auxotrophs with D-arginine (6). However, the activity of *P. aeruginosa* arginine racemase has never been demonstrated in vitro, presumably because of the instant decomposition of both L- and D-arginine in extracts.

Under aerobic conditions, L-arginine is preferentially catabolized by the arginine succinyltransferase (AST) pathway, followed by the ATA pathway (7, 11). Enzymes of the AST pathway are encoded by the *aruCFGDBE* operon (12), which is induced by exogenous L-arginine in the presence of a functional arginine regulator, ArgR (13). The ArgR protein belongs to the AraC family of transcriptional regulators. Depending on the location of its binding sites, ArgR serves as a repressor or activator of ArgR regulon in arginine and glutamate metabolism. Thus, when the AST pathway is absent or remains uninduced (e.g., in the *argR* mutant), the ATA pathway then takes charge as the auxiliary route of L-arginine utilization.

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**Table 1. Microarray analysis of representative genes for arginine metabolism in *P. aeruginosa* PAO1**

Gene no.*	Gene name	Absolute signal value	
		Glu <sup>†</sup>	Glu + D-arginine <sup>‡</sup>
Group I: D-Arginine-specific			
PA3862	dauB	199	9209
PA3863	dauA	80	3247
PA3864	dauR	185	2194
PA3865	dauT	173	4840
Group II: The AST pathway			
PA0895	aruC	925	5442
PA0896	aruF	442	4071
PA0897	aruG	419	3680
PA0898	aruD	350	2805
PA0899	aruB	637	3965
PA0901	aruE	786	2835
PA3068	gdhB	1681	7026
Group III: The ATA pathway			
PA0265	gabD	796	1500
PA0266	gabT	943	1315
PA1421	gbuA	78	943
PA4976	aruH	169	152
PA4977	aruI	93	105
PA5312	kauB	1099	1357
Group IV: Arginine and glutamate biosynthesis			
PA3537	argF	1715	267
PA4588	gdhA	373	56

\*Gene numbers are from the *Pseudomonas* Genome Project ([www.pseudomonas.com](http://www.pseudomonas.com)).

<sup>†</sup>Cells were grown in minimal medium P with L-glutamate.

<sup>‡</sup>Cells were grown in minimal medium P with L-glutamate and D-arginine.

Genes of the ATA pathway are composed of several modules that are subjected to sequential induction by different intermediate compounds in the pathway (6). Expression of the *aruHI* genes encoding the first 2 enzymes of the ATA pathway is controlled by the *AruRS* 2-component systems (7) in response to L-arginine, and the *gbuA* gene encoding 4-guanidinobutyrate is controlled by *GbuR* and 4-guanidinobutyrate (14).

From DNA microarray analysis, a 4-gene locus (PA3862 to PA3865 of the annotated genome project) of unknown functions was found highly inducible by D-arginine in *P. aeruginosa* PAO1. We provide evidence that these genes participate in D-arginine catabolism. The promoters of PA3862–PA3864 and the convergent PA3865 are inducible by exogenous D-arginine but not by L-arginine. PA3863 encodes a D-arginine dehydrogenase, and in concert with PA3862 encoding an anabolic L-arginine dehydrogenase, these 2 genes play essential roles in D- to L-arginine conversion. As a result, this unique racemization reaction is the prerequisite for D-arginine utilization through L-arginine catabolic pathways. From here on, PA3862–PA3865 are designated as *dauBART*, for D-arginine utilization.

## Results

**DNA Microarray Analysis.** *P. aeruginosa* grows on D-arginine as the sole source of carbon and nitrogen. To understand the metabolic flow of D-arginine, we conducted DNA microarray experiments and compared the transcriptional profiles of strain PAO1 grown in the minimal medium in the absence or presence of D-arginine. As shown in Table 1, the *dauBART* genes were significantly induced by D-arginine. Surprisingly, D-arginine exerts the same effects as L-arginine (15) on all genes in the *ArgR* regulon (as represented by genes in group II for the activation effect and genes in group IV for the repression effect of Table 1).

Deamination by a variety of enantiomer-specific enzymes is the

**Table 2. Effect of D- or L-arginine on *dauB* and *dauT* promoter activities in PAO1**

Plasmid (promoter)	C & N source		Specific activities, nmol/min per mg*
	Glu	Glu + D-arg	
pCR1 ( <i>dauB</i> )	Glu		34
	Glu + D-arg		680
	Glu + L-arg		63
pZY4 ( <i>dauT</i> ) (7)	Glu		619
	Glu + D-arg		6006
	Glu + L-arg		233

C & N, carbon and nitrogen; Glu, cells were grown in MMP-glutamate; Glu + D-arg, cells were grown in MMP-glutamate supplemented with 5 mM D-arginine; and Glu + L-arg, cells were grown in MMP-glutamate supplemented with 20 mM L-arginine.

\*Specific activities of  $\beta$ -galactosidase expressed from plasmids were averages of 2 measurements for each growth condition, with standard errors of less than 5%.

most common first step of D-amino acid catabolism. In the case of D-arginine, 2-ketoarginine has been identified as a product of an inducible D-arginine dehydrogenase in PAO1 (6). Therefore, one would expect that genes in the ATA pathway (group III of Table 1) of 2-ketoarginine utilization must be induced by D-arginine; however, that was not the case. These results provide the first line of evidence to support the hypothesis of D-to-L racemization, instead of deamination, as the prerequisite of D-arginine catabolism.

**Bioinformatic Analysis.** Based on genome annotations ([www.pseudomonas.com](http://www.pseudomonas.com)), *DauBARs* were all hypothetical proteins of unknown functions. By Pfam database predictions, *DauB* belongs to the ornithine cyclodeaminase/mu-crystallin family (PF02423; *E* value 9e-26) and exhibits a limited sequence similarity to an alanine dehydrogenase from an archeobacterium (TIGRFAM accession no. 02371). *DauA* was predicted as an FAD-dependent oxidoreductase (PF01266; *E* value 6e-24), and *DauR* possesses a PAS domain (PF08348; *E* value 2e-32) of cell signaling at the N terminus. By using a BLAST search against the Protein Data Bank, we found that the C terminus of *DauR* (R162-E204) exhibits limited similarity to a helix-turn-helix DNA-binding domain (R144-E181) of  $\gamma\delta$  resolvase (16). Downstream of the putative *dauBAR* operon, the convergent *dauT* gene encodes a polypeptide that shows 80% sequence similarity to the *AotJ* protein (17), a periplasmic arginine/ornithine-binding protein of an ABC transport system in *P. aeruginosa*. The *dauBART* locus is conserved among 4 strains of *P. aeruginosa* and 2 strains of *Pseudomonas fluorescens* with a completed genomic sequence. Without *dauT*, a putative *dauBAR* operon can also be found in *Pseudomonas mendocina*, but not in *Pseudomonas putida*, *Pseudomonas stutzeri*, and *Pseudomonas syringae*.

**Promoter Induction by D-Arginine.** To examine the possible effects of exogenous arginine on *dauBART* expression, *lacZ* fusion plasmids carrying the putative promoter regions of *dauB* and *dauT* were constructed and introduced into strain PAO1, and the cells were grown in the minimal medium in the absence and presence of exogenous L- and D-arginine. As shown in Table 2, both *dauB* and *dauT* promoters were significantly induced by D-arginine. In comparison, exogenous L-arginine exerted a repression effect on the *dauT* promoter but showed no effect on the *dauB* promoter. These results support the hypothesis that *DauBART* could be related to D-arginine catabolism and uptake.

**Growth Phenotypes.** The knockout mutants of *dauBART* were constructed to investigate the potential functions of these genes in D-arginine catabolism. As shown in Table 3, the parental strain PAO1 grew on D-arginine as the sole carbon source. Growth on

**Table 3. Growth phenotype of PAO1 and its mutants**

Strains (genotype)	D-Arg		L-Arg
	C source*	N source <sup>†</sup>	C & N source <sup>‡</sup>
PAO1 (wild type)	+	+	+
PAO5801 ( <i>dauB</i> )	– <sup>§</sup>	+	+
PAO5802 ( <i>dauA</i> )	–	–	+
PAO5803 ( <i>dauR</i> )	+	+	+
PAO5804 ( <i>dauT</i> )	+	+	+
PAO4558 ( <i>aruF</i> )	+	+	+
PAO5602 ( <i>aruF aruH</i> )	–	+	–
PAO5603 ( <i>aruF aruI</i> )	–	+	–

\*D-Arginine as the sole carbon (C) source. Cells were grown in minimal medium P with 5 mM NH<sub>4</sub>Cl and 10 mM D-arginine.

<sup>†</sup>D-Arginine as the sole nitrogen (N) source. Cells were grown in minimal medium P with 20 mM glucose and 5 mM D-arginine.

<sup>‡</sup>L-Arginine as the sole carbon and nitrogen (C&N) source, cells were grown in minimal medium P with 20 mM L-arginine.

<sup>§</sup>No growth for at least 48 h.

D-arginine was completely abolished in either *dauB* or *dauA* mutants but was sustained in *dauR* and *dauT* mutants. When supplied as the sole nitrogen source, only the *dauA* mutant showed no growth on D-arginine. In comparison, none of these mutants exhibited any growth defects on L-arginine or L-glutamate.

**Effects of *dau* Mutations on D-Arginine Dehydrogenase Activity.** The results from growth phenotype analysis suggested that the *dau* genes are involved in D-arginine utilization. Haas and coworkers reported the presence of D-arginine-inducible D-arginine dehydrogenase (DADH) activity in *P. aeruginosa* (6) to generate 2-ketoarginine and ammonia (Fig. 1). We therefore conducted experiments to measure DADH activities in PAO1 and congenic *dau* mutants.

Consistent with a previously published report (6), the DADH of PAO1 was specifically induced by the addition of D-arginine (Table 4); no induction by L-arginine could be observed (data not shown). The *dauT* mutant retained the induction effect of D-arginine on DADH. However, in the *dauB* and *dauA* mutants that lost the capability to grow on D-arginine as the sole carbon source, no D-arginine-inducible DADH could be detected. In contrast, the *dauR* mutant exhibited a high basal level of DADH in the absence of D-arginine. In conjunction with genome annotations as described above, we hypothesized that DADH might be composed of DauA and/or DauB, and that DauR serves as a transcriptional repressor of the *dauBAR* operon.

**DauA Exhibits DADH Activity.** To test the proposed biochemical function of DauA and DauB, we constructed recombinant strains of *E. coli* for overexpression of DauA and DauB separately, with a

**Table 4. Effect of D-arginine on DADH activity of PAO1 and its mutants**

Strain (genotype)	Specific activities, nmol/min per mg*	
	Glu	Glu + D-arg
PAO1 (wild type)	20	261
PAO5801 ( <i>dauB</i> )	12	11
PAO5802 ( <i>dauA</i> )	ND	ND
PAO5803 ( <i>dauR</i> )	140	174
PAO5804 ( <i>dauT</i> )	22	251

Glu, cells were grown in glutamate minimal medium; Glu + D-arg, cells were grown in glutamate minimal medium supplemented with 5 mM D-arginine; ND, not detectable.

\*Specific activities were averages of 2 measurements for each growth condition, with standard errors of less than 5%.

hexa-histidine tag fused to the N terminus. Although DauA and DauB tended to form inclusion bodies when overexpressed, at least 50% of recombinant proteins remained soluble. Measurements of the DADH activity from the crude extracts of these recombinant strains clearly indicated that DauA but not DauB possesses DADH activity (data not shown, but see below).

The recombinant DauA protein was purified to homogeneity, and the results of SDS/PAGE and size exclusion column chromatography revealed that the histidine-tagged DauA protein is monomeric in its native form. The purified DauA had a distinct yellowish color, suggesting the presence of a bound chromophore. The specific activity of the recombinant DauA was estimated to be 18.8 μmol/min per mg when D-arginine (20 mM) serves as substrate, and it was negligible with L-arginine as substrate.

Generation of 2-ketoarginine from the proposed reaction was demonstrated by MS analysis, with phenazine methosulfate (PMS) as the artificial electron acceptor. PMS can be recycled by transferring the electron to oxygen and forming hydrogen peroxide. In the mass spectrum of the reaction mixture with active DauA alone (Fig. 2A), a molecular ion peak of *m/z* 146.1 was observed, which corresponds to that of 4-guanidinobutyric acid (4-GBA), the decarboxylation product of 2-ketoarginine by hydrogen peroxide (18). With the addition of catalase (Fig. 2C) to reduce the amount of hydrogen peroxide, another peak of *m/z* 174.1 appeared, which is identical to that of the authentic 2-ketoarginine. As expected, neither 4-GBA nor 2-ketoarginine was detected in the negative controls with heat-inactivated DauA (Fig. 2B and D). It is essential to include PMS in the reaction to ensure DauA remains active with FAD; no product signal can be detected without PMS (data not shown).

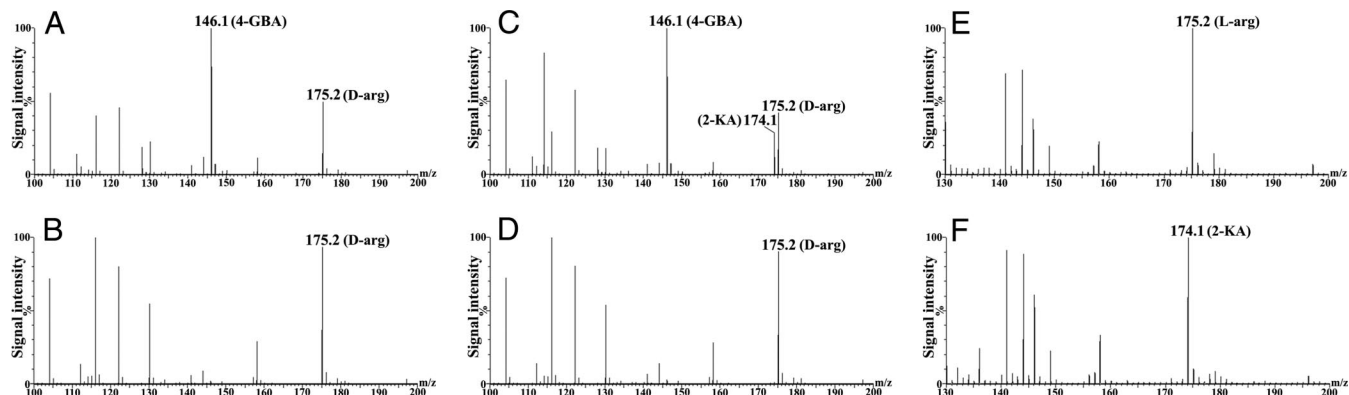
#### Participation of the AST and ATA Pathways in D-Arginine Catabolism.

Under aerobic conditions, the AST and ATA pathways are 2 major routes for L-arginine utilization as the sole source of carbon and nitrogen. Arginine succinyltransferase (encoded by *aruF*) and arginine transaminase (encoded by *aruH*) catalyze the first reaction of the AST and ATA pathways, respectively, and both enzymes take L-arginine, but not D-arginine, as substrate (8, 19). Because D-arginine is converted into 2-ketoarginine by DauA, one might expect that 2-ketoarginine can be further catabolized by 2-ketoarginine decarboxylase (encoded by *aruI*), and subsequent enzymes of the ATA pathway into succinate (Fig. 1). Accordingly, D-arginine utilization was tested in a series of mutants with lesions in the affected pathways, and the results are shown in Table 3.

Interestingly, growth on D-arginine as the sole carbon source was partially retarded in strain PAO4558 (*aruF*) (20) devoid of the AST pathway. No growth on D-arginine could be observed in strains PAO5602 (*aruF, aruH*) or PAO5603 (*aruF, aruI*) (7) in which both AST and ATA pathways were blocked. The growth defects of PAO4558 and PAO5602 on D-arginine do not fit into the proposed model of D-arginine utilization through 2-ketoarginine and the ATA pathway. Instead, these results suggest that D-arginine is mainly converted into L-arginine before being channeled into the AST and/or ATA pathways.

#### Role of *dauA* and *dauB* on the Induction of the AST Pathway by D-Arginine.

To test the hypothesis of D-to-L racemization of arginine, we conducted experiments to measure the promoter activity of *aruC*, the first gene of the *aruCFGDBE* operon for the AST pathway. As shown in Table 5, exogenous D-arginine exerted an induction effect slightly less than that by L-arginine in the parental strain PAO1; it was not clear whether this observed minor difference is of physiological significance. In strains PAO5802 (*dauA*) and PAO5801 (*dauB*), the induction effect of D-arginine was greatly abolished, whereas the *aruC* promoter remained inducible by L-arginine. The residual 2-fold induction by D-arginine in *dauA* and *dauB* mutants might imply possible cross-activation by D-arginine on ArgR, the transcriptional regulator of the *aruC* promoter. These



**Fig. 2.** ESI-MS analysis of the reaction products of DauA and DauB. (A and C) Analysis of reaction mixtures with active DauA without (A) or with (C) catalase. (E) Analysis of reaction mixture with active DauB. (B, D, and F) Corresponding controls with heat-denatured DauA or DauB. The signals with  $m/z$  values of 146.1, 175.2, and 174.1 were in accordance with the positive ions of 4-guanidinobutyric acid (4-GBA; A and C), D-arginine (D-Arg; A–D), or L-arginine (L-arg; E), and 2-ketoarginine (2-KA; C and F).

findings strongly suggest that DauA and DauB are essential for the proposed D-to-L racemization of arginine.

**Growth Complementation of an Arginine Auxotroph by D-Arginine Requires DauA and DauB.** To further investigate the proposed racemization, the growth phenotypes of an arginine auxotroph PAO303 (*argB*) (21) and its *dauA* and *dauB* mutants were determined. As expected, the growth defect of these 3 strains in the minimal medium due to a mutation on *argB* of arginine biosynthesis was complemented by exogenous L-arginine. When D-arginine was added as supplement, only PAO303 resumed growth, but its *dauA* and *dauB* mutants failed to grow. This is consistent with the proposed function of DauA and DauB in arginine racemization.

**Anabolic L-Arginine Dehydrogenase Activity of DauB.** In the proposed racemization reaction, 2-ketoarginine and ammonia derived from D-arginine in the DauA-catalyzed reaction would be taken by DauB to regenerate L-arginine in a second anabolic dehydrogenase reaction. To test this hypothesis, we overexpressed and purified a histidine-tagged DauB. With the purified enzyme we were able to demonstrate the proposed reaction with NADH or NADPH as the reducing power. The reaction was 2-ketoarginine-dependent and ammonia-dependent; consumption of NADH or NADPH could

only be observed when both substrates were present (Fig. 3A). We also tested the proposed reaction of DauB in the reverse direction and found that significant activity could only be detected when L-arginine instead of D-arginine was used as substrate (Fig. 3B).

Generation of L-arginine from 2-ketoarginine and ammonia was also demonstrated by MS analysis. Although the ion peak of 2-ketoarginine ( $m/z$  value of 174.1) was the predominant one in the control sample of heat-inactivated DauB (Fig. 2F), it was almost diminished in the mass spectrum of the reaction mixture with active DauB (Fig. 2E). Instead, one molecular ion peak was observed with an  $m/z$  value of 175.2, which is identical to that of the authentic L-arginine.

## Discussion

Several lines of evidence were presented to indicate that *P. aeruginosa* PAO1 performs D-to-L racemization of arginine through 2 collaborative enzymes: a catabolic D-arginine dehydrogenase (encoded by *dauA*) and an anabolic L-arginine dehydrogenase (encoded by *dauB*). The proposed biochemical reactions of these 2 enzymes were demonstrated with purified DauA and DauB, and the importance of *dauAB* in arginine racemization was supported by the finding that growth complementation of an L-arginine auxotroph by exogenous D-arginine was completely abolished in strains with a *dauA* or *dauB* lesion.

We also established that D-to-L racemization is in fact a prerequisite for D-arginine utilization as the carbon source through L-arginine catabolic pathways. Without converting into L-arginine, D-arginine by itself is not able to induce genes of the AST pathway, and most likely genes of the ATA pathway as well. Therefore, exogenous D-arginine either cannot be used at all, as in strain PAO5802 (*dauA*), or it can only be used as nitrogen source, as in strain PAO5801 (*dauB*). When D-to-L inversion is blocked in the *dauB* mutant, ammonia released from the DauA-catalyzed reaction is sufficient to support growth on D-arginine as a nitrogen source, and accumulation of 2-ketoarginine is anticipated. Although 2-ketoarginine may be channeled into the ATA pathway, contribution of this route in the *dauB* mutant is minimized as the ATA pathway is induced by L-arginine. It has been reported that strain PAO1 grows well on exogenous 2-ketoarginine as a sole source of carbon and nitrogen, and this compound is able to induce DADH as well as 4-guanidinobutyraldehyde dehydrogenase and guanidinobutyrase of the ATA pathway (Fig. 1). Perhaps this discrepancy is due to racemization, and it suggests that 2-ketoarginine may be the signal of *dauBART* induction.

Genome annotations suggest DauA as an FAD-dependent D-amino acid oxidase. We have observed that addition of FAD to the purified DauA indeed can increase the specific activity about

**Table 5.** Effect of D- or L-arginine on the *aruC* promoter activity in PAO1 and its mutants

Strain (genotype)	C & N source	Specific activities, nmol/min per mg*	Fold change
PAO1 (wild type)	Glu	501	1.0
	Glu + D-arg	7007	14.0
	Glu + L-arg	11104	22.2
PAO5801 ( <i>dauB</i> )	Glu	349	1.0
	Glu + D-arg	750	2.2
	Glu + L-arg	6950	19.9
PAO5802 ( <i>dauA</i> )	Glu	380	1.0
	Glu + D-arg	610	1.6
	Glu + L-arg	7345	19.3

Glu, cells were grown in glutamate minimal medium; Glu + D-arg, cells were grown in glutamate minimal medium supplemented with 5 mM D-arginine; Glu + L-arg, cells were grown in glutamate minimal medium supplemented with 20 mM L-arginine.

\*Specific activities of  $\beta$ -galactosidase expressed from the *aruC::lacZ* fusion plasmid pSC500 [Park SM, Lu CD, Abdelal AT (1997) *J Bacteriol* 79:5309–5317] were averages of 2 measurements for each growth condition, with standard errors of less than 5%.



SM10 (25) and then mobilized into the desired recipient strains of *P. aeruginosa* by biparental plate mating (25). After incubation at 37°C for 6 h, transconjugants were selected on LB plates supplemented with tetracycline and streptomycin. The genotypes of all of the mutants constructed were verified by colony PCR.

**Expression of *dauB* and *dauA* in *E. coli*.** The expression vector pBAD-His-6, containing a 6-histidine tag followed by a *Sma*I site, was derived from pBAD-HisA (Invitrogen) by replacing the *Nco*I-*Xho*I region of pBAD-HisA with the following sequence: 5'-CC ATG GGT CAT CAT CAT CAT CCC GGG CTC GAG-3'. The *dauB* and *dauA* structural genes were amplified by PCR from the genomic DNA of PAO1 using the following primer pairs: 5'-AGC GCC GCC ACT CCC CTG ATC-3' and 5'-CCCAAG CTT CAG CCT GCG TGG CGC AG-3' for *dauB*; and 5'-ATC GAA GCG GAT TAC CTC-3' and 5'-CCC AAG CTT CAG GGG GAC AGG CGG CG-3' for *dauA*. The resulting PCR products were digested with *Hind*III, which is a unique restriction site at the 3' end introduced by the primers, and cloned into the *Sma*I and *Hind*III sites of the expression vector so that the N terminus of *DauA* and *DauB* was fused in frame with the 6-histidine tag preceded by a ribosomal binding site and an arabinose-inducible promoter in the plasmid. The resulting plasmids, pCR2 and pCR3, were introduced into *E. coli* Top10. For overexpression of *DauB* and *DauA*, the recombinant strains of *E. coli* were grown in LB medium containing ampicillin (100 µg/mL) at 30°C until the optical density at 600 nm reached 0.5, at which point 0.2% arabinose (wt/vol; final concentration) was added to the cultures for induction. Culture growth was continued for another 3 h under the same conditions, and cells were harvested by centrifugation.

**Purification of Hexa-Histidine-Tagged *DauB* and *DauA*.** The cell pellets were suspended in the phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4) plus 1 mM PMSF as a protease inhibitor, and the cells were ruptured by an Aminco French pressure cell at 17,000 psi. Cell debris was removed by centrifugation at 20,000 × g for 1 h, and the resulting cell-free crude extracts were further precipitated by streptomycin sulfate and centrifuged again. The supernatants from this step were applied to a HisTrap HP column (GE Healthcare) equilibrated with the sodium phosphate buffer described above. After washing off of the unbound proteins with equilibration buffer, His-tagged *DauB* or *DauA* was eluted with a linear gradient of 20–500 mM imidazole over 10 column volumes. For further purification, *DauA* protein was subjected to anion-exchange chromatography using a Mono Q HR 5/5 column (GE Healthcare) followed by gel filtration chromatography using a Superdex 200 HR 10/30 column (GE Healthcare).

**Enzyme Assays.** For the measurements of enzyme activities with the crude extracts, cell cultures in the logarithmic phase were collected by centrifugation, and the cell pellets were washed once with 20 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.6) and resuspended in the same buffer. Cells were broken with a French pressure cell at 17,000 psi, and soluble cell extracts were

prepared after centrifugation at 16,000 × g for 30 min. Protein concentration was determined by the method of Bradford with BSA as standard. For the measurements of β-galactosidase activity, o-nitrophenyl-β-D-galactopyranoside was used as substrate. D-Arginine dehydrogenase activity was assayed with the addition of artificial electron acceptors, PMS and INT, as described previously (6). One unit of D-arginine dehydrogenase activity was defined as the amount of enzyme that led to the reduction of 1 nmol idonitrotetrazolium chloride per minute under the standard assay conditions.

The forward activity of *DauB* in generation of L-arginine (Fig. 1) was determined by monitoring the consumption of NADH or NADPH at 340 nm using Cary 3E (Varian). The reaction mixture contained the following components in a volume of 2 mL: 50 mM Tris-Cl (pH 8.7), 0.35 mM NADH or NADPH, 40 mM NH<sub>4</sub>Cl, 10 mM 2-ketoarginine (KA) (for sample) or ddH<sub>2</sub>O (for control), and 20 µg of the purified *DauB*. The reaction mixtures without NH<sub>4</sub>Cl were preincubated at 37°C for about 6 min, and the reaction was initiated by adding NH<sub>4</sub>Cl. The reverse reaction of *DauB* in taking L- or D-arginine as the substrate was measured by monitoring the formation of NADH or NADPH at 340 nm using Cary 3E (Varian). The reaction mixture contained 10 mM L- or D-arginine, 0.5 mM NAD<sup>+</sup> or NADP<sup>+</sup>, and 20 µg of the purified *DauB* in 50 mM Tris-Cl (pH 8.7) in a total volume of 2 mL. The mixtures without L- or D-arginine were preincubated at 37°C for about 9 min, and the reaction was initiated by adding the missing substrate.

2-KA was synthesized by following the published protocol (6) and lyophilized. Quality and purity of thusly prepared 2-KA has been analyzed by HPLC in a previous report (7) and was confirmed by electrospray ionization (ESI)-MS in this study (Fig. 2F). The 2-KA solution was prepared by dissolving an appropriate amount of the lyophilized powder in water, assuming more than 95% purity and a molecular weight of 174 g/mol.

**ESI-MS Analysis of the Reaction Products.** For *DauA*, the reaction mixtures (500 µL) containing 5 µg of purified *DauA*, 5 mM D-arginine, and 0.8 mM PMS in 10 mM Tris-Cl buffer (pH 8.7) with or without catalase (50 µg) were incubated for 1.5 h at 37°C. For *DauB*, a reaction mixture (500 µL) containing 48 µg of purified *DauB*, 5 mM 2-ketoarginine, 40 mM NH<sub>4</sub>Cl, and 5 mM NADH in 10 mM Tris-Cl buffer (pH 8.7) was incubated overnight at 37°C. The samples were boiled for 10 min after incubation and centrifuged at 16,000 × g for 30 min. The resulting supernatants were submitted for ESI-MS analysis at the MS facility of Georgia State University. In the negative control experiments, heat-denatured recombinant *DauA* or *DauB* was used to prepare the reaction mixture. Samples were diluted in 50% methanol with the addition of 0.1% formic acid for positive ion analysis by TOF mass spectrometry.

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