Regulation of the *dauBAR* operon and characterization of D-amino acid dehydrogenase DauA in arginine and lysine catabolism of *Pseudomonas aeruginosa* PAO1

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A unique D-to-L racemization of arginine by coupled arginine dehydrogenases DauA and DauB encoded by the *dauBAR* operon has been recently reported as a prerequisite for D-arginine utilization as the sole source of carbon and nitrogen through L-arginine catabolic pathways in *P. aeruginosa*. In this study, enzymic properties of the catabolic FAD-dependent D-amino acid dehydrogenase DauA and the physiological functions of the *dauBAR* operon were further characterized with other D-amino acids. These results establish DauA as a D-amino acid dehydrogenase of broad substrate specificity, with D-Arg and D-Lys as the two most effective substrates, based on the kinetic parameters. In addition, expression of *dauBAR* is specifically induced by exogenous D-Arg and D-Lys, and mutations in the *dauBAR* operon affect utilization of these two amino acids alone. The function of DauR as a repressor in the control of the *dauBAR* operon was demonstrated by *dauB* promoter activity measurements *in vivo* and mobility shift assays with purified His-tagged protein *in vitro*. The potential effect of 2-ketoarginine (2-KA) derived from D-Arg deamination by DauA as a signal molecule in *dauBAR* induction was first revealed by mutation analysis and further supported by its *in vitro* effect on alleviation of DauR–DNA interactions. Through sequence analysis, putative DauR operators were identified and confirmed by mutation analysis. Induction of the *dauBAR* operon to the maximal level was found to require the L-arginine-responsive regulator ArgR, as supported by the loss of inductive effect by L-Arg on *dauBAR* expression in the *argR* mutant and binding of purified ArgR to the *dauB* regulatory region *in vitro*. In summary, this study establishes that optimal induction of the *dauBAR* operon requires relief of DauR repression by 2-KA and activation of ArgR by L-Arg as a result of D-Arg racemization by the encoded DauA and DauB.

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INTRODUCTION

Although L-enantiomers of amino acids are selected by nature as building blocks of proteins, D-amino acids do exist in living organisms from bacteria to humans to serve a variety of specific functions. For example, D-amino acids are components in the synthesis of cell wall peptidoglycans and secondary metabolites (antibiotics, siderophores, etc.) in micro-organisms, and D-serine (D-Ser) plays an important role in the central nervous system of humans (Schell *et al.*, 1995).

In living organisms, biosynthesis of free-form D-amino acids is catalysed by racemases with L-enantiomers as

substrates. Peptidyl D-amino acids arise either by taking free D-amino acid as substrate, as in cell wall synthesis (Vollmer *et al.*, 2008), or by L-to-D epimerization, as in non-ribosomal peptide synthesis (Challis & Naismith, 2004), in micro-organisms. In higher eukaryotic organisms, this process is infrequently catalysed by enzyme-driven post-translational isomerization (Takahashi *et al.*, 2008). Amino acid racemization can also occur at an accelerated rate by physical and/or chemical treatments. For example, racemization of L-lysine at elevated temperatures has great potential in the commercial process of D-lysine (D-Lys) production (Takahashi *et al.*, 1997).

Although D-amino acids are required for very specialized biological purposes, it is apparently not in the best interest of cells to maintain high concentrations of these rare amino acids. In the plant *Arabidopsis thaliana*, D-alanine (D-Ala)

Abbreviations: AST, arginine succinyltransferase; ATA, arginine transaminase; DADH, D-arginine dehydrogenase; INT, iodinitrotriazolium chloride; 2-KA, 2-ketoarginine; PMS, phenazine methosulfate.

and D-Ser exert a toxic effect on growth, and introducing a recombinant D-amino acid oxidase to plants not only alleviates the toxic effect but also promotes growth (Erikson *et al.*, 2004). In bacteria, D-Ala and D-glutamic acid (D-Glu), and in some cases D-Lys, are the natural ingredients of cell wall peptidoglycan. However, when they are provided in the growth medium, *Escherichia coli* is able to incorporate other D-amino acids into peptidoglycan, which results in severe alterations to cell wall structure (Caparros *et al.*, 1992). Therefore, organisms with the capacity to degrade D-amino acids should have an advantage in surviving these potentially adverse challenges.

The biochemistry of D-amino acid catabolism has not been intensively studied in comparison with those of L-amino acids, and therefore information is still fragmentary. In general, D-amino acids are metabolized either directly or after conversion into the L-enantiomers. Many racemases from different organisms have been reported. It is the general notion that amino acid racemases serve in the biosynthesis of D-amino acids by taking L-isomers as substrates. In bacteria, alanine racemase and glutamate racemase are essential enzymes due to their functions in the biosynthesis of D-Ala and D-Glu, and hence in cell wall synthesis. Most amino acid racemases require pyridoxal 5'-phosphate (PLP) as coenzyme, with a few exceptions. The structures and functions of the PLP-dependent and PLP-independent amino acid racemases have been clarified at the molecular level (Yoshimura & Esak, 2003).

Our group recently reported a new type of D- to L-arginine racemization by coupled dehydrogenases (Fig. 1b): an FAD-dependent catabolic D-arginine (D-Arg) dehydrogenase DauA and a NADH/NADPH-dependent anabolic L-arginine dehydrogenase DauB (Li & Lu, 2009). These two enzymes are encoded in the *dauBAR* operon, which is induced by exogenous D-Arg but not by L-Arg, and the conversion of D-Arg to L-Arg is the prerequisite for D-Arg utilization as the sole source of carbon and nitrogen through L-Arg catabolic pathways in *Pseudomonas aeruginosa*. This operon and its encoded enzymes are conserved among pseudomonads (Li & Lu, 2009) and many other related bacteria.

There are four catabolic pathways for L-Arg utilization in *P. aeruginosa* (Lu, 2006). The arginine decarboxylase pathway serves to supply putrescine, and hence spermidine, when arginine is abundant (Nakada & Itoh, 2003). The arginine deiminase pathway provides ATP under anaerobic conditions (Vander Wauven *et al.*, 1984), and its induction is mainly controlled by Anr, the homologue of *E. coli* Fnr, in response to oxygen status, as well as by ArgR, the L-Arg-responsive regulator, as auxiliary factor in the presence of L-Arg. The arginine succinyltransferase (AST) pathway is the major route of L-Arg utilization as the carbon and nitrogen source under aerobic conditions (Jann *et al.*, 1986), and its induction by L-Arg requires a functional ArgR (Fig. 1a). When the AST pathway is blocked, the arginine transaminase (ATA) pathway can still support cell

growth on L-Arg, but with a longer generation time (Yang & Lu, 2007). Induction of the ATA pathway is controlled by the AruSR two-component system (Fig. 1a), independent of ArgR. Mutants without AST and ATA pathways show no apparent growth on L-Arg as the sole source of carbon and nitrogen.

In this study, the enzymic properties of DauA, encoding a catabolic FAD-dependent D-amino acid dehydrogenase, and the physiological functions of the *dauBAR* genes were further characterized with a group of 19 D-amino acids. Regulation of the *dauBAR* operon was analysed by a combination of genetics and biochemical approaches with a variety of mutants and purified transcriptional regulators DauR and ArgR.

METHODS

Strains and growth conditions. Strains and plasmids used in the study are listed in Table 1. Luria-Bertani (LB) medium was used for strain construction with the following supplements as required: ampicillin at 100 µg ml⁻¹ (*E. coli*); carbenicillin at 100 µg ml⁻¹ (*P. aeruginosa*). Minimal medium P (MMP) (Haas *et al.*, 1977) was used for the growth of *P. aeruginosa* strains, with specific carbon (C) and nitrogen (N) sources as indicated (20 mM, unless specified otherwise).

Enzyme assays. DauA was purified as previously described (Li & Lu, 2009); the purified protein appeared as a mixture of monomer and dimer when analysed by SDS-PAGE under reducing conditions, which may be the result of non-covalent interactions mediated by the exposure of hydrophobic patches upon boiling (Shin *et al.*, 2002). The substrate specificity of DauA was measured by D-amino acid dehydrogenase activity by a published method (Li & Lu, 2009). A total of 18 D-amino acids was used, including D-phenylalanine (D-Phe), D-Lys, D-Glu, D-tryptophan (D-Trp), D-leucine (D-Leu), D-ornithine (D-Orn), D-asparagine (D-Asn), D-histidine (D-His), D-tyrosine (D-Tyr), D-Ser, D-glutamine (D-Gln), D-proline (D-Pro), D-methionine (D-Met), D-threonine (D-Thr), D-valine (D-Val), D-aspartic acid (D-Asp), D-Ala and D-Arg. D-Cysteine (D-Cys) was not included because the -SH group of D-Cys causes reduction of iodonitrotetrazolium chloride (INT). The reaction mixture contained 100 mM Tris/HCl (pH 8.7), 10 mM KCN, 0.2 mM FAD, 0.8 mM phenazine methosulfate (PMS), 0.8 mM INT, 2.05 µg DauA ml⁻¹, and 20 mM of the tested D-amino acid in a total volume of 0.5 ml (Li & Lu, 2009). The reaction was initiated by the addition of DauA, and was continued for 3 min at 37 °C before being stopped by 4 M HCl (50 µl). After addition of 5 µl Triton X-100 to dissolve the precipitates and appropriate dilution, A₅₀₀ readings were taken. The background reading with double-distilled H₂O substitution of D-amino acid in a parallel control reaction was subtracted from the readings with D-amino acids and the specific activities were calculated thereafter. One unit of D-amino acid dehydrogenase activity was defined as the amount of enzyme that led to the reduction of one nanomole of INT per minute under the standard assay conditions.

After initial screening, seven good substrates (D-Arg, D-Lys, D-Phe, D-Met, D-Orn, D-His and D-Tyr) were further analysed to determine the catalytic parameters. The assay was carried out with the same procedure as described above, except that different concentrations of D-amino acids were used. The final concentration of purified protein used in the reaction was 2.37 µg ml⁻¹. The *K_m*, *k_{cat}* and *k_{cat}/K_m* values were determined by non-linear regression data analysis using SigmaPlot 9.0.

Fig. 1. Arginine catabolic pathways in *P. aeruginosa* PAO1. (a) Relevant intermediates of the ATA pathway, the AST pathway and D- to L-arginine racemization are shown in the biochemical reactions, and genes involved in these pathways are displayed as transcriptional units. The genes encoding transcriptional factors are marked as grey arrows, and promoters subject to activation and repression are labelled as arrowheads and solid bars, respectively. (b) Proposed functions of DauA and DauB in D-Arg and D-Lys catabolism. PMS and INT are artificial electron acceptors in the assays for DauA, as described in Methods, while the physiological electron acceptor remains unknown. Also shown are spontaneous chemical reactions of the keto acids derived from D-Arg and D-Lys.

Measurements of β -galactosidase activity were conducted in strains carrying plasmid pCR1 (*dauB::lacZ*) to analyse the effect of exogenous D-amino acids on the *dauB* promoter activity. Cells were grown at 37 °C in MMP supplemented with 20 mM L-Glu in the absence or presence of 5 mM D-amino acids, as indicated. Cells in the exponential phase were harvested by centrifugation and washed once with 20 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.6) and resuspended in the same buffer plus 1 mM PMSF. Cells were broken with a French press at 17 000 p.s.i. (117 300 kPa), and soluble cell extracts were prepared after centrifugation at 16 000 g for

30 min. Protein concentration was determined by the Bradford method with BSA as standard (Bradford, 1976). For the measurements of β -galactosidase activity, ONPG was used as substrate (Miller, 1972).

Expression and purification of hexa-histidine-tagged DauR and ArgR. The structural genes *dauR* and *argR* were amplified by PCR from the genomic DNA of strain PAO1 using the following primer pairs: 5'-AGC CCG AGC CAA GAC CCC AGC-3' and 5'-CCC AAG CTT CAG TCG CCG CCT TCC TTC-3' for *dauR*; 5'-TCC CCC GGG

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description*	Source or reference
<i>E. coli</i> strains		
DH5 α	F ϕ 80 <i>dlac</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (r _K m _K) <i>supE44</i> λ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Bethesda Research Laboratories
TOP10	F [−] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>araA-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen
<i>P. aeruginosa</i> strains		
PAO1	Wild-type	Gallegos <i>et al.</i> (1997)
PAO5801	<i>dauB</i> ::Tc ^r derivative of PAO1	Nishijyo <i>et al.</i> (1998)
PAO5802	<i>dauA</i> ::Tc ^r derivative of PAO1	Haas <i>et al.</i> (1977)
PAO5803	<i>dauR</i> ::Tc ^r derivative of PAO1	Li & Lu (2009)
PAO501	<i>argR</i> ::Gm ^r	Park <i>et al.</i> (1997)
PAO5011	<i>dauB</i> ::Tc ^r derivative of PAO501	This study
PAO5012	<i>dauA</i> ::Tc ^r derivative of PAO501	This study
PAO5013	<i>dauR</i> ::Tc ^r derivative of PAO501	This study
PAO0315	<i>argG9</i> <i>Chl-2</i> derivative of PAO1	ATCC
PAO0362	<i>argH32</i> <i>Chl-2</i> derivative of PAO1	ATCC
PAO0372	<i>argH32</i> <i>Lys-58</i> <i>Chl-2</i> derivative of PAO1	ATCC
Plasmids		
pRTP2	Ap ^r Sm ^s conjugation vector derived from pRTP1 with deletion of <i>EcoRI</i> site	Li & Lu (2009)
pQF50	<i>bla lacZ</i> transcriptional fusion vector	Li & Lu (2009)
pQF52	<i>bla lacZ</i> translational fusion vector	Li & Lu (2009)
pBAD-His6	Vector modified from pBAD-HisA	Farinha & Kropinski (1990)
pCR1	<i>dauB</i> :: <i>lacZ</i> transcriptional fusion of pQF50	Park <i>et al.</i> (1997)
pCR3	For expression of His-tagged DauA	Li & Lu (2009)
pCR4	For expression of His-tagged DauR	This study
pCR5	For expression of His-tagged ArgR	This study
pZY1	<i>gbuA</i> :: <i>lacZ</i> fusion of pQF52	Li & Lu (2009)
pZY5	PA4980:: <i>lacZ</i> fusion of pQF52	Li & Lu (2009)

*Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Sm^s, streptomycin sensitivity.

ACT GCC CAA CCC CAA CGC ATC-3' and 5'-CCC AAG CTT CAG CCG CGT TCG ACG GGA GTG-3' for *argR*. The resulting PCR products were digested with *HindIII* (*dauR*) or *SmaI* plus *HindIII* (*argR*), which cut at unique restriction sites introduced by the primers, and cloned into the *SmaI* and *HindIII* sites of the expression vector pBAD-His6 (Li & Lu, 2009). The resulting plasmids, designated pCR4 and pCR5, were introduced into *E. coli* TOP10.

For overexpression of DauR, *E. coli* TOP10 containing pCR4 was grown in LB medium containing ampicillin (100 μ g ml^{−1}) at 37 °C until the OD₆₀₀ reached 0.5, at which point 0.2% arabinose (w/v, final concentration) was added. After induction for 3 h, the cells were harvested by centrifugation, resuspended in phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) plus 1 mM PMSF and ruptured by an Aminco French press at 17 000 p.s.i. (117 300 kPa). Cell debris was removed by centrifugation at 20 000 *g* for 1 h, and the resulting cell-free extract was further treated with streptomycin sulphate. After centrifugation, the resulting supernatant was applied to a HisTrap HP column (GE Healthcare) equilibrated with the above phosphate buffer. After washing off the unbound proteins, His-tagged DauR was eluted with a linear gradient of 20–500 mM imidazole over 10 column volumes. Fractions containing DauR, as indicated by SDS-PAGE, were pooled, desalted by an Aminco Ultra-15 centrifugal filter unit (molecular mass cutoff 10 kDa, Millipore) and further purified by anion-

exchange chromatography through a Mono Q HR5/5 column (GE Healthcare) equilibrated with 20 mM Tris/HCl (pH 7.6; buffer A). The DauR protein was eluted by a linear gradient of 0–1 M KCl in buffer A over 20 column volumes. Target fractions that were homogeneous, as determined by visual inspection after SDS-PAGE, were pooled, desalted and concentrated using an Aminco Ultra-15 centrifugal filter unit (molecular mass cutoff 10 kDa, Millipore). The protein concentration in the final preparation was determined by the Bradford method with BSA as standard.

For overexpression of ArgR, *E. coli* TOP10 containing pCR5 was grown in LB medium containing ampicillin (100 μ g ml^{−1}) at 30 °C. The steps described above were employed to prepare protein samples. After being applied to a HisTrap HP column (GE Healthcare), His-tagged ArgR was eluted out at 300 mM imidazole, as indicated by SDS-PAGE. Fractions with the desired purity were pooled and concentrated as described above.

Gel retardation analysis. The regulatory region of *dauB* was PCR-amplified from plasmid pCR1 with the following primers: 5'-TAC AAG CTT CTG TAC AAC CTG GTG GCG CAG-3' and 5'-AAA CAA TTT GTC CAT TCT GGA CTT TAA GGT-3'. Probe *dauB*-Mu containing the same region with mutations on the predicted DauR operator site (from GTCCAN₃TGGAC to GTGGAN₃TCCAC) was amplified with the same forward primer and the following reverse

primer: 5'-AAA CAA TTT GTG GAT TCT CCA CTT TAA GGT-3'. The resulting PCR products of 602 bp were purified by gel extraction and used as probes in gel retardation analysis.

The binding reactions were carried out as follows. The *dauB* or *dauB-Mu* DNA probe was allowed to interact with different concentrations of DauR or ArgR in a mixture of 20 μ l containing 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 5% (v/v) glycerol, 200 μ g BSA ml^{-1} and 4 mM DTT. When indicated, 10 mM of specified compounds [2-ketoarginine (2-KA), L-Arg or D-Arg] was added to the reaction to analyse their potential effects on nucleoprotein complex formation. Reaction mixtures were incubated for 20 min at room temperature and an aliquot of 10 μ l was loaded on a 6% polyacrylamide gel while the gel was running. The gel was stained with SYBR Green I (1:10 000 dilution, Invitrogen) for 20 min, washed twice with deionized H_2O , and scanned by an Omega 10GD Imaging System (UltraLum) with a setting for excitation at 473 nm and emission at 520 nm.

RESULTS

Substrate specificity and catalytic parameters of DauA

We have recently reported that purified DauA possesses a D-arginine dehydrogenase (DADH) activity (Li & Lu, 2009). Fig. 2 summarizes the substrate specificity of DauA. With a final concentration of 20 mM in the reaction, several D-amino acids including D-Lys, D-Met, D-Phe, D-Tyr, D-Orn and D-His served as good substrates in addition to D-Arg. In fact, DauA even exhibited a higher activity towards D-Lys than towards D-Arg, which is due to a strong substrate inhibition effect of D-Arg at high concentrations as described below. In comparison, DauA showed very low

or undetectable activity toward D-Thr, D-Asn, D-Glu, D-Gln, D-Ala, D-Asp and D-Ser.

The k_{cat} , K_m , K_i (if applicable) and k_{cat}/K_m values of the seven good substrates as mentioned above were determined as shown in Table 2. It was apparent that k_{cat}/K_m values (Koshland, 2002) of D-Arg and D-Lys are far better than those of the other substrates. In addition, substrate inhibition was observed for D-Arg and D-Lys, with the former ($K_i=24.1$ mM) exerting a much stronger effect than the latter ($K_i=150.1$ mM). No substrate inhibition was observed for the other five D-amino acids.

Effects of D-amino acids on the *dauB* promoter activity

Since DauA possesses broad substrate specificity, the effect of exogenous D-amino acids on expression of the *dauBAR* operon was analysed in strain PAO1 carrying pCR1 (*dauB::lacZ*) grown overnight in glutamate minimal medium with or without D-amino acid supplements at 5 mM. Among the 19 D-amino acids tested, only D-Arg and D-Lys exerted 4.2-fold and 2.9-fold induction effects, respectively, while the other D-amino acids exhibited either no apparent effects or even inhibition effects (data not shown).

Effects of *dauA* and *dauB* on D-amino acid utilization

The physiological functions of *dauA* and *dauB* in D-amino acid utilization were analysed by comparing the growth phenotypes of PAO5801 (*dauB*) and PAO5802 (*dauA*) with

Fig. 2. Substrate specificity of DauA. The substrate specificity of DauA was determined from the D-amino acid dehydrogenase activity of the purified enzyme. The relative activity towards each D-amino acid was calculated with respect to the activity towards D-Arg, which was taken as 100%.

Table 2. Catalytic parameters of DauA

Values represent the average and range of two measurements.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	K_i (mM)
D-Arginine	11.1 ± 0.7	0.08 ± 0.02	1.5×10^5	24.1 ± 7.1
D-Lysine	9.2 ± 0.3	0.19 ± 0.02	4.8×10^4	150.1 ± 53.0
D-Methionine	6.8 ± 0.3	1.43 ± 0.24	4.8×10^3	
D-Ornithine	6.2 ± 0.1	1.48 ± 0.13	4.2×10^3	
D-Phenylalanine	5.3 ± 0.2	1.13 ± 0.14	4.7×10^3	
D-Tyrosine	3.6 ± 0.1	0.40 ± 0.07	9.1×10^3	
D-Histidine	3.6 ± 0.1	3.52 ± 0.15	1.0×10^3	

that of the parental strain PAO1 on D-amino acids, as listed in Table 3. PAO1 was able to grow on D-Arg/Lys and several other D-amino acids as sole nitrogen source, but only D-Arg/Glu/Gln/Ala can support its growth efficiently as the sole source of carbon and nitrogen. A lesion in *dauA* or *dauB* abolished utilization of D-Arg and D-Lys specifically. Without a functional *dauA*, PAO5802 lost

the capability to grow on these two D-amino acids as the sole nitrogen source. PAO5801 (*dauB*) grew similarly to the parental strain PAO1 on D-Arg or D-Lys as sole nitrogen source, but its growth on D-Arg as carbon source was abolished. Consistent with the induction effect of exogenous D-Arg and D-Lys on the *dauB* promoter, these results support the physiological functions of DauA and

Table 3. Growth phenotype of *dauA* and *dauB* mutants on D-amino acids

Cultures were inoculated with fresh cells with a starting OD₆₀₀ of 0.02, and growth was continued for 4 days before measurements of final OD₆₀₀. –, OD₆₀₀ < 0.1; +, 0.1 ≤ OD₆₀₀ < 0.2; ++, 0.2 ≤ OD₆₀₀ < 0.6; +++, OD₆₀₀ ≥ 0.6.

D-Amino acid	PAO1 (wild-type)		PAO5801 (<i>dauB</i>)		PAO5802 (<i>dauA</i>)	
	N source*	C&N source†	N source	C&N source	N source	C&N source
D-Arginine	+++	++	+++	–	–	–
D-Lysine	+++	–	+++	–	+	–
D-Glutamic acid	+++	++	+++	++	+++	++
D-Glutamine	+++	++	+++	++	+++	++
D-Alanine	+++	++	+++	++	+++	++
D-Asparagine	+++	+	+++	–	+++	–
D-Proline	+++	–	+++	–	+++	–
D-Threonine	+++	–	+++	–	+++	–
D-Ornithine	++	–	+++	–	++	–
D-Histidine	++	–	+++	–	++	–
D-Serine	++	–	+++	–	+++	–
D-Phenylalanine	+	–	+++	+	++	–
D-Valine	+++	+	+++	+++	+++	+++
D-Aspartic acid	–	–	–	–	–	–
D-Cysteine	–	–	–	–	–	–
D-Leucine	–	–	–	–	+	–
D-Methionine	–	–	–	–	+	–
D-Tryptophan	–	–	–	–	–	–
D-Tyrosine	–	–	–	–	–	–

*N source: cells were grown in glucose (20 mM) MMP with 5 mM D-amino acids as nitrogen source.

†C&N source: cells were grown in MMP with 10 mM D-amino acids (5 mM for D-tyrosine) as carbon and nitrogen source.

DauB on D-Arg and D-Lys catabolism. In contrast, the other five D-amino acids that were considered to be good substrates of DauA cannot induce the *dauB* promoter. Except for D-Arg and D-Lys, mutations in the *dauBAR* operon did not abolish utilization of the other D-amino acids tested in this study. Moreover, we noticed that utilization of D-Phe as nitrogen source and D-Val as carbon source was greatly enhanced in the *dauA* and *dauB* mutants. This suggests potential interactions of DauA/DauB with enzymes yet to be identified for D-Phe/Val utilization.

Growth complementation tests of arginine and lysine auxotrophs by D-Arg and D-Lys

The physiological function of DauA and DauB in D-to-L racemization of arginine was supported by growth complementation of an arginine auxotroph (a lesion in *argB*) by D-Arg in our previous report (Li & Lu, 2009). Here we employed two more arginine auxotrophs (PAO0315 and PAO0362) and one arginine/lysine auxotroph (PAO0372) to test growth complementation by D-Arg and D-Lys. Consistent with the previous results, arginine auxotrophy can be complemented by either L-Arg or D-Arg (Li & Lu, 2009). In contrast, lysine auxotrophy can only be complemented by L-Lys but not D-Lys. Since exogenous D-Arg/Lys were both able to induce expression of *dauBA* as described above, the lack of growth complementation by D-Lys would therefore indicate the absence of D-to-L racemization of lysine through coupled DauA/DauB reactions.

Effects of *dauBAR* on *dauB* promoter activity

Expression of the *dauB* promoter was analysed by measurement of β -galactosidase activity in the *dau* mutants harbouring pCR1 (*dauB::lacZ*). As shown in Table 4, mutations of these *dau* genes exerted different effects on the *dauB* promoter activity in response to exogenous D-

Arg. In PAO1, the promoter activity and DADH activity were induced by 22.2-fold and 27-fold, respectively, when 5 mM D-Arg was included in the growth medium. In the *dauA* mutant, the induction effect of D-Arg on the promoter activity was completely abolished. Surprisingly, in the *dauB* mutant the promoter was still inducible by D-Arg, but to a lesser extent, although the DADH level showed no induction. These results suggest that 2-KA, the catalytic product of DauA on D-Arg, but not D-Arg per se, serves as an internal signal of *dauBAR* induction by exogenous D-Arg. Consistent with the proposed regulatory function of DauR, the basal level of the *dauB* promoter activity in the *dauR* mutant was 5.7-fold higher than that in PAO1; however, exogenous D-Arg and L-Arg did further induce the promoter activity by 2.4-fold and 3.2-fold, respectively. This pattern of promoter activity was also reflected in the levels of DADH activity: the basal level of DADH in the *dauR* mutant was 8.1-fold higher than that in PAO1, and exogenous D-Arg and L-Arg further induced promoter activity by about 2.8- and 3.3-fold, respectively. The induction effects of D-Arg and L-Arg on the promoter activity and DADH activity in *dauR* mutant suggested regulator(s) other than DauR in regulation of the *dauBAR* operon, and L-Arg (the racemization product of D-Arg by DauAB) might be the signal.

Participation of ArgR in regulation of the *dauBAR* operon

The potential effect of ArgR (the L-Arg-responsive regulator) in control of the *dauBAR* operon was analysed in *dau* mutants derived from PAO501 (*argR*) and the results are shown in Table 4. In comparison with PAO1, the induction effect of D-Arg on promoter activity and DADH activity was much lower in the *argR* mutant, suggesting the involvement of ArgR in *dauBAR* regulation. Consistent with the prediction of L-Arg as the second signal compound in *dauBAR* expression, the induction effect of D-Arg and L-Arg in the *dauR* mutant was completely

Table 4. Effect of *dauBAR* and *argR* on regulation of *dauB* promoter activity

Strain (genotype)	pCR1 (<i>dauB::lacZ</i>)*			DADH*		
	Glu	Glu + D-Arg	Glu + L-Arg	Glu	Glu + D-Arg	Glu + L-Arg
PAO1 (wild-type)	35	776	68	49	1323	106
PAO5801 (<i>dauB</i>)	35	233	149	27	35	36
PAO5802 (<i>dauA</i>)	13	33	56	ND	ND	ND
PAO5803 (<i>dauR</i>)	198	479	632	398	1097	1318
PAO501 (<i>argR</i>)	26	190	49	29	393	57
PAO5011 (<i>dauB argR</i>)	20	181	67	32	33	29
PAO5012 (<i>dauA argR</i>)	10	23	17	ND	ND	ND
PAO5013 (<i>dauR argR</i>)	150	163	192	290	328	342

*Specific activity (nmol min⁻¹ mg⁻¹): strains harbouring pCR1 were grown in MMP supplemented with 20 mM Glu plus 5 mM D-Arg or L-Arg. *dauB* promoter activity was determined with ONPG as the substrate and measurement of A₄₂₀. DADH activity was determined with D-Arg at 20 mM as the substrate and measurement of A₅₀₀. ND, Not detectable.

abolished in the *dauR argR* mutant. The function of L-Arg/ArgR in *dauBAR* regulation was further supported by the observation that the promoter activity can be further induced in the *dauB* mutant (in which the D-to-L racemization is blocked) when L-Arg is added to the growth medium in addition to D-Arg (233 versus 571 nmol min⁻¹ mg⁻¹). However, this additive effect of L-Arg did not exist in the *dauB argR* mutant (181 versus 150 nmol min⁻¹ mg⁻¹).

Binding of DauR and ArgR to the regulatory region of *dauB*

To further support the hypothesis of DauR and ArgR as transcriptional regulators of the *dauBAR* operon, gel

retardation experiments were carried out with the purified recombinant His-tagged DauR or ArgR protein and DNA fragments carrying the regulatory region of *dauB*. As shown in Fig. 3(b), DauR interacts specifically with the regulatory region of *dauB*, as evidenced by the formation of nucleoprotein complexes with slower mobility. When 2-KA at 10 mM was added in the reaction, an inhibitory effect on nucleoprotein complex formation was observed, while addition of D-Arg or L-Arg showed no apparent effect. These results supported the predicted function of 2-KA as a signal compound for *dauBAR* expression through DauR.

Through genome comparison, a highly conserved palindromic sequence was identified in the *dauB* regulatory region of *P. aeruginosa*, *Pseudomonas fluorescens* and

Fig. 3. Gel retardation assays of DauR and ArgR with the *dauB* regulatory region. (a) Electromobility shift analysis of *dauB* and *dauB-Mu* probes with recombinant His-tagged DauR. The *dauB* and *dauB-Mu* probes have identical sequences except for the DauR operator site (GTCCAGAATGGAC for probe *dauB* and GTGGAGAATCCAC for probe *dauB-Mu*). These probes at 0.27 nM were titrated with DauR at the indicated concentrations. (b) Effect of different compounds on DauR-*dauB* interactions. The *dauB* probe at 0.13 nM was added to the reaction at the indicated concentrations of DauR in the absence or presence of 2-KA, D-Arg and L-Arg at 10 mM. (c) Effect of different compounds on ArgR-*dauB* interactions. The *dauB* probe at 0.07 nM was allowed to interact with ArgR at the indicated concentrations in the absence or presence of the same set of compounds as in panel (b).

Pseudomonas mendocina (Fig. 4b). We propose this sequence to be the putative DauR operator site. To test this hypothesis, mutations on the proposed operator site in PAO1 were introduced, and this DNA probe (*dauB*-Mu of Fig. 3a) carrying the mutated operator site was unable to form a nucleoprotein complex with DauR.

The results of genetic studies strongly suggested that ArgR is a transcriptional activator of the *dauBAR* operon in response to the presence of L-Arg. In addition, sequence analysis of the *dauB* regulatory region identified a putative ArgR binding site (Fig. 4c). Binding of ArgR to the regulatory region of *dauB* was further demonstrated by electromobility shift analysis as shown in Fig. 3(c), and formation of ArgR-dependent nucleoprotein complex was not affected by the presence of 2-KA, L-Arg or D-Arg.

Effects of D-Arg on the ATA pathway

To serve as a carbon source, D-Arg needs to be converted into L-Arg through DauA and DauB (Li & Lu, 2009) before being channelled into the AST or ATA pathway (Fig. 1). We have reported that genes of the AST pathway are induced by exogenous D-Arg, and this induction effect of D-Arg requires a complete D-to-L racemization. In contrast, D-Arg did not induce genes of the ATA pathway, except *gbuA*, encoding 4-guanidinobutyrase (Fig. 1a). To further substantiate this finding, the promoter activities of *gbuA* and PA4980 (which drives transcription of *aruH* and *aruI* in the ATA pathway) were analysed in PAO1 and *dau* mutants in response to exogenous D-Arg and L-Arg.

As shown in Table 5, the promoter activity of PA4980 was significantly induced by exogenous L-Arg, while the effect of exogenous D-Arg was barely detectable in PAO1, and this pattern of expression was not affected by mutations in the *dauBAR* genes. In contrast, the *gbuA* promoter in PAO1 was induced by exogenous D-Arg but not by L-Arg, and this induction effect was completely abolished in the *dauA* mutant but sustained in the *dauB* mutant. These results were consistent with the previous observations with DNA microarrays (Li & Lu, 2009), and their implications for the regulatory mechanisms of PA4980 and *gbuA* promoters are discussed below.

DISCUSSION

The *dauBAR* operon is designated for D-Arg and D-Lys catabolism

The *dauBAR* operon has been recently reported to participate in a unique D-to-L racemization of arginine by the coupled arginine dehydrogenases DauA and DauB (Li & Lu, 2009). In this study, the enzymic properties of DauA, encoding a catabolic FAD-dependent D-amino acid dehydrogenase, and the physiological functions of the *dauBAR* genes were further characterized with a group of 19 D-amino acids. The substrate specificity assay with the purified His-tagged DauA clearly demonstrated DauA to be a D-amino acid dehydrogenase of broad substrate specificity, with D-Arg and D-Lys being the two most

Fig. 4. ArgR and DauR binding sites in the *dauB* regulatory region. (a) Nucleotide sequence of the intergenic region between the ATG initiation codon of *dauB* and the TGA stop codon of its upstream *rhl* gene. The proposed -10 and -35 regions of the *dauB* promoter, the ArgR binding site and the DauR binding site are labelled. Also shown, by divergent arrows, is a putative rho-independent transcriptional terminator. (b) Multiple sequence alignment of DauR operators in the *dauB* regulatory regions of pseudomonads. The putative DauR operator sites are highlighted, followed by the number of nucleotides before the ATG initiation codon of *dauB*. PAO1, *P. aeruginosa* PAO1; PF5, *P. fluorescens* Pf-5; Pmen, *P. mendocina* ymp. (c) Conservation of the ArgR binding site in the *dauB* regulatory region. Nucleotides identical to those of the consensus sites are shown in bold type. The nucleotide sequence shown here corresponds to the complementary strand to that shown in panel (a).

Table 5. Effect of L-Arg and D-Arg on PA4980 and *gbuA* promoter activity

Strain (genotype)	pZY5 (PA4980::lacZ)*			pZY1 (<i>gbuA</i> ::lacZ)*		
	Glu	Glu + D-Arg	Glu + L-Arg	Glu	Glu + D-Arg	Glu + L-Arg
PAO1 (wild-type)	2269	2260	23 364	12	105	12
PAO5801 (<i>dauB</i>)	2236	3607	13 657	8	229	10
PAO5802 (<i>dauA</i>)	2051	3628	15 954	7	11	9

*Strains harbouring pZY5 or pZY1 were grown in MMP supplemented with 20 mM Glu plus 5 mM D-Arg or 20 mM L-Arg. The promoter activities were determined with ONPG as the substrate and measurement of A_{420} . The results [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] are the average of two measurements with SD lower than 5 %.

effective substrates based on the kinetic parameters (Fig. 2, Table 2). In addition, *dauB* promoter activity measurements with different D-amino acids suggested that expression of *dauBAR* is specifically induced by exogenous D-Arg and D-Lys. The physiological functions of *dauBAR* in D-Arg and D-Lys catabolism were further supported by growth phenotype analysis, which demonstrated that mutations in *dauBAR* affect utilization of these two amino acids alone (Table 3). Therefore, we conclude that the *dauBAR* operon, which is conserved in pseudomonads, functions in D-Arg and D-Lys catabolism.

D-Lys catabolism

P. aeruginosa PAO1 growing on D-Lys as the sole nitrogen source requires DauA. This is supported by the fact that in the *dauA* mutant, utilization of D-Lys as the sole nitrogen source was almost abolished (Table 3). DauA catalyses oxidative deamination of D-Lys to ammonia and 2-ketolysine (Fig. 1b). Non-enzymically, 2-ketolysine is in equilibrium with cyclic Δ^1 -piperidine-2-carboxylate predominantly, and possibly with other compounds (Friede & Henderson, 1976; Kamio *et al.*, 2009). While *Pseudomonas putida* has been proposed to possess enzymes for subsequent degradation of Δ^1 -piperidine-2-carboxylate in D-Lys catabolism as carbon source (Revelles *et al.*, 2007), these enzymes seem to be missing in *P. aeruginosa* PAO1, as D-Lys cannot be used as sole carbon source in this organism. Moreover, since D-Lys does not complement the L-Lys requirement of a lysine auxotroph, it seems to have no D-to-L lysine racemization, either by coupled DauA/DauB reactions or by the traditional racemases.

D-Arg catabolism

In *P. aeruginosa* PAO1, D-Arg is capable of serving as the sole source of carbon and nitrogen. To be utilized as carbon source, D-Arg needs to be converted into L-Arg by DauA and DauB before being channelled into a complicated catabolic network, which is composed of several different pathways that are conserved among pseudomonads (Lu, 2006). With a lesion in *dauA* or *dauB*, the mutant strains failed completely to utilize D-Arg as the sole source of carbon (Table 3). D-to-L racemization of arginine

is also evidenced by growth complementation of arginine auxotrophs with D-Arg.

Although racemization is also blocked in the *dauB* mutant, D-Arg is still able to serve as the sole source of nitrogen due to the release of ammonia from DauA-catalysed oxidative deamination; however, 2-KA as a second product from this reaction apparently cannot serve as the carbon source in a *dauB* mutant. This observation is somewhat contradictory to a report that PAO1 is able to grow on exogenous 2-KA as sole source of carbon and nitrogen (Jann *et al.*, 1988). Perhaps this discrepancy may depend on the supply of 2-KA. One possibility is that DauB facilitates electron transport of FADH₂ in DauA and hence recycles the enzymic activity of DauA. Hence, in the *dauB* mutant, without a functional DauB to recycle the enzymic activity of DauA, the production of 2-KA is so low that it cannot support cell growth. The other possibility may relate to instability of 2-KA inside the cells. Like other keto acids, 2-KA is liable to degrade into 4-guanidinobutyrate (4-GBA) in the presence of H₂O₂ (Li & Lu, 2009; Vlessis *et al.*, 1990), which is abundant in actively growing cells under aerobic conditions. The latter hypothesis also explains another obscure observation that among genes of the ATA pathway for L-Arg catabolism, only *gbuA*, encoding guanidinobutyrase of the ATA pathway, was induced by D-Arg. It has been reported that 4-GBA serves as an inducer signal of *gbuA* (Nakada & Itoh, 2002). As shown in Table 5, D-Arg per se does not induce *gbuA*; it is 2-KA, and most likely 4-GBA derived from 2-KA degradation, that causes the observed induction.

Why is D-Arg inefficient in the induction of the ATA pathway?

We have reported that exogenous D-Arg and L-Arg are similar in induction of the *aruCFGBDE* operon of the AST pathway (Li & Lu, 2009). In contrast, only exogenous L-Arg, but not D-Arg, was able to induce the PA4980 promoter, and hence *aruHI* of the ATA pathway (Table 5). Once transported into the cells, D-Arg is converted into L-Arg by DauA and DauB, but apparently the increment of L-Arg through this process has a dramatic discrepancy in induction of two L-Arg catabolic pathways. We considered

that this discrepancy may be due to the nature of two regulatory mechanisms. For the *aru* operon (AST pathway), induction by L-Arg requires a functional ArgR (Park *et al.*, 1997), a transcriptional regulator of the AraC family that most likely resides in the cytoplasm. For *aruHI* and the PA4980 promoter (ATA pathway), induction by L-Arg is controlled by the AruS/AruR two-component system (Yang & Lu, 2007), with the sensor AruS anchored on the inner membrane and the response regulator AruR inside the cells, based on sequence analysis. Therefore, it is conceivable that the increment of intracellular L-Arg through D-Arg racemization inside the cells can be detected by ArgR but is completely missed by AruSR.

Functions of DauR and ArgR on *dauBAR* regulation

Expression of the *dauBAR* operon is subject to control by two transcriptional regulators, DauR as repressor and ArgR as activator. Positions of DauR and ArgR operator sites relative to the putative σ^{70} -dependent *dauB* promoter are shown in Fig. 4(a). Consistent with the proposed functions, the DauR binding site overlaps completely with the -10 region, and the ArgR binding site is located upstream of the -35 region. Primary sequence analysis revealed features of DauR as a transcriptional regulator. Specifically, the N terminus of DauR encloses an YheO-like PAS domain and the C terminus has a helix–turn–helix FIS domain which comprises a three-helix bundle motif, generally found in site-specific recombinases (Yang & Steitz, 1995). Through multiple sequence alignment, the putative DauR operator sites were identified by genome comparison and confirmed by mutagenesis analysis in this study. The consensus DauR binding site (5′-GTCCAN₃TGGAC-3′) consists of two half-sites with a palindrome arrangement. The first half-site sequences are relatively more conserved than the second half-site sequences.

In this study, we also established 2-KA, instead of D-Arg, to be the signal compound that interacts with DauR to alleviate its repression effect. Participation of 2-KA in control of *dauBAR* operon was supported by the finding that exogenous 2-KA or a *dauR* lesion increases the basal level of the *dauB* promoter (Table 4). *In vitro*, inclusion of 2-KA tends to inhibit the DauR–DNA interactions (Fig. 3b). Once D-Arg was successfully converted into L-Arg by DauAB-dependent racemization inside the cells, the ArgR regulon was turned on in response to the elevated intracellular L-Arg pool, as revealed by transcriptome analysis (Li & Lu, 2009).

ArgR, the arginine-responsive regulator protein, is auto-induced from the *aot-argR* operon for arginine uptake and regulation in *P. aeruginosa* (Nishijyo *et al.*, 1998). ArgR has been found to participate in the regulation of a wide range of genes in arginine catabolic and anabolic metabolism. In the present study, the participation of ArgR in *dauBAR* operon regulation was demonstrated by *dauB* promoter activity measurement as well as electromobility shift

analysis. The function of L-Arg as a signal compound for ArgR regulation was observed in the analysis of promoter activity. However, L-Arg did not affect the *in vitro* binding of ArgR to the *dauB* probe in the electromobility shift analysis, which has been shown to be the case for other ArgR operators in earlier studies (Park *et al.*, 1997; Nishijyo *et al.*, 1998). The other two compounds tested (D-Arg and 2-KA) also showed no effect on the binding of ArgR to the *dauB* operator. The ArgR protein of *P. aeruginosa* belongs to the AraC/XylS family of transcriptional regulators (Nishijyo *et al.*, 1998), and therefore it is likely that binding of the signal ligand (L-Arg) induces a conformational change of ArgR for its interactions with the RNA polymerase in promoter activation. In summary, this study established that optimal induction of the *dauBAR* operon requires relief of DauR repression by 2-KA and activation of ArgR by L-Arg as a result of D-Arg racemization by the encoded DauA and DauB arginine dehydrogenases.

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