Functional characterization of the \textit{dguRABC} locus for d-Glu and d-Gln utilization in \textit{Pseudomonas aeruginosa} PAO1

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\textbf{INTRODUCTION}

D-Amino acids are naturally synthesized in all living organisms to serve in a variety of specific functions. Examples include the well-known D-Ala and D-Glu as essential components of the bacterial cell wall. Recently, D-amino acids have been reported to control growth phase-dependent cell wall remodelling (Lam \textit{et al.}, 2009) and to trigger biofilm disassembly (Kolodkin-Gal \textit{et al.}, 2010). Therefore, it is important to understand how bacterial cells regulate D-amino acid homeostasis.

In general, free D-amino acids are generated from their cognate L-enantiomers by amino acid racemases, e.g. MurI for Glu racemase (Doublet \textit{et al.}, 1993), and Alr and DadX for Ala racemases (Galakatos \textit{et al.}, 1986; Wild \textit{et al.}, 1985). Recently, we reported D-to-L racemization of Arg in \textit{Pseudomonas aeruginosa} through a pair of enzymes – catabolic D-Arg dehydrogenase DauA and anabolic L-Arg dehydrogenase DauB (Li & Lu, 2009a). Although DauA was able to utilize D-Lys and many other D-amino acids as substrate (Fu \textit{et al.}, 2010; Li \textit{et al.}, 2010), the racemization function of DauAB was limited to Arg only, most likely due to a very restricted substrate specificity of DauB. Although \textit{Pseudomonas putida} does not possess the \textit{dauBA} operon in its genome, a unique periplasmic racemase was reported in this group of bacteria to utilize basic amino acids (Lys, Arg and Orn) as preferred substrates (Wu \textit{et al.}, 2012).

When in excess, many D-amino acids can be utilized by bacteria as sources of carbon or nitrogen. Taking \textit{P. aeruginosa} PAO1 as the model organism, it was found that this bacterium could grow efficiently on the following four...
D-amino acids as the sole source of carbon and nitrogen: D-Ala, D-Arg, D-Glu and D-Gln. Our group has reported characterization of two FAD-dependent D-amino acid dehydrogenases (DadA and DauA) that are essential for D-Ala and D-Arg catabolism (He et al., 2011; Li et al., 2010). Although these two enzymes exhibit broad substrate specificity \textit{in vitro}, neither DadA nor DauA showed activities on D-Glu and D-Gln. As growth on D-Glu and D-Gln was not affected in the dadA and dauA mutants, the presence of additional enzymes of this family was proposed to support growth on these two D-amino acids.

In this study, DNA microarrays were employed to identify the \textit{dguR–dguABC} locus for D-Glu utilization. Through several lines of genetic and biochemical evidence, we reported DguA as a new member of the FAD-dependent D-amino acid dehydrogenase family. Regulation of the \textit{dguABC} operon by the DguR transcriptional activator in response to D-Glu was demonstrated. In addition, the AatJMP transporter for acidic amino acid uptake (Singh & Röhm, 2008; Sonawane et al., 2003) was shown to be inducible by D-Glu and essential for growth on this amino acid.

METHODS

Strains and growth conditions. Bacterial strains used in the study included \emph{Escherichia coli} DH5\textsubscript{x} and Top10 (Invitrogen), and \textit{P. aeruginosa} PA01. Mutants derived from PA01 were constructed as described below or acquired from the transposon mutant library at the University of Washington. Luria–Bertani (LB) medium was routinely used for bacterial growth with the following supplements as required: ampicillin (100 µg ml\textsuperscript{-1}) for \emph{E. coli}, and cabenecillin (100 µg ml\textsuperscript{-1}), streptomycin (400 µg ml\textsuperscript{-1}) and gentamicin (100 µg ml\textsuperscript{-1}) for \textit{P. aeruginosa}. Minimal medium P (Haas et al., 1977) was used for the growth of \textit{P. aeruginosa}, supplemented with specific carbon and nitrogen sources as indicated.

RNA preparation and DNA microarray analyses. Two independent sets of \textit{P. aeruginosa} PA01 cultures were grown aerobically in pyruvate minimal medium P in the presence of 10 mM L-Arg, L-Glu or d-Glu. The cells were harvested at OD\textsubscript{600} 0.5–0.6 by centrifugation for 5 min at 4 °C. Standard protocols for RNA isolation (Qiagen) and sample preparations of GeneChip (Affymetrix) were followed according the manufacturers’ suggestions. Data were processed by Microarray Suite 5.0 software, normalizing the absolute expression signal values of all chips to a target intensity of 500. Only genes showing consistent expression profiles in duplicates were selected for further analysis. Processed microarray data files have been deposited in the Gene Expression Omnibus database under access number GSE46603.

Construction of knockout mutants. The protocol for gene replacement and excision by the Flp–FRT (Flp recombinase target) recombination system (Hoang et al., 1998) was used to generate unmarked \textit{dguRABC} mutants of PA01. In general, the flanking regions of the intended knockout gene were amplified by PCR, and the restriction enzyme sites were introduced into the primers so that the PCR products possessed configurations of 5’-BamHI-[left arm]-Sad-3’ and 5’-Sad-[right arm]-HindIII-3’. After restriction enzyme digestions, these two DNA fragments were ligated into the BamHI and HindIII sites of pRTP2 (Li & Lu, 2009b). The FRT-Gm\textsuperscript{R}–FRT cassette cut from pPS856 (Hoang et al., 1998) was Sad digestion was inserted into the two arms with the same site. The final plasmid construct was introduced into \emph{E. coli} SM10 to serve as the donor in the biparental conjugation, with a spontaneous streptomycin-resistant mutant of PA01 as recipient. Expected deletions in the resulting mutants were confirmed by PCR.

Construction of \textit{PdguA::lacZ} fusions. For the construction of \textit{lacZ} fusions, the regulatory regions of \textit{dguA} were amplified by PCR with specific primers from the genomic DNA of \textit{P. aeruginosa} PA01. After restriction digestion of the purified PCR products, these DNA fragments were cloned into the corresponding restriction sites of pQF50 (Farinha & Kropinski, 1990) before they were transformed into \emph{E. coli} DH5\textsubscript{x}. The positive clones were selected on LB plates containing ampicillin and X-Gal. The nucleotide sequence of the inserts was confirmed by DNA sequencing.

Expression of DguA and DguR proteins in \textit{E. coli}. The structural genes of \textit{dguA} and \textit{dguR} were amplified by PCR from the genomic DNA of \textit{P. aeruginosa} PA01 using the following primer pairs: 5’-TCCCTCCGCGCTCACCGGCTTGGCA-3’/5’-CCCAAGTCTTCGACGCA-GCCGCGCGG-3’ and 5’−AACGTCGGCCATCATGGAA3’/ 5’−GGCAATATCCATCAGCTCAAGCCGCGGCAA3’, respectively. The resulting PCR products were digested with proper enzymes and cloned into the expression vector pBAD-HisD (Li & Lu, 2009a) so that the N terminus of DguA and DguR was fused in-frame with the His\textsubscript{6}-tag preceded by a ribosome-binding site and an arabinoside-inducible promoter in the plasmid. The resulting plasmids, pDGUA and pDGRU, were introduced into \emph{E. coli} Top10 (Invitrogen). For overexpression of DguA and DguR proteins, the recombinant strains of \emph{E. coli} were grown in LB medium containing ampicillin at 30 °C until OD\textsubscript{600} 0.5, at which point 0.2 % arabinose (w/v; final concentration) was added to the culture for induction. Culture growth was continued for another 3 h under the same conditions before harvesting by centrifugation.

Purification of His\textsubscript{6}-tagged DguA and DguR. The cell pellets were suspended in phosphate buffer A (20 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 M NaCl, 20 mM imidazole, pH 7.5) with PMSF (1 mM) as a protease inhibitor and the cells were ruptured by an Aminco French press. Cell debris was removed by centrifugation at 20 000 g for 30 min. The supernatant was applied to a HisTrap HP column (GE Healthcare) equilibrated with the same buffer. After the unbound proteins were washed off with equilibration buffer, His-tagged proteins were eluted at 50 % of buffer B (20 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 M NaCl, 1 M imidazole, pH 7.5). The target fractions were pooled together and concentrated using an Aminco Ultra-15 centrifugal filter unit (molecular mass cut-off, 10 kDa; Millipore) to change the buffer to 20 mM Tris/HCl (pH 7.5).

Enzyme assays. For the measurements of D-amino acid dehydrogenase activity of DguA, the method described previously (Li et al., 2010) was followed, employing phenazine methosulphate and iodonitrotetrazolium chloride (INT) as artificial electron acceptors and the chromogenic reagent to ensure recycle of FAD and to maintain the enzymic activity of DguA in the reaction. Cell-free crude extracts were prepared from \emph{E. coli} Top10 carrying either pDGUA for DguA or the cloning vector pBAD-HisD as negative control. Equal amounts of total proteins from these two samples were added into the reaction containing D-amino acids (5 mM) as substrates. One unit of D-amino acid dehydrogenase activity was defined as the amount of enzyme that led to the reduction of 1 nmol INT min\textsuperscript{-1} under the standard assay conditions. For the measurements of \textit{b}-galactosidase activities, the standard protocol with ONPG as substrate was followed. The cells were grown at 37 °C in minimal medium supplemented with L-Glu (10 mM) and other amino acids (5 mM) as indicated.

Electrophoretic mobility shift assays. A 118 bp DNA fragment covering the regulatory region of \textit{dguA} as in pDGU3 was PCR-amplified with specific pairs of oligonucleotide primers. A DNA fragment of
248 bp covering the region of the dadA promoter (He et al., 2011) was used as a negative control. For the binding reactions, the DNA probe (5.0 ng) was allowed to interact with different concentrations of purified DguR in a mixture of 20 μl containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 4 mM DTT, 5 % (v/v) glycerol, negative-control DNA (5.0 ng) and acetylated BSA (300 μg ml⁻¹). After incubation for 20 min at room temperature, 10 μl of each reaction mixture was loaded on a polyacrylamide gel (5 %) in Tris/borate-EDTA buffer (pH 8.7). The gels were stained with SYBR Green I solution, scanned with an imaging system (Omega UltraLum) with a setting for excitation at 473 nm and emission at 520 nm.

**RESULTS**

### Transcriptional profiling in response to exogenous D-Glu and L-Glu

DNA microarrays experiments were conducted to obtain a snapshot of gene expression in *P. aeruginosa* PAO1 grown exponentially in pyruvate minimal medium supplemented with D-Glu, L-Glu or L-Arg. For data analysis, we first compared D-Glu to L-Glu and genes that displayed over threefold difference with significant signal levels in duplicates were selected. Second, taking the same approach, genes were selected for comparison of L-Glu to L-Arg, excluding those in the ArgR regulon of Arg metabolism (Lu et al., 2004). As L-Arg is converted into L-Glu through the Arg succinyltransferase pathway, the intracellular L-Glu concentration was expected to be high under this growth condition and therefore only genes responding to exogenous L-Glu were picked up by this approach. Genes from these two analyses were then compiled as shown in Table 1. As described below, these genes could be divided into three major groups based on their functions: D-Glu catabolism, D/L-Glu uptake and L-Glu biosynthesis.

### dguR–dguABC locus

Amongst the genes listed in Table 1, the PA5084–PA5082 gene cluster exhibited the highest fold induction by D-Glu. These genes were designated dguABC due to their potential functions in D-Glu utilization. As shown in Fig. 1(a), the dguABC genes form a putative operon. The DguA protein (PA5804) exhibited 40 % sequence identity to the D-Ala dehydrogenase DadA of *P. aeruginosa* (He et al., 2011). The dguB gene encoded a small peptide of the YigF family (Lambrecht et al., 2012), whilst dguC produced a periplasmic solute binding protein showing 65 % sequence identity to AatJ (PA1334; Table 1) for acidic amino acid transport (Singh & Roehm, 2008). Although not inducible by D-Glu, PA5085 divergently transcribed upstream of dguA encoded a transcriptional regulator of the LysR family and was designated dguR for its role in the control of dguABC expression.

### Gene knockout and complementation tests

Markerless deletion mutants of dguR, dguA and dguC were constructed successfully by the Flp–FRT recombination system followed by biparental conjugation as described in Methods. Another set of these mutants was obtained from the transposon mutant library (University of Washington). Growth of these mutants on D/L-Glu and D/L-Gln as the sole source of carbon and/or nitrogen was checked on minimal media containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 4 mM DTT, 5 % (v/v) glycerol.

#### Table 1. The gene list from DNA microarray analysis of D-Glu metabolism and uptake

<table>
<thead>
<tr>
<th>PA no.</th>
<th>Gene name</th>
<th>Signal intensity</th>
<th>Descriptions</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td><strong>D-Glu</strong></td>
<td><strong>L-Glu</strong></td>
</tr>
<tr>
<td>PA1335</td>
<td>aauR</td>
<td>750</td>
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<td>aauS</td>
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<tr>
<td>PA1337</td>
<td>ansB</td>
<td>13 183</td>
<td>12 780</td>
</tr>
<tr>
<td>PA1338</td>
<td>ggt</td>
<td>8886</td>
<td>8667</td>
</tr>
<tr>
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<td>aatP</td>
<td>10 889</td>
<td>11 966</td>
</tr>
<tr>
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<td>6663</td>
<td>7498</td>
</tr>
<tr>
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<td>aatM</td>
<td>6473</td>
<td>6600</td>
</tr>
<tr>
<td>PA1342</td>
<td>aatJ</td>
<td>17 650</td>
<td>20 386</td>
</tr>
<tr>
<td>PA5082</td>
<td>dguC</td>
<td>13 395</td>
<td>269</td>
</tr>
<tr>
<td>PA5083</td>
<td>dguB</td>
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<td>98</td>
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<td>PA5036</td>
<td>glutB</td>
<td>3583</td>
<td>1144</td>
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The PA identification numbers were taken from the *P. aeruginosa* PAO1 genome annotation project (www.pseudomonas.com). GeneChip raw data are mean values from two independent sets of cultures. The cells were grown in pyruvate minimal medium P supplemented with D-Glu, L-Glu or L-Arg (5 mM) as the sole source of nitrogen.
The DguA protein was proposed as a D-Glu dehydrogenase due to its high sequence homology to the D-Ala dehydrogenase DadA and the growth phenotype of the dguA mutant on D-Glu as described above. Plasmid pDGUA was constructed for the expression of DguA with a His<sub>6</sub>-tag at its N terminus in E. coli Top10. Although this recombinant DguA was overexpressed and the proposed FAD-dependent enzymic activity (Fig. 1b) could be detected in the soluble fraction of crude extracts by the enzyme assays described in Methods, the purified proteins detected in the soluble fraction of crude extracts by the enzyme assays described in Methods, the purified proteins could be observed in pDGU2 with a shorter insert. By comparing the upstream regions covered by pDGU2 and pDGU3, it was conceivable to expect a potential DguR-binding site in a region of 26 bp at the 5′ end of dguA–dguA intergenic region (Fig. 2).

Surprisingly, D-Glu-dependent induction of dguA promoter activity was also abolished in the strain PAO1 carrying pDGU5. As pDGU5 carries not only the dguA promoter but also the cognate gene, it may potentially generate a truncated DguR protein that contains the N-terminal DNA-binding domain but no C-terminal ligand-binding domain. When expressed from pDGU5, the truncated DguR, either in

Table 2. Growth phenotypes of P. aeruginosa mutants in D-Glu metabolism and uptake

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Growth phenotype</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>L-Glu</td>
</tr>
<tr>
<td>MPAO1</td>
<td>WT</td>
<td>+++</td>
</tr>
<tr>
<td>PW3424</td>
<td>aauR</td>
<td>–</td>
</tr>
<tr>
<td>PW3427</td>
<td>aauS</td>
<td>+</td>
</tr>
<tr>
<td>PW3430</td>
<td>ansB</td>
<td>+++</td>
</tr>
<tr>
<td>PW3433</td>
<td>gtt</td>
<td>++++</td>
</tr>
<tr>
<td>PW3434</td>
<td>aatP</td>
<td>–</td>
</tr>
<tr>
<td>PW3437</td>
<td>aatQ</td>
<td>–</td>
</tr>
<tr>
<td>PW3441</td>
<td>aatI</td>
<td>–</td>
</tr>
<tr>
<td>PW8726</td>
<td>gdhA</td>
<td>++++</td>
</tr>
<tr>
<td>PW9459</td>
<td>gdhD</td>
<td>+++</td>
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<tr>
<td>PW9461</td>
<td>gdhB</td>
<td>++</td>
</tr>
<tr>
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<td>++++</td>
</tr>
<tr>
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<td>dguA</td>
<td>++++</td>
</tr>
<tr>
<td>PW9533</td>
<td>dguR</td>
<td>++++</td>
</tr>
<tr>
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<td>ΔdguC</td>
<td>++++</td>
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<tr>
<td>PAO5731</td>
<td>ΔdguA</td>
<td>++++</td>
</tr>
<tr>
<td>PAO5730</td>
<td>ΔdguR</td>
<td>++++</td>
</tr>
</tbody>
</table>

The cells were grown in minimal medium P with the indicated amino acids (20 mM) as the sole source of carbon and nitrogen. Growth under each condition was recorded after incubation for 36 h at 37 °C: +,++, strong growth in 15 h; +,+, strong growth in 36 h; +, weak growth in 36 h; –, no growth in 36 h. The PW strains of transposon insertion mutants were derived from MPAO1 and obtained from the stock centre at the University of Washington. The PAO strains of deletion mutants were constructed in this study as described in Methods.

Induction of the dguA promoter by D-Glu and D-Gln

As shown in Fig. 2, four P<sub>dguA::lacZ</sub> fusion plasmids (pDGU2, pDGU3, pDGU4 and pDGU5) were constructed that carried serial deletions of the DNA sequence upstream of dguA. Once introduced into PAO1, the promoter activities of these fusions in response to the presence and absence of D-Glu were measured. It was found that the dguA promoter in pDGU3 or pDGU4 was inducible by exogenous D-Glu. On the contrary, no induction by D-Glu could be observed in pDGU2 with a shorter insert. By comparing the upstream regions covered by pDGU2 and pDGU3, it was conceivable to expect a potential DguR-binding site in a region of 26 bp at the 5′ end of dguR–dguA intergenic region (Fig. 2).

Medium plates. It was found that the dguR and dguA mutants were completely defective in growth on D-Glu and severely retarded on D-Gln, but grew normally on L-Glu and L-Gln (Table 2). Complementation of dguR and dguA mutants with plasmids carrying the cognate gene restored growth on D-Glu and D-Gln (data not shown). These results indicated the importance of the dguA and dguR genes in D-Glu and D-Gln utilization.

D-Glu dehydrogenase activity of DguA

The DguA protein was proposed as a D-Glu dehydrogenase due to its high sequence homology to the D-Ala dehydrogenase DadA and the growth phenotype of the dguA mutant on D-Glu as described above. Plasmid pDGUA was constructed for the expression of DguA with a His<sub>6</sub>-tag at its N terminus in E. coli Top10. Although this recombinant DguA was overexpressed and the proposed FAD-dependent enzymic activity (Fig. 1b) could be detected in the soluble fraction of crude extracts by the enzyme assays described in Methods, the purified proteins from the nickel column lost their activity completely. Instead, crude extracts of the recombinant strain were used to test substrate specificity and the background activities on each substrate were measured with the crude extract of the host cells carrying the cloning vector pBAD-HisD. Among 17 different D-amino acids tested (20 mM; excluding D-Cys and D-Ile), DguA exhibited the highest activity towards D-Glu (2622 U mg<sup>−1</sup>) followed by D-Pro (1276 U mg<sup>−1</sup>) and D-Gln (372 U mg<sup>−1</sup>), whilst negligible or no activity could be detected with D-Asp, D-Asn and others.

Fig. 1. (a) Genetic organization of the aat and dgu loci for D-Glu transport and catabolism. (b) Proposed pathways for D-Glu catabolism and L-Glu biosynthesis in P. aeruginosa PAO1. The proposed gene functions in the aat and dgu loci are described in the text: grey arrows, components of an ATP-binding cassette (ABC) transporter; black arrows, transcriptional regulators; white arrows, enzymes. Murl, Glu racemase; DguA, D-Glu dehydrogenase; GlnA, L-Gln synthetase; GdhA, anabolic L-Glu dehydrogenase; GltBD, Glu synthase.
homomeric form or in heteromeric form with the authentic DguR, may turn into a constitutively active repressor when losing the capacity to sense D-Glu and authentic DguR, may turn into a constitutively active homomeric form or in heteromeric form with the corresponding D-amino acids tested, only D-Glu and D-Asp were found to exert a significant induction effect on the DguA promoter. Furthermore, no induction of the DguA promoter by these two D-amino acids could be detected in the DguR mutant. The induction effect of exogenous D-Glu was retained in the DguA mutant that could not degrade this amino acid. These results supported DguR as a transcriptional activator of the DguA promoter in response to D-Glu in vivo.

Effect of D-Glu on the DNA-binding activity of DguR

Sequence comparison of DguR orthologues in different species of Pseudomonas led us to speculate that the authentic DguR should in fact start from an ATG codon 16 residues shorter than the original annotated version in the PAO1 website (Winsor et al., 2011). In addition, the initial attempt to produce the peptide from the first ATG codon failed. Instead, the second construct, pDGUR, was able to synthesize a recombinant DguR starting from the proposed second ATG codon with a His6-tag at its N terminus. After purification from a nickel column, the recombinant DguR protein was used to demonstrate its interactions with the DguA promoter region by the electrophoretic mobility shift assay. As shown in Fig. 4(a), DguR bound specifically to a DNA fragment covering the DguA promoter region, but not the DNA fragment of the negative control. However, several nucleoprotein complexes of retarded mobility could be detected. When D-Glu was included in the reaction mixture, DguR formed only one distinct nucleoprotein complex with the DguA probe. In comparison, inclusion of D-Asp showed no effect on the pattern of nucleoprotein complexes.

Identification of the DguR-binding site

As shown in Fig. 2, the results of Pdgua::lacZ fusions strongly indicated the presence of a cis-acting site for DguR-dependent induction in the 26 bp region that existed in pDGU3 but not in pDGU2. Through comparative genomics, it was apparent that the divergent DguR–dguABC operons were highly conserved in many Pseudomonas species (Fig. 5a) and therefore the same regulatory mechanism for activation of the DguA promoter in P. aeruginosa PAO1 may also apply to other cases. Along this line, we conducted in silica analysis of the DguR–DguA intergenic region among seven representative strains of different species of Pseudomonas to further pinpoint this

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**Fig. 3.** Expression profile of the DguA promoter in P. aeruginosa PAO1. The DguA promoter activities were monitored by measurements of the β-galactosidase activity in PAO1, PAO5730 (ΔdguR) and PAO5731 (ΔdguA) carrying pDGU3 grown in L-Glu (10 mM) minimal medium in the absence and presence of the indicated D-amino acids (5 mM). Values represent the mean±sd of four measurements for each growth condition.

**Fig. 2.** Schematic presentation of the dguA promoter–lacZ fusions and expression. The regions carried by four different fusions (pDGU2, pDGU3, pDGU4 and pDGU5) are depicted, and the promoter activities of these four constructs in P. aeruginosa PAO1 in response to L-Glu and D-Glu are shown on the right. The numbers in parentheses indicate the size of promoter regions covered by each construct, starting from a common 3′ end from the ATG initiation codon of dguA. Also shown is the nucleotide sequence in the intergenic region of dguR and dguA. Activities are expressed as OD_{420}/OD_{600} min^{-1}×1000.
cis-acting element. A highly conserved 19 bp sequence motif of partial dyad symmetry was revealed from the results of multiple sequence alignment (Fig. 5b). This sequence motif was intact in pDGU3, but was truncated in pDGU2 (Fig. 2). Therefore, we hypothesized this motif as the DguR-binding site.

To test this hypothesis, the putative DguR operator was reconstituted from a pair of 19 bp synthetic oligonucleotide primers and then cloned into the SmaI site of vector pUC18. Through PCR amplification, DNA fragments carrying the multiple cloning site region of pUC18 with or without the cloned DguR operator were prepared for the mobility shift assays with the purified DguR protein. As shown in Fig. 4(b), a distinct DguR–DNA complex could be detected with the probe containing the putative DguR operator, but not with the control DNA, and this nucleoprotein complex was more apparent when D-Glu (5 mM) was included in the reaction. Addition of D-Glu, D-Asp or D-Asn had no effect on the formation of DguR–DNA complex (data not shown).

Growth phenotypes of the aatJMOP–ggt–ansB–aauSR mutants on D-Glu and D-Gln

DNA microarrays analysis revealed that the PA1335–PA1342 locus (Fig. 1a) was inducible by exogenous D-Glu and L-Glu, but not by L-Arg. The counterpart of this locus in P. putida has been reported (Singh & Röhm, 2008) to encode an ATP-binding cassette (ABC) transporter for acidic amino acids (AatJMP; PA1342–PA1339) and its two-component regulatory system (AauSR; PA1336–PA1335). Two additional genes are included in this locus of P. aeruginosa: ggt (PA1338) for a putative γ-glutamyltranspeptidase and asnB (PA1337) for a putative glutaminase-asparaginase (Sonawane et al., 2003).

To assess the importance of these genes in Glu and Gln utilization, a set of knockout mutants was obtained from the two-allele transposon mutant library at the University of Washington, and growth of these mutants on DL-Glu and DL-Gln as the sole source of carbon and/or nitrogen was tested. As shown in Table 2, any lesion in aatJMOP was able to abolish growth completely on DL-Glu as the carbon and nitrogen source, and growth on L-Glu as the sole nitrogen source was retarded severely. The aauSR mutants also displayed growth defects on DL-Glu, most likely due to their roles in expression of the AatJMP transport system (Singh & Röhm, 2008). In comparison, the aatJMOP and aauSR mutants also exhibited significant growth defects when D-Gln served as the sole source of carbon and nitrogen. These mutants grew normally on L-Gln as the nitrogen source, and growth was only slightly affected when L-Gln served as the sole source of carbon and nitrogen. The ggt and asnB mutants grew normally on DL-Glu and DL-Gln.

Effects of aatJM and aauS mutants on D-Glu induction of the dguA promoter

To further demonstrate the importance of the Aat transport system of D-Glu uptake, we measured the dguA::lacZ expression of pDGU3 in the aatJ, aatM and aauS mutants, and in their parental strain MPAO1. As shown in Fig. 6, the D-Glu-dependent induction of the dguA promoter was abolished completely in the aatJ and aatM mutants, whilst the level of induction was reduced by twofold in the aauS mutant in comparison with that in the MPAO1 strain. These results were consistent with the growth phenotype analysis of these mutants on D-Glu (Table 2), supporting the conclusion that the Aat transport system was essential for D-Glu uptake.
L-Glu biosynthesis

Several genes in L-Glu biosynthesis, including gltBD for Glu synthase and gdhA for anabolic Glu dehydrogenase, exhibit higher levels of expression when grown in pyruvate minimal medium with D-Glu as the sole source of nitrogen. As shown in Fig. 1(b) and described above, D-Glu deamination catalysed by the D-Glu dehydrogenase DguA was supposed to make \( \alpha \)-ketoglutarate and ammonia, which serve as carbon and nitrogen sources to support growth. In addition, L-Glu can be generated via reactions of GdhA and GltBD. We have reported that gltBD and gdhA are subjected to repression by exogenous L-Glu and L-Arg in response to L-Glu accumulation inside the cells (Hashim et al., 2004; Lu et al., 2004), and therefore no repression by D-Glu implied the absence of L-Glu excess under this growth condition. Growth phenotypes of the gltBD and gdhA mutants on DL-Glu and DL-Gln are shown in Table 2. It was found that growth on D-Glu as the sole source of carbon and/or nitrogen was abolished completely in the gltBD mutants, but the gdhA mutant grew normally. These results suggested Glu synthase as the major route for L-Glu synthesis during growth on D-Glu.

DISCUSSION

DguA-dependent deamination is the essential route of D-Glu catabolism

Through genetic and biochemical studies, DguA was demonstrated as a D-Glu dehydrogenase in this study. The ability to grow on D-Glu as the sole source of carbon or nitrogen was abolished completely in the dguBD mutant without a functional DguA or in the dguR mutant devoid of D-Glu-dependent induction of dguA. These results established the oxidative deamination by DguA as the essential route for D-Glu catabolism. Deamination of D-Glu by DguA...
Although we were able to express and purify a recombinant boxylic acid cycle and the ammonia assimilation pathway, utilized as carbon and nitrogen sources through the tricarboxylic acid cycle and the ammonia assimilation pathway, respectively.

As a member of the LysR family of transcriptional regulators, DguR may sense the presence of intracellular D-Glu through its C-terminal ligand-binding domain, which results in a conformational change of DguR to activate the dguA promoter. This working model was supported by the findings that D-Glu-dependent induction of the dguA promoter is retained in the dguA mutant (Fig. 3), and that the induction effect was abolished completely when there is no D-Glu uptake in the aatJ and aatM mutants (Fig. 6). In addition, formation of a distinct nucleoprotein complex by DguR and the dguA regulatory region was enhanced specifically by the presence of D-Glu in vitro. Although we also observed induction of dguABC genes and the dguA promoter by exogenous D-Gln in vitro, formation of DguR-dependent nucleoprotein complexes was not affected by D-Gln in vitro. The discrepancy of the effects of D-Gln in vitro and in vivo might be related to conversion of D-Gln to D-Glu, as discussed below.

**D-Gln utilization**

One route for D-Gln catabolism is to convert D-Gln to D-Glu (e.g. glutaminase), followed by oxidative deamination of D-Glu by DguA. The fact that growth on D-Gln was retarded severely in the dguA mutant supports the physiological function of this route, but the residual growth of the dguA mutant on D-Gln also indicates the presence of an alternative pathway yet to be identified. In comparison with other species of Pseudomonas, only P. aeruginosa possesses two additional genes (ggt and ansB) between the aatJQMP and aauSR loci. As ggt and ansB were also induced by Dl-Glu to levels comparable with those of aatJQMP, it is likely that these six genes form an operon. The ansB gene encodes a putative periplasmic glutaminase-asparaginase that is highly conserved in pseudomonads (Sonawane et al., 2003). In P. putida, although the genetic location of ansB is physically distant from aat and aau, its expression is also subject to regulation by AauSR (Singh & Röhm, 2008). Whilst the ansB knockout mutant of P. putida lost the ability to grow on L-Gln, a lesion in ansB of P. aeruginosa PAO1 did not affect growth on DL-Gln (Table 1). This result would imply the presence of another glutaminase or other routes of Gln catabolism in PAO1.

**Uptake via the AatJMQP transporter is equally important for growth on DL-Glu**

The AatJMQP transporter of P. putida KT2440 has been characterized in detail (Singh & Röhm, 2008) for acidic amino acid uptake. In P. aeruginosa, it has been reported that growth on L-Glu as the carbon source was abolished by mutations of the aatMQP genes and the expression levels of aatQP genes were induced by exogenous L-Glu (Johnson et al., 2008). In this study, we found the AatJMQP transporter was indispensable for the growth of P. aeruginosa PAO1 on both D-Glu and L-Glu as the sole source of carbon and nitrogen. In addition, the importance of this transport system extends to DL-Gln; growth of the aat mutants on D-Gln was retarded severely, but only affected slightly on L-Gln. These results indicate that the AatJMQP transport system takes both enantiomers of Glu and Gln as substrates. Although exogenous D-Glu was able to induce the expression of dguC encoding an AatJ homologue (65% sequence identity) and the dguC deletion mutant indeed showed retarded growth on D-Glu (Table 2), the substrate specificity of DguC remains to be clarified. However, it is reasonable to speculate that DguC may function in D-Glu uptake via interactions with AatMQP and that the induced DguC expression by exogenous D-Glu provides another avenue to increase growth efficiency on this amino acid.
The notion that DL-amino acid uptake is mediated by the same transport system may in fact apply over a broader scope. The BraCEDFG transport system for the branched-chain amino acids in *P. aeruginosa* was reported to be essential for the growth on DL-Ala as the carbon source (Johnson et al., 2008). Deletion of two ABC transporter systems for L-Arg uptake (Lu et al., 2004; Nishijyo et al., 1998) retarded growth on L-Arg and D-Arg (unpublished data). Analogous to DguC on D-Glu uptake, a putative D-Arg periplasmic binding protein DauT was induced by exogenous D-Arg (Li & Lu, 2009a), which in turn may work with other ABC transporters of L-Arg to streamline the uptake and utilization of D-Arg. To our knowledge, no transport system taking only D-amino acids as substrate has been reported so far.

### Regulatory mechanism in control of *aat*J*Q*M*P* expression

In *P. putida*, control of the *aat*J*MQ*P operon is mediated by the AauSR two-component system (Sonawane et al., 2003) and binding of AauR to its cis-acting site upstream of the $E^{34}$ promoter of *aat* has been demonstrated (Singh & Röhm, 2008). As these cis- and trans-acting regulatory elements in control of *aat*J*MQ*P expression are conserved highly in *Pseudomonas* spp., it is conceivable that the same regulatory mechanism may apply to these organisms, including *P. aeruginosa* PAO1. From the results of transcriptome analysis, we found that expression of *aat*J*MQ*P genes was induced to a comparable level by exogenous D-Glu and L-Glu (Table 1) as well as by D-L-Gln (unpublished data). Based on the current model of the AauS/AauR two-component system in the control of *aat*J*MQ*P expression, our results suggest strongly that the membrane-anchored AauS sensor may not be able to differentiate the enantiomers of Glu and possibly Gln. This is in sharp contrast to the case of DguR, which has a more stringent selection on D-Glu as its native ligand and activates the *dugABC* operon accordingly.

In summary, a complete set of genetic elements for D-Glu catabolism and uptake was revealed in this study. This new information is beneficial to our understanding of molecular mechanisms in the maintenance of D-Glu homeostasis. The intracellular concentration of D-Glu needs to be monitored tightly to ensure cell wall synthesis and to prevent any potential adverse effects in translation when D-Glu is in excess. As an essential component of peptidoglycan, biosynthesis of D-Glu requires the Glu racemase MurI. Whilst DguA prevents D-Glu accumulation inside the cells, its expression needs to be monitored closely to prevent overdrainage of D-Glu. Along this line, perturbing D-Glu metabolism through MurI inhibition or DguA overexpression might be potential strategies of antibiotics development.

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