Characterization of a *Pseudomonas putida* ABC transporter (AatJMQP) required for acidic amino acid uptake: biochemical properties and regulation by the Aau two-component system

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We describe an ATP-binding cassette (ABC) transporter in *Pseudomonas putida* KT2440 that mediates the uptake of glutamate and aspartate. The system (AatJMQP, for acidic amino acid transport) is encoded by an operon involving genes PP1071–PP1068. A deletion mutant with inactivated solute-binding protein (KTaatJ) failed to grow on Glu and Gln as sole sources of carbon and nitrogen, while a mutant lacking a functional nucleotide-binding domain (KTaatP) was able to adapt to growth on Glu after an extended lag phase. Uptake of Glu and Asp by either mutant was greatly impaired at both low and high amino acid concentrations. The purified solute-binding protein AatJ exhibited high affinity towards Glu and Asp ($K_d=0.4$ and $1.3\ \mu\text{M}$, respectively), while Gln and Asn as well as dicarboxylates (succinate and fumarate) were bound with much lower affinity. We further show that the expression of AatJMQP is controlled by the σ^{54} -dependent two-component system AauRS. Binding of the response regulator AauR to the *aat* promoter was examined by gel mobility shift assays and DNase I footprinting. By *in silico* screening, the AauR-binding motif (the inverted repeat TTCGGNNNNCCGAA) was detected in further *P. putida* KT2440 genes with established or putative functions in acidic amino acid utilization, and also occurred in other pseudomonads. The products of these AauR-responsive genes include the H^+ /Glu symporter GltP, a periplasmic glutaminase/asparaginase, AnsB, and phosphoenolpyruvate synthase (PpsA), a key enzyme of gluconeogenesis in Gram-negative bacteria. Based on these findings, we propose that AauR is a central regulator of acidic amino acid uptake and metabolism in pseudomonads.

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INTRODUCTION

The fate of micro-organisms in the environment depends critically on their ability to utilize as many carbon and nitrogen sources as possible and to secure a constant supply of nutrients in the presence of competitors. Therefore, a wide repertoire of selective and efficient uptake systems affords a marked advantage in the struggle for survival. Transporters of the ATP-binding cassette (ABC) superfamily have been identified in all organisms (prokaryotes as well as eukaryotes). In bacteria, they constitute a central part of the nutrient uptake system, translocating a wide variety of solutes (sugars, amino acids, metals, growth factors, ions and others) across the cell membrane. Other members of the ABC transporter family are involved in signal transduction, protein secretion, drug and antibiotic resistance, antigen presentation, bacterial pathogenesis and sporulation (Higgins, 1992).

ABC transporters are typically composed of four functional modules: two transmembrane permease domains, each of them spanning the membrane four to eight times, and two nucleotide-binding domains (NBDs). In eukaryotes, these modules are frequently fused to yield a single polypeptide chain, while bacterial ABC transporters are assembled from individual subunits. With most bacterial importers, a highly specific solute-binding protein is also part of the transport machinery, whereas ABC exporters lack the solute-binding protein (Fath & Kolter, 1993; Davidson & Chen, 2004). In Gram-negative organisms, solute receptors are dissolved in the periplasm, while in archaea and Gram-positive micro-organisms, they are membrane-anchored lipoproteins (Higgins, 1992; Hediger, 1994; Schneider & Hunke 1998; Saurin & Dassa, 1994). The specificity of ABC transporters mainly depends on the selective binding of the solute by the periplasmic receptor. They not only ensure effective solute capture but also act as essential activators of the cytoplasmic nucleotide-binding domains (Davidson & Chen, 2004; Saurin *et al.*, 1999).

Abbreviations: ABC, ATP-binding cassette; PAAT, polar amino acid uptake transporter.

Pseudomonas putida KT2440 is a well-characterized Gram-negative soil micro-organism with potential applications in bioremediation and biocontrol. Like other pseudomonads, strain KT2440 is physiologically very versatile, growing on a wide range of carbon and nitrogen sources, including the acidic amino acids and their amides (Sonawane *et al.*, 2003a). The ABC transporter characterized in the present study exhibits a marked selectivity for glutamate and aspartate. We therefore named the coding operon *aat* (for acidic amino acid transporter). *P. putida* KT2440 *AatJMQP* is encoded by genes PP1068–PP1071. Closely related orthologues exist in *Pseudomonas aeruginosa* (PA1339–PA1342), *Pseudomonas fluorescens* (PFI1048–PFI1051) and *Pseudomonas syringae* (PS4171–PS4174).

Like other bacterial ABC transporters, the *aat* system involves a periplasmic solute-binding protein *AatJ* (encoded by PP1071), two permease domains, *AatQ* and *AatM* (PP1070–PP1069), and an ATP-binding subunit, *AatP* (PP1068). In the *P. putida* KT2440 genome, *aat* is immediately adjacent to *aau*, an operon that encodes the two-component system *AauRS* (see Fig. 1). The *aau* system was recently identified as a regulator of acidic amino acid utilization in *P. putida*. As judged by sequence similarity, the response regulator *AauR* is a DNA-binding helix–turn–helix protein belonging to the Fis family (Morett & Bork, 1998). We have further shown that expression of *aat* depends on σ^{54} , an alternative sigma factor typically involved in the transcription of genes related to nitrogen metabolism (Sonawane *et al.*, 2006).

In the present communication we provide evidence that indicates that *AauR* directly controls the expression of *aat* and other glutamate-related genes or operons by binding to a well-conserved 16 bp inverted repeat. We further define the binding specificity of *AatJ* and provide dissociation constants for binding of various amino acids.

METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are shown in Table 1. *P. putida* KT2440 was grown

in LB broth (Gibco-BRL), in M9 minimal medium (Sambrook *et al.*, 1989) or on LB agar plates at 30 °C.

Escherichia coli strains were grown in LB broth or on LB agar at 37 °C. Ampicillin (100 µg ml⁻¹), kanamycin (40 µg ml⁻¹) and carbenicillin (100 µg ml⁻¹) were used for mutant selection. To examine growth kinetics and amino acid transport, the cells were pre-grown for 15 h on M9⁺ medium (M9 supplemented with 20 mM NH₄Cl and 22 mM glucose), collected by centrifugation at 4500 r.p.m., washed twice with M9, and then suspended in M9 containing (i) 5 mM amino acids as sole source of carbon and nitrogen or (ii) 5 mM amino acids as nitrogen source and 22 mM glucose as carbon source. Bacterial growth was monitored by turbidity measurements at 595 nm.

Construction of deletion mutants *KTaatP* and *KTaatJ*. Genes *aatP* (PP1068) and *aatJ* (PP1071) were inactivated by a strategy described previously in detail (Sonawane *et al.*, 2006). Briefly, internal gene fragments of about 300 bp were amplified from the KT2440 genome using primers that introduce single base-pair frameshifts and cloned into pK18, a suicide vector that cannot multiply inside *P. putida* unless integrated into the genome by homologous recombination. The resulting mutants contain fragments of the target gene separated by the plasmid sequence, i.e. (1) a short truncated fragment or (2) a fragment containing the frameshift mutation at the 5' end. The frameshift mutation was necessary, as in the second case, the transconjugants might contain a wild-type copy of the genes after recombination. In the case of *aatP*, a 261 bp fragment (408–147 bp relative to the translation start site) was amplified using primers PP1068_For and PP1068_Rev (Table 2). In the case of PP1071 (*aatJ*), a 284 bp internal fragment (189–473 bp) was generated using primers PP1071_For and PP1071_Rev. The amplified fragments were cloned into pK18 (Pridmore, 1987) and the resulting plasmids electroporated into *P. putida* KT2440, followed by selection of kanamycin-resistant clones. Successful recombination was verified by PCR using specific primers. All further work with these mutants was performed in the presence of kanamycin (40 µg ml⁻¹).

Complementation experiments. Mutants *KTaatJ* and *KTaatP* were complemented with intact copies of the respective functional genes as follows. The coding sequence and the promoter of gene *aatJ* were amplified from KT2440 genomic DNA using primers *CaatJ_For* and *CaatJ_Rev*. A stop codon was inserted into *CaatJ_For* immediately after the restriction site to block expression from the *tac* promoter of the vector; thus *aatJ* used its own promoter for expression. *AatP* was amplified in a similar way using primers *CaatP_For* and *CaatP_Rev*. The amplified fragments were cloned into the *Bam*HI/*Eco*RI-digested pMMB67EH (Fürste *et al.*, 1986), a low-copy-number expression

Fig. 1. Genetic organization of the *aat* and *aau* operons in the genomes of different pseudomonads (see text).

Table 1. Strains and plasmids used in this work

Strain or plasmid	Genotype, origin	Reference or source
<i>P. putida</i> strains		
KT2440	<i>mt-2</i> , <i>hsdR1</i> ($r^- m^+$)	Bagdasarian & Timmis (1982)
<i>KtaatP</i>	<i>aatP</i> derivative of KT2440	This work
<i>KTaatP</i> ⁺	<i>KTaatP</i> complemented with functional <i>aatP</i>	This work
<i>KTaatJ</i>	<i>aatJ</i> derivative of KT2440	This work
<i>KTaatJ</i> ⁺	<i>KTaatJ</i> complemented with functional <i>aatJ</i>	This work
<i>E. coli</i> strains		
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>ara-leu</i>)7687, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen
BL21	F ⁻ , <i>ompT</i> , <i>hsdS</i> ($r_B^- m_B^-$), <i>gal</i> , <i>dcm</i>	Amersham Biosciences
HB101	<i>supE44</i> , <i>hsdS20</i> ($r_B^- m_B^-$), <i>recA13</i> , <i>ara-14</i> , <i>proA21</i> , <i>acY1</i> , <i>galK2</i> , <i>rpsL20</i>	Amersham Biosciences
Plasmids		
pUC19	Cloning vector, <i>amp</i> ^r	Pridmore (1987)
pK18	Cloning vector, <i>kan</i> ^r	
pMMB67EH	Cloning and expression vector, <i>amp</i> ^r	Fürste <i>et al.</i> (1986)
pTrcHisA	Expression vector, pBR322 <i>ori</i> , <i>lacI</i> ^q , <i>ptac</i> , <i>lacO</i> , <i>amp</i> ^r	Invitrogen
pFLAG-ATS	Expression vector, <i>ompA</i> , <i>ptac</i> , <i>lacI</i> , <i>amp</i> ^r	Sigma

vector commonly used for complementation experiments with *Pseudomonas* spp. The sequenced vectors were electroporated into *KTaatJ* and *KTaatP*, and complemented clones were isolated by carbenicillin selection, yielding strains *KTaatJ*⁺ and *KTaatP*⁺.

Analysis of the *aatJMPQ* promoter region. Various segments of the upstream promoter region of *aatJMPQ* (−83 to −343 bp relative to the transcription start site) were amplified from the KT2440 genome using forward primer PromFor and various reverse primers, as summarized in Table 2. The amplified fragments were cloned in pUC19, and the resulting plasmids transformed into *E. coli* Top10 for

multiplication. The vectors were then digested with *EcoRI* and *HindIII* and the excised fragment purified by agarose gel electrophoresis using the Qiagen gel extraction kit. The purified fragments were labelled with ³³P in a 50 µl reaction volume containing 10 µl 5 × forward reaction buffer, 5 pmol DNA, 25 U T4 Polynucleotide Kinase (Stratagene) and 5 µl [γ -P³³]ATP (10 µCi µl^{−1}; 110 TBq mmol^{−1}). After incubation at 37 °C for 1 h, the labelled fragment was separated from free [γ -P³³]ATP on a Microspin S-300 HR column (GE Healthcare). An unspecific DNA fragment of about the same size was labelled accordingly. Specific radioactivities of the resulting fragments were estimated by liquid scintillation counting.

Table 2. PCR primers used for DNA amplification and introduction of frame-shift mutations

Primer	Sequence
PP1068_For*	5'-CGCggaatccTCGGGCAAGTCCACCCT-3'
PP1068_Rev*	5'-CCCaaagcttTACACGGTCGAGCAGCGCCA-3'
PP1071_For*	5'-CGCggaatccTCGCATGACAATCCAGCTGGC-3'
PP1071_Rev*	5'-CCCaaagcttGTCAACACGTTCTTGCCGGCC-3'
AatJ_For†	5'-CGCaaagcttGAGGAGCTCACCGGCACCCT-3'
AatJ_Rev†	5'-CCCgaattcTCAGGACTTCTTCTCGTCCGCGAG-3'
CaatJ_For‡	5'-CGCgaattcTAGCGCATCGAGCCTTTCCGGTTG-3'
CaatJ_Rev‡	5'-CCCggaatccTCAGGACTTCTTCTCGTCCGCGAGCC-3'
CaatP_For‡	5'-CGCgaattcGTGGCTGGTCAAGCGCCTGCA-3'
CaatP_Rev‡	5'-CCCggaatccTCAGTGCTGCAGGATCTTGCTGAGGAG-3'
aauR_For‡	5'-CGCggaatccATGAACCAAGCGCCTCTTAC-3'
aauR_Rev‡	5'-CCGgaattcTCAGGCGAGCCGTATTTTTTC-3'
PromFor	5'-CCCGGATCCCGCATCGAGCCTTTCCGGTTGGGC-3'
PromRev_83	5'-CCCAGGCTTCTCCGGGTTTCGCCAGGCCGAAT-3'
PromRev_117	5'-GCTCGTTTCGGTTATCCGAACGCATGGGC-3'
PromRev_136	5'-CGCATGGGCTGCCAACCTATCCCGG-3'
PromRev_157	5'-CCGGTATCGCTTGCAGTAACATGCAGTGC-3'
PromRev_202	5'-ACGTAGTGAGCTTTGCTCTTTGCCTGGAGAG-3'

**Bam*HI and *Hind*III sites in lower-case type; inserted base in bold/underlined type.

†*Hind*III and *Eco*RI sites in lower-case type.

‡*Bam*HI and *Eco*RI sites in lower-case type.

Gel mobility shift assay. Binding of AauR to *aatJMPQ* promoter fragments was monitored by a published procedure (Banerjee & Kundu, 2003). Labelled DNA probe (5 fmol) was added to 10–100 nM of AauR in HGMKE buffer (25 mM HEPES, pH 8, with 5%, v/v, glycerol, 10 mM MgCl₂, 20 mM KCl and 0.1 mM EDTA). The mixtures were incubated at 37 °C for 20 min and separated in 6% native polyacrylamide gels. The gels were dried and analysed by a Phosphorimager (Molecular Dynamics) after a 24 h exposure.

DNase I protection assay. DNase I footprinting was performed according to Licht & Brantl (2006). The labelled DNA fragment was digested with *Bam*HI to remove the 5' ³²P label and purified by the phenol/chloroform method. Binding reactions were carried out in a final volume of 20 µl containing HGMKE buffer, 0.05 g l⁻¹ salmon sperm DNA, 50 ng labelled probe and 20–100 nmol AauR. The reaction mixtures were incubated at 37 °C for 20 min and digested with 0.05 U DNase I (Fermentas) for 2 min at 37 °C. Digestion was stopped by extraction with phenol and subsequent ethanol precipitation. The pellets were separated on a polyacrylamide sequencing gel (8% urea) along with standard sequencing ladders (G + A and C + A) produced by Maxam–Gilbert chemical sequencing, as described by Sambrook *et al.* (1989). The dried gel was exposed to a phosphor screen for 1 week and analysed by phosphor imaging.

Expression and purification of His-tagged AauR. The gene encoding the response regulator AauR (PP1066) was amplified by PCR using primers aauRFor and aauRRev (Table 2) and ligated with the expression vector pTrcHis-A (Invitrogen) to generate pTrcAauR. After transformation into *E. coli* BL21, single colonies were grown in LB/Amp medium to OD₅₉₅ 0.8–0.9 before expression was induced by addition of 1 mM IPTG. After another 6 h, the cells were collected at 5000 r.p.m. for 10 min, washed with 100 ml cold His-tag protein-binding buffer (20 mM HEPES, pH 7.5, with 500 mM NaCl and 100 mM imidazole) and resuspended in 20 ml of the same buffer. The cell pellets were disrupted by sonication and the homogenate cleared by centrifugation (20 000 r.p.m., 30 min). The supernatant was loaded on an equilibrated Ni-NTA column (HisTrap-FF, GE Healthcare Biosciences) and protein was eluted with a linear imidazole gradient (100–500 mM). AauR-containing fractions were pooled and subjected to gel filtration on Superdex 200 (GE Healthcare Biosciences) equilibrated with the same buffer. Finally, the purified protein was dialysed against 50 mM HEPES, pH 7.5, containing 500 mM NaCl, and concentrated by a Centricon cartridge [10 kDa molecular weight cut-off (MWCO); Millipore].

Overexpression and purification of AatJ. The *aatJ* gene is 918 bp long, with base pairs 1–72 encoding the signal peptide for periplasmic transport. A truncated form of *aatJ* lacking this leader sequence was amplified by using AatJ_For and AatJ_Rev (Table 2). The amplified fragment was cloned into expression vector pFLAG-ATS (Sigma). This vector already contains an *ompA* signal peptide coding gene and a small flag sequence that mediates transport of the expressed protein into the periplasmic space. *E. coli* BL21 cells containing the recombinant plasmid were grown in LB/Amp medium at 37 °C to OD₅₉₅ 0.8–1. Expression was induced by 1 mM IPTG and cultivation continued for another 6 h at 37 °C. The cells were harvested by centrifugation at 4 °C and the periplasmic protein fraction liberated by osmotic shock, as described by Harms *et al.* (1991). The periplasmic proteins were purified on a Q-Sepharose anion exchange column (GE Healthcare Biosciences) equilibrated with 50 mM Tris/HCl, pH 7.8, and eluted by a linear NaCl gradient (0–500 mM). AatJ-containing fractions were pooled and further purified by gel filtration on a Sephacryl S-100 HR column equilibrated with 50 mM Tris/HCl buffer, pH 7.8, containing 100 mM NaCl. The relevant fractions were pooled, concentrated using a Centricon cartridge (10 kDa MWCO), assayed for protein using a bicinchoninic acid (BCA) assay kit (Pierce) and stored at 4 °C.

[¹⁴C]Glu/[¹⁴C]Asp uptake assay. The rates of Glu and Asp uptake were measured by a conventional filter assay (Hosie *et al.*, 2001). Cells were grown overnight, washed twice with M9 salt solution, suspended in 50 ml M9 and stored at 4 °C until the start of the experiment. Then the cells were starved for amino acids by gentle agitation for 30 min at 30 °C. The uptake assay was initiated by adding [¹⁴C]-labelled L-Glu or L-Asp (200 mCi mmol⁻¹; Hartmann Analytic) to a final concentration of 5 µM. Then, 100 µl aliquots were removed at regular time intervals and added to 400 µl stop solution (M9 salt solution containing 50 mM of the respective unlabelled amino acid). In competition experiments, 50 µM unlabelled amino acids was added 2 min before addition of the labelled compounds. The cultures were filtered through nitrocellulose membranes (0.45 µm pore-size), and the filters were washed with 500 µl stop solution (M9 salt solution with 0.5 mM unlabelled amino acids) twice. After drying at room temperature for 12 h, the filter discs were immersed in 6 ml scintillation cocktail and counted on a Beckman Coulter LS 6500 scintillation counter. The protein content of the cells was measured by BCA assay kit (Pierce) using BSA as a standard. The kinetics of amino acid uptake from the medium was analysed by quantitative amino acid analysis, as described by Sonawane *et al.* (2006).

[¹⁴C]Glu/[¹⁴C]Asp binding assay by equilibrium microdialysis.

The binding of amino acids and other potential ligands to AatJ was studied by equilibrium dialysis. Purified AatJ was dialysed against ligand-binding buffer (50 mM HEPES, pH 7.5, 100 mM NaCl) and concentrated to a protein concentration of 10 µM using Centricon concentrators. The working solutions consisted of 5 µM AatJ protein and 0.1–25 µM [¹⁴C]L-Glu or [¹⁴C]L-Asp (200 mCi mmol⁻¹; 7.4 GBq mmol⁻¹) in 50 µl ligand-binding buffer. In competitive binding experiments, 50 µM unlabelled ligand was added together with the labelled amino acid. Disposable micro dialyser cells (DispoEquilibrium Dialyser, Harvard Apparatus) were loaded with sample solution on one side and buffer–ligand on the other, and allowed to equilibrate for 4–5 h at 25 °C with gentle rocking. Then, separately, the contents of the sample and buffer sides were mixed with 2 ml liquid scintillation cocktail and counted. The fraction of bound ligand was estimated from the difference of the two counts. As, in the present case, the ligand dissociation constants K_d^L are comparable to the concentration of the receptor (AatJ), ligand depletion had to be taken into account. In such cases, the concentration of the ligand–receptor complex [RL] is given by:

$$[RL] = \frac{([R] + [L] + K_d^L) - \sqrt{([R] + [L] + K_d^L)^2 - 4[R][L]}}{2}$$

where [R] and [L] are the total concentrations of receptor and ligand, respectively (Copeland, 2000). Dissociation constants were estimated by fitting the above equation to the binding data. Binding constants K_d^A of unlabelled ligands (Asn, Gln and dicarboxylates) were estimated from their competitive effects on Glu and Asp binding, based on the equation:

$$[RL] = \frac{[R] \cdot n}{1 + K_d^L/[L] \cdot (1 + [A]/K_d^A)}$$

where n is the number of ligand-binding sites in R, and [A] is the total concentration of competing unlabelled ligand. The respective calculations were performed using the SigmaPlot 8.0 software (SPSS).

RESULTS

The Aat transporter is indispensable for growth on Glu and Gln as sole carbon and nitrogen sources

Many amino acids, especially the acidic amino acids and their amides (Glu, Gln, Asp and Asn), support rapid

growth of wild-type *P. putida* KT 2440, even in the absence of additional carbon sources (Sonawane *et al.*, 2003a). The efficient utilization of these amino acids, however, depends on an intact AatJMQP system. Fig. 2 shows typical growth curves observed with wild-type and mutant strains of KT2440 cultured on minimal medium supplemented with these amino acids as sole organic nutrients. Quantitative growth data obtained with these and other amino acids are summarized in Table 3. The table shows that mutants lacking a functional solute-binding protein AatJ (KTaatJ) completely lost the ability to proliferate on Glu and Gln as sole organic nutrient, whereas growth on Asp and Asn was only slightly impaired. Interestingly, KTaapP (a strain defective in the Aat nucleotide-binding domain) was still able to grow on Glu or Gln, although with a markedly longer lag phase and reduced growth yields, while growth of KTaapP on Asp and Asn was almost unaffected. Growth on His, Lys, Pro, Arg was unaffected by *aat* inactivation (Table 3). Branched-chain amino acids do not support growth of *P. putida* KT2440 when supplied as the sole source of carbon and nitrogen. Glucose supplied as an additional carbon source restored normal growth of both Aat mutants on any of the amino acids examined (data not

shown). As certain Glu uptake systems are coupled to a Na^+ gradient (see below), we also examined whether growth of KTaapP on Glu was affected by increasing the Na^+ concentration in the medium from 30 to 80 mM, but did not detect any positive growth effect (data not shown). As shown by Fig. 2, complementation of the mutations introduced into *aatJ* and *aatP* restored normal growth on any of the amino acids examined, indicating that the observed growth defects of KTaapJ and KTaapP were not caused by polar effects on downstream genes.

AatJMQP is a Glu/Asp importer

Next, we examined the uptake of Glu and Asp by wild-type KT2440 and the *aat* deletion mutants (KTaatJ and KTaapP). High-affinity transport at micromolar ligand concentrations was studied by a conventional filter assay using ^{14}C -labelled amino acids, whereas uptake at millimolar amino acid concentrations was monitored by measuring the disappearance of Glu and Asp from the medium by amino acid estimation using HPLC analysis. As shown in Fig. 3(a), high-affinity uptake of [^{14}C]Glu and [^{14}C]Asp by wild-type KT2440 proceeded at the same

Fig. 2. Growth of wild-type *P. putida* KT2440 and mutants on acidic amino acids and their amides. Cells were pre-grown on M9⁺ medium overnight and then transferred to M9 minimal medium supplemented with 5 mM amino acid as sole source of carbon and nitrogen. Growth is expressed as change in OD₅₉₅ (ΔOD_{595}). □, Wild-type; ●, KTaapJ; ○, KTaapJ⁺ (complemented); ■, KTaapP; □, KTaapP⁺ (complemented).

Table 3. Growth of *P. putida* KT2440 wild-type and mutants on amino acids as sole carbon and nitrogen sources

Growth curves (see Fig. 2) were fitted by a logistic model (Zwietering *et al.*, 1990) to estimate maximal absorption in the stationary state ($A_{\max}$ in milliabsorption units, mAU), maximal growth rate ($\mu_{\max}$, mAU h⁻¹) and duration of the lag phase (Lag, h). ND, Not determined.

Amino acid	Wild-type			KTaatJ			KTaatJ ⁺			KTaatP			KTaatP ⁺		
	$A_{\max}$ (mAU)	$\mu_{\max}$ (mAU h ⁻¹)	Lag (h)	$A_{\max}$ (mAU)	$\mu_{\max}$ (mAU h ⁻¹)	Lag (h)	$A_{\max}$ (mAU)	$\mu_{\max}$ (mAU h ⁻¹)	Lag (h)	$A_{\max}$ (mAU)	$\mu_{\max}$ (mAU h ⁻¹)	Lag (h)	$A_{\max}$ (mAU)	$\mu_{\max}$ (mAU h ⁻¹)	Lag (h)
Glu	1081	182	3.78	—	—	—	1090	171	5.6	586	33	3.8	1045	156	5.5
Gln	854	72	4.5	—	—	—	869	76	4.6	480	28	5.7	849	64	4.1
Asp	721	55	3.3	490	35	4.6	742	61	4.2	519	47	4.1	784	50	3.5
Asn	878	195	3.6	829	110	2.9	833	216	3.9	876	166	3.4	872	180	3.3
His	1089	118	6.0	1102	116	6.1	ND	ND	ND	1103	105	6.6	ND	ND	ND
Lys	553	72	9.7	561	78	10	ND	ND	ND	566	71	9.6	ND	ND	ND
Pro	1145	126	4.0	1148	119	4.5	ND	ND	ND	1107	115	4.5	ND	ND	ND
Arg	842	87	6.1	855	87	6.1	ND	ND	ND	851	100	7.9	ND	ND	ND

velocity. With both mutants (KTaatP and KTaafJ), uptake rates were reduced to 10–15 % of the wild-type values. As expected, addition of an excess of unlabelled Glu or Asp (50 μ M) inhibited the uptake of the respective labelled amino acid by 80–90 %, whereas 50 μ M unlabelled amides (Gln and Asn) did not significantly compete with the import of [¹⁴C]Glu or [¹⁴C]Asp (data not shown).

Similar results were obtained at millimolar Glu and Asp concentrations (Fig. 3b). Again, both amino acids were taken up from the medium at roughly the same rate. Under the conditions of the experiment, wild-type cells consumed the available amounts of Glu and Asp within 6–8 h, while the uptake by both mutants gradually slowed down to cease after 8–10 h.

Fig. 3. Kinetics of Glu and Asp uptake by *P. putida* KT2440 wild-type (○) and mutants KTaafJ (▲) and KTaafP (▼) at low and high amino acid concentrations. (a) ¹⁴C-labelled amino acids were added at zero time to a final concentration of 5 μ M. Aliquots were removed from the cultures at 30 s intervals, mixed with stop solution and filtered through nitrocellulose membranes. The washed and dried filters were analysed by liquid scintillation counting. The data shown represent mean $\pm$ SD of three experiments. (b) After adding unlabelled Glu or Asp to a final concentration of 5 mM at zero time, the decrease of amino acid concentration in the medium was followed by HPLC with pre-column derivatization (for details see text). Cumulative uptake is expressed as micromoles of amino acid consumed per millilitre of medium.

Ligand-binding properties of the AatJ solute receptor

As already mentioned, the selectivity of ABC solute importers is principally dependent on the binding characteristics of the cognate solute-binding protein (Davidson & Chen, 2004). To examine the binding specificity of the Aat system, we therefore overexpressed and purified the solute receptor AatJ for equilibrium binding studies. The system used allowed the transport of the overexpressed protein into the periplasmic space, from where it was conveniently released by osmotic shock. Two subsequent purification steps (ion exchange chromatography on Q-Sepharose and gel filtration on Sephacryl-S100) provided 6–7 mg of highly enriched product per litre of culture (Fig. 4). By analytical gel filtration, the resulting preparation was found to be a monomer with the expected mass of about 34 kDa (data not shown).

For quantitative assays of amino acid binding to AatJ the equilibrium dialysis technique was chosen. Saturation curves for Glu and Asp binding to AatJ are depicted in Fig. 5 (a semilogarithmic plot is shown for clarity). As the AatJ concentration used was in the same range as the dissociation constant of the receptor–amino acid complex, ligand depletion had to be taken into account (see Methods). By fitting such a model to the data, dissociation constants, K_d , of 0.3 μM for Glu and 1.5–2 μM for Asp were obtained. Since Gln and Asn were not available in radiolabelled form, a competition method was applied to determine their binding characteristics. In this approach, the reduction in binding of labelled ligand in the presence of a large excess of unlabelled amino acid is used to estimate

Fig. 4. Expression in *E. coli* BL21 and purification of the solute-binding protein AatJ. Samples were analysed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue R-250. M, marker proteins. Lanes: 1, cell lysate before induction; 2, cell lysate 6 h after induction; 3, crude periplasmic fraction; 4, after ion exchange chromatography (for details see Methods).

the dissociation constant of the competing unlabelled ligand. Constants calculated in this way are summarized in Table 4. In general, the values obtained by direct binding experiments and competition assays were in good agreement, with the exception of Asp, for which the competition assay yielded somewhat higher dissociation constants than the direct assay. Gln and the dicarboxylates fumarate and succinate were very weak ligands, with dissociation constants too high to be estimated with certainty.

The response regulator AauR binds to the *aat* promoter

As the AauR–AauS two-component system has been shown to induce the expression of *aatJMQP* (Sonawane *et al.*, 2006), it was of interest to delineate *cis*-acting elements in the *aat* promoter that are required for this activation. A promoter deletion series was prepared involving sequences between –83 and –202 relative to the transcription start site (+1). These fragments (schematically depicted in Fig. 6) were amplified from the KT2440 genome using primer PromFor and various PromRev primers (Table 2). The resulting PCR products were purified, labelled with ^{32}P and used to analyse AauR binding by gel mobility shift assays. The results, summarized in Fig. 6, show that binding of AauR to the *aat* operon promoter is specific. A non-specific DNA fragment of about the same length did bind to AauR (data not shown). Only those DNA

Fig. 5. Binding of [^{14}C]Glu (●) and [^{14}C]Asp (○) to AatJ. Binding curves of fractional saturation (moles ligand bound/moles monomeric protein) versus the logarithm of total ligand concentration are shown. Solid lines represent best fits of the model equation employed (see Methods). Mean values $\pm$ SD of three experiments are shown. Dissociation constants, K_d , estimated from the data are summarized in Table 4.

Table 4. Calculated dissociation constants, K_d , of AatJ ligand complexes

Abbreviations: ED, determined by equilibrium dialysis with ^{14}C -labelled ligand; C_{Glu} , estimated from inhibitory effect on Glu binding; C_{Asp} , estimated from inhibitory effect on Asp binding (see text).

Compound	K_d ($\mu\text{mol l}^{-1}$)	Estimated from:
L-Glutamate	0.3 ± 0.1	ED
	0.4	C_{Glu}
	0.3	C_{Asp}
L-Aspartate	1.5 ± 0.2	ED
	7	C_{Glu}
	9	C_{Asp}
L-Glutamine	>100	C_{Glu}
	80	C_{Asp}
L-Asparagine	18	C_{Glu}
	15	C_{Asp}
Succinate	>100	C_{Glu}
	>100	C_{Asp}
Fumarate	>100	C_{Glu}
	>100	C_{Asp}

fragments that contained base pairs -117 to -136 were shifted, indicating that this partial sequence of the *aat* promoter interacts with AauR.

The *P. putida* KT2440 genome contains multiple AauR recognition motifs

To define the AauR-binding site more precisely, a DNase I footprint assay was performed, using a promoter fragment

comprising base pairs -83 to -246 . As shown by Fig. 7(a), a 19 bp sequence located between -119 and -137 was efficiently and selectively protected by AauR. The footprint contains the conspicuous inverted repeat TTCGGNNNN-CCGAA. The overall length and spacing of this motif exactly meet the requirements for binding sites of dimeric helix–turn–helix proteins, of which AauR is a member (Tzou & Hwang, 1999). In addition, a typical $-12/-24$ motif (Barrios *et al.*, 1999), which is required for recruitment of the alternative sigma factor σ^{54} to the initial transcription complex, was found in the *aat* promoter.

In order to identify further *aau*-controlled genes, we used the Genomes Similarity Search tool at EMBL-EBI (<http://www.ebi.ac.uk/fasta33/genomes.html>) to screen the intergenic regions of the entire *P. putida* KT2440 genome for additional occurrences of the AauR-binding inverted repeat. In addition to the expected positives (*aat* and *ansB*), the search revealed additional promoters that contain a clear-cut AauR-binding motif (see Fig. 8). An especially intriguing hit was the monocistronic gene PP0137, which, by sequence homology, encodes a glutamate: H^+ symporter of the GltP type (Raunser *et al.*, 2006). Another putative AauR-binding motif was detected in the intergenic region of the operon *braCDEFG* (PP1141–PP1137), assigned as an ABC transporter for branched-chain amino acids. However, this assigned solute specificity needs to be substantiated by experiment, as the homologous Bra system of *Rhizobium leguminosarum* encodes a general amino acid permease with a broad range of solutes including L-Glu (Hosie *et al.*, 2001). Two further genes with possible AauR-binding sites are not directly involved in amino acid uptake: the product of the *dsbC* gene (PP1469) assists in the periplasmic formation of disulfide bonds and has been shown to play an important

Fig. 6. Gel mobility shift assay of AauR binding to *aat* promoter fragments. Lengths and positions (relative to the transcription start site) of fragments A–E are depicted schematically at the top of the figure. Within each block, the leftmost lane contains 5 fM ^{33}P -labelled DNA without AauR, while lanes 2–5 contain labelled probe DNA plus increasing amounts of AauR (2–10, 3–20, 4–30 and 5–50 nM). A, -202 to -343 ; B, -157 to -343 ; C, -136 to -343 ; D, -117 to -343 ; and E, -83 to -343 . Mobility was analysed by 6% native PAGE and autoradiography.

Fig. 7. (a) DNase I footprint of AauR on the *aatJ* promoter. Fifty nanograms of ^{32}P -labelled DNA fragments corresponding to positions -83 to -246 were incubated with 0 – 100 nmol AauR, treated with 0.05 U DNase I for 2 min at 37°C , and separated on an 8% polyacrylamide sequencing gel. Lanes: 1, G + A sequencing ladder; 2, C + A sequencing ladder; 3 and 4, promoter fragment without AauR; 5–9, promoter sequence with 20 – 100 nmol AauR. (b) DNA sequence of the *aat* promoter region showing the σ^{54} -binding site (-12 – -24), and the transcription and translation start sites. The inverted repeat within the -119 to -137 region protected by AauR is highlighted by white type on a black background.

role in the maturation of extracellular enzymes (Urban *et al.*, 2001), while *ppsA* (PP2082) encodes phosphoenolpyruvate synthase (PpsA), a key enzyme in gluconeogenesis from α -keto acids (Chao *et al.*, 1993).

Sequence alignments of *aat* with homologous genes from other *Pseudomonas* spp. revealed more than 95% similarity. As shown in Fig. 8(b) the AauR-binding motif (TTTCGGNNNNCCGAA) is also well conserved in the *aat* homologues of other pseudomonads. The same holds for further genes with a putative AauR recognition site (*ansB*, *gltP*, *bra* and *dsbC*; data not shown).

DISCUSSION

The acidic amino acids (Asp, Glu) and their amides (Asn, Gln) are excellent growth substrates for *P. putida* KT2440, being comparable to NH_4^+ as nitrogen sources or to glucose as carbon sources (Sonawane *et al.*, 2003a). By contrast, *E. coli* is unable to proliferate on Glu as sole carbon source unless the capacity of its Glu uptake systems is increased by mutations (Booth *et al.*, 1989). Again, contrary to *P. putida*, *E. coli* also grows more slowly on

gluconeogenic carbon sources (e.g. succinate) than on glucose itself. Here, we show that the ability of *P. putida* KT2440 to proliferate on Glu or Gln as sole carbon and nitrogen source largely depends on the ABC transporter AatJMQP. The role of AatJMQP in Gln utilization is an indirect one, as *P. putida* KT2440 does not contain a Gln transporter; Gln is first hydrolysed by a periplasmic glutaminase/asparaginase (AnsB) to yield Glu, which is then taken up by the Aat system. *P. putida* mutants lacking a functional AnsB, therefore, are unable to grow on Gln (Sonawane *et al.*, 2003a). Utilization of Asp and Asn by strain KT2440 does not depend on a functional Aat system, indicating that additional uptake systems for Asp and/or Asn exist. The presence of a selective Asn transporter in *P. putida* has not been established; it is known, however, that pseudomonads contain a cytosolic hydrolase with selectivity for Asn that could convert imported Asn to Asp (Sonawane *et al.*, 2003a).

As judged by sequence similarity, AatJMQP is a typical receptor-dependent ABC transporter, belonging to the polar amino acid uptake transporter (PAAT) family (Saier, 2000). The closest orthologue of the *P. putida* Aat system in *E. coli* is the ABC transporter GltIJKLM (Linton & Higgins,

(a)

Gene		Function/annotation
PP1071	ATTGCTCCTTCGGTTATCCGAACGCA	Glu/Asp ABC transporter (<i>aat</i>)
PP0137	TGGTCTTCTTCGGGATTCCGAATGTT	Glu/Asp:H ⁺ symporter (<i>gltP</i>)
PP1141	CTTCGGTTTTCGGCTTCCGAAGTGC	Branched-chain aa transporter (<i>bra</i>)
PP1469	GTGCGGCTTTCGGGTTACCGAACTGG	Thiol/disulfide exchange protein (<i>dsbC</i>)
PP2082	ATGATAACTTCGGTTTGCCGAATGCG	Phosphoenolpyruvate synthase (<i>ppsA</i>)
PP2453	GCATTGCATTTCGTAATTCCGAACAAA	Glutaminase/asparaginase motif 1 (<i>ansB</i>)
PP2453	AAAAGGTCTTCGGTTTTTCCGAATAAG	Glutaminase/asparaginase motif 2 (<i>ansB</i>)

(b)

PP1071	ATTGCTCCTTCGGTTATCCGAACGCA	<i>P. putida</i> KT2440
PFL4871	CATGCGCCTTCGGTTTTCGAATGGC	<i>P. fluorescens</i> Pf-5
PA1342	GCATGGCCTTCGGTTTTCGAACGTT	<i>P. aeruginosa</i> PA01
PSPT04171	CGACTGCATTTCGGCTTTCGAACGCG	<i>P. syringae</i> DC3000
PSYR3908	CCCTTCCATTTCGGTTTTCGAACAAT	<i>P. syringae</i> pv. B728a
PSPPH3902	CCGTTGCATTTCGGTTTTCGAACATT	<i>P. syringae</i> pv. <i>phaseolicola</i>
PSEEN1193	ATATGGCCTTCGGAAATCCGAATGAG	<i>P. entomophila</i> L48

Consensus CTTCGGNNNNCCGAAC.....

Fig. 8. Alignment of verified and putative AauR-binding sites. While the AauR-binding motif within the *P. putida* KT2440 *aat* gene was experimentally identified in this work, all others were detected by *in silico* genome screening (see text). (a) AauR-binding motifs (white type on black background) in *P. putida* KT2440 genes related to acidic amino acid transport and metabolism; (b) AauR-binding motifs in *aat* promoters of other pseudomonads.

1998). Although this system is still poorly characterized, the available data suggest that its amino acid-binding properties are similar to those reported here for Aat. Furlong and co-workers reported apparent K_d values for the *E. coli* Glu/Asp-binding protein of 0.7 μ M for Glu and 1.2 μ M for Asp, respectively (Willis & Furlong, 1975; Schellenberg & Furlong, 1977), i.e. values that are in close agreement with our data (Table 4).

In addition to GltJKLM, *E. coli* contains at least two additional transport systems for Glu and/or Asp, i.e. an H⁺ symporter for both Glu and Asp (GltP) and an Na⁺-dependent Glu-specific uptake system (GltS). GltP from *E. coli* has been studied in considerable detail, and has been shown to catalyse an electrogenic cotransport of Glu and Asp with H⁺ ions (Tolner *et al.*, 1995; Raunser *et al.*, 2006), while GltS is a binding protein-independent Glu:Na⁺ symporter (Reizer *et al.*, 1994) with selectivity for L- and D-glutamate, homocysteate and α -methylglutamate (Kalman *et al.*, 1991). Bacterial uptake systems with a strict selectivity for Asp seem to be uncommon. However, many organisms express C₄-dicarboxylate carriers that not only import dicarboxylates such as succinate or fumarate but also Asp, although with lower affinity (Janausch *et al.*, 2002). The *E. coli* genome encodes several such C₄-dicarboxylate carriers, most of which are only expressed under anaerobic conditions. The main system responsible for aerobic Asp uptake, DctA, operates as an H⁺ or Na⁺:dicarboxylate symporter and catalyses the uptake of succinate, fumarate, malate, aspartate and other carboxylic acids.

All of the Glu/Asp uptake systems mentioned above are also encoded by the *P. putida* KT2440 genome. A comparison of the respective loci in strain KT2440 and *E. coli* K12 is shown in Table 5. Mutual sequence identities of 50 % or more and similarities between 60 and 86 % suggest that the gene products of *gltP*, *gltS* and *dctA* in *P. putida* are functional counterparts of the respective *E. coli* systems. The growth characteristics and amino acid uptake properties of the *P. putida* strains described here indicate that AatJMQP is the most important Glu uptake system under the conditions in our experiments. Note that the growth curves shown in Fig. 2 and Table 3 and the data summarized in Fig. 3 were measured with cells pre-grown on NH₄⁺/glucose, i.e. under conditions where the Aat system was not upregulated by amino acids. Thus, even at its basal level of activity, AatJMQP appears to account for about 85 % of the capacity for high-affinity Glu transport. Cells lacking a functional AatJ did not proliferate on Glu at all, while a mutant with a defective nucleotide-binding domain AatP did, although with a lag phase of 6–8 h and reduced cell yields (Fig. 2). An explanation for this unexpected finding is suggested by data indicating that nucleotide-binding domains of ABC transporters may interact not only with their cognate transporter but also with other ABC-type permeases. So Quentin *et al.* (1999) proposed that in *Bacillus subtilis* three ATPase subunits energize 10 different transporters. Similar observations have also been made with carbohydrate importers of the ABC type (Schneider, 2001). Thus, nucleotide-binding domains of other PAAT-family transporters of *P. putida*

Table 5. Established or putative Glu/Asp transporters of *E. coli* and their *P. putida* KT2440 homologues

Sequence identities and similarities were derived from FASTA3 alignments (see text).

Uptake system	<i>E. coli</i> K12	Identity (%)	Similarity (%)	<i>P. putida</i> KT2440	Function
ABC transporter (Glu/Asp)	GltI	55	69	AatJ (PP1071)	Solute-binding protein
	GltJ	49	68	AatM (PP1070)	Permease subunit
	GltK	52	66	AatQ (PP1069)	Permease subunit
	GltL	70	79	AatP (PP1068)	ATP-binding domain
Glu/Asp:H ⁺ symporter	GltP	62	73	GltP (PP0137)	Permease
Glu:Na ⁺ symporter	GltS	50	60	GltS (PP0996)	Permease
C ₄ -dicarboxylate/Asp:H ⁺ symporter	DctA	73	86	DctA (PP1188)	Permease

may partially compensate for the loss of AatP to sustain growth of strain KTaatP.

We have shown previously that the *aat* operon and the glutaminase-encoding *ansB* gene of *P. putida* KT2440 are upregulated by the AauRS two-component system (Sonawane *et al.*, 2003b). Here we provide evidence indicating that at least four additional genes are under the control of AauR, i.e. the Glu/Asp importer GltP, the enzyme phosphoenolpyruvate synthase (PpsA), a thiol-disulfide exchange protein (DsbC) and the *braCDEFG* operon (PP1141–PP1137). Assuming that all of these genes are indeed regulated by *aau*, a consistent picture of Glu/Asp utilization by *P. putida* KT2440 and the regulatory role of the AauR–AauS two-component system begins to

emerge (Fig. 9). Acidic amino acids and their amides present in the medium first activate the AauR–AauS system, which then upregulates (1) glutaminase/asparaginase [AnsB, enzyme (1)] to allow for the extracellular conversion of Asn and Gln to Asp and Glu, respectively; (2) Glu/Asp transporters (AatJMQP, and possibly also GltP and Bra; see below) to facilitate uptake of the Asp and/or Glu; and (3) phosphoenolpyruvate synthase [PpsA, enzyme 8] to stimulate gluconeogenesis from Asp and Glu (the gluconeogenic pathway becomes essential for the synthesis of carbohydrates and lipids if amino acids are supplied as sole sources of carbon and nitrogen). The enzymes numbered 2–7 in Fig. 9 all catalyse reactions that channel the carbon skeletons of Glu or Asp into the tricarboxylic acid cycle or from there into pyruvate.

Fig. 9. Simplified scheme of acidic amino acid utilization and metabolism in *P. putida* KT2440 (see text). Metabolites are shown in italic type, while proteins known or thought to be under the control of the *aau* two-component system are rendered in bold type. Important enzymes are indicated by numbers as follows: 1, periplasmic glutaminase/asparaginase (AnsB); 2, glutamate dehydrogenase; 3, aspartate aminotransferase; 4, aspartate lyase (aspartase); 5, aspartate oxidase; 6, oxaloacetate decarboxylase; 7, malic enzyme; 8, phosphoenolpyruvate synthase.

At present, the function of thiol–disulfide isomerase (DsbC) remains a matter of speculation. The DsbC protein may act as a chaperone in the folding of periplasmic proteins required for Glu/Asp uptake, such as AatJ or AnsB. Indeed, several periplasmic solute-binding proteins as well as the periplasmic *E. coli* asparaginase isoenzyme contain disulfide bonds. The roles of GltP and Bra in Glu uptake by *P. putida* are still unresolved. Although the respective genes contain an AauR-binding motif, their protein products do not compensate for an inactivated AatJMQP, at least under our experimental conditions. The ABC transporters BraCDEFG as well as the related AapJQMP were shown to catalyze not only amino acid uptake but also the export of Glu from *R. leguminosarum* as part of an overflow pathway (Hosie *et al.*, 2001). We have provided evidence to indicate that the BraCDEFG transporter of *P. putida* KT2440 might fulfil a similar role. While *bra* was down-regulated during growth on Glu, a Glu-grown KTaauR mutant lost Glu by efflux (Sonawane *et al.*, 2006). We therefore hypothesize that binding of AauR to the *P. putida bra* promoter does not induce but rather inhibits expression of *bra* and thus helps to prevent losses of Glu via the overflow pathway. Further experiments are required to clarify these points.

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