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 $1.3$ μM, 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.

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 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* (PFl1048–PFl1051) 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 $\sigma^4$, 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 NH$_4$Cl 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 KTaatU.** Genes *aatP* (PP1068) and *aatU* (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 (*aatU*), 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 KTaatP and KTaatU were complemented with intact copies of the respective functional genes as follows. The coding sequence and the promoter of gene *aatU* were amplified from KT2440 genomic DNA using primers *CaatU* For and *CaatU* Rev. A stop codon was inserted into *CaatU* For immediately after the restriction site to block expression from the tac promoter of the vector; thus *aatU* 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 BamHI/EcoRI-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).](image-url)
vector commonly used for complementation experiments with *Pseudomonas* spp. The sequenced vectors were electroporated into KT2440 and KTaatP, and complemented clones were isolated by carbenicillin selection, yielding strains KTaatP* 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 *Hind*III and the excised fragment purified by agarose gel electrophoresis using the Qiagen gel extraction kit. The purified fragments were labelled with $^{33}$P in a 50 μl reaction volume containing 10 μl 5× forward reaction buffer, 5 pmol DNA, 25 U T4 Polynucleotide Kinase (TruBio) and 5 μl [γ-$^{33}$P]ATP ($^{33}$P 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 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, origin</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. putida strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KT2440</td>
<td><em>mt</em>-2, <em>hsdR1</em> ($r^-$ $m^+$)</td>
<td>Bagdasarian &amp; Timmis (1982)</td>
</tr>
<tr>
<td>KtaatP</td>
<td>aatP derivative of KT2440</td>
<td>This work</td>
</tr>
<tr>
<td>KtaatP*</td>
<td>KTaatP complemented with functional aatP</td>
<td>This work</td>
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<tr>
<td>KtaatP†</td>
<td>KTaatP derivative</td>
<td>This work</td>
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<tr>
<td>KtaatP‡</td>
<td>KTaatP complemented with functional aatP</td>
<td>This work</td>
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<td><strong>E. coli strains</strong></td>
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<td>Amersham Biosciences</td>
</tr>
<tr>
<td>HB101</td>
<td><em>supE44</em>, <em>hsdS20</em> ($r^-$ $m^+$), <em>recA</em>, <em>ara</em>-14, <em>proA21</em>, <em>acY1</em>, <em>galK2</em>, <em>rpsL20</em></td>
<td>Amersham Biosciences</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pTrcHisA</td>
<td>Expression vector, pBR322 <em>ori</em>, <em>lac</em>O, <em>lacZ</em>, <em>tac</em>, <em>lacO</em>, <em>amp</em></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pFLAG-ATS</td>
<td>Expression vector, <em>ompA</em>, <em>p</em>ta, <em>lacI</em>, <em>amp</em></td>
<td>Sigma</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PP1068_For*</td>
<td>5′-CGCggattcTCGGGCAAGGTCCACCCT-3′</td>
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<tr>
<td>PP1068_Rev*</td>
<td>5′-CCCAagcttATACAGGCGTCGCCACAGC-3′</td>
</tr>
<tr>
<td>PP1071_For*</td>
<td>5′-CCGggattcTCGCAATGCAATCCAGCGTGCC-3′</td>
</tr>
<tr>
<td>PP1071_Rev*</td>
<td>5′-CCGagcttGTCAACAGTTCGTGCCGCC-3′</td>
</tr>
<tr>
<td>AatJ_For†</td>
<td>5′-CGCGgattcGAGGAGCTCAGCGCCACCT-3′</td>
</tr>
<tr>
<td>AatJ_Rev†</td>
<td>5′-GGCCggattcCTAGGACTTTCTCCGAGCAG-3′</td>
</tr>
<tr>
<td>CaatJ_For‡</td>
<td>5′-CCGggattcTAGGGCATGCGGCCCCCTCTCCGAGG-3′</td>
</tr>
<tr>
<td>CaatJ_Rev‡</td>
<td>5′-CCGGggattcTCAGGACTTTCTCCGAGCGG-3′</td>
</tr>
<tr>
<td>CaatP_For‡</td>
<td>5′-CGGGggattcGTGGCTGTCAAGCGGCCAGCAG-3′</td>
</tr>
<tr>
<td>CaatP_Rev‡</td>
<td>5′-CGGGggattcATGTTGCGATGAGGAATCGTCCAGGAGG-3′</td>
</tr>
<tr>
<td>aauR_For‡</td>
<td>5′-CGGggattcATGAACCAAGGCCTTCTTAC-3′</td>
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<td>PromRev_117</td>
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</tr>
<tr>
<td>PromRev_202</td>
<td>5′-ACGGATGCGGCCCACCCATTACCTCCGGC-3′</td>
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</table>

*BanHI and *Hind*III sites in lower-case type; inserted base in bold/underlined type.
†*Hind*III and EcoRI sites in lower-case type.
‡*BanHI and EcoRI 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 BamHI to remove the 5′-3′ 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 1 mM IPTG. After another 6 h, the cells were collected at 5000 g, washed with 100 mM 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) and protein was eluted with a linear gradient of 0–1 M NaCl. The AauR-containing fractions were pooled and concentrated using a Centricon cartridge (10 kDa MWCO), pooled, concentrated using a Centricon cartridge (10 kDa MWCO), and stored at 4 °C.

**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, KTaatP (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 KTaatP 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 KTaatP 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 KTaatJ and KTaatP 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 KTaatP). High-affinity transport at micromolar ligand concentrations was studied by a conventional filter assay using ¹⁴C-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 [¹⁴C]Glu and [¹⁴C]Asp by wild-type KT2440 proceeded at the same

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### 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 (ΔOD₅⁹⁵). □, Wild-type; ■, KTaatJ; ○, KTaatJ⁺ (complemented); ●, KTaatP; □, KTaatP⁺ (complemented).
velocity. With both mutants (KTaatP and KTaatJ), uptake rates were reduced to 10–15% of the wild-type values. As expected, addition of an excess of unlabelled Glu or Asp (50 μM) inhibited the uptake of the respective labelled amino acid by 80–90%, whereas 50 μM 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.

**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 (μ_max, mAU h⁻¹) and duration of the lag phase (Lag, h). ND, Not determined.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Wild-type</th>
<th>KTaatJ</th>
<th>KTaatJ⁺</th>
<th>KTaatP</th>
<th>KTaatP⁺</th>
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<tbody>
<tr>
<td></td>
<td>A_max (mAU)</td>
<td>μ_max (mAU h⁻¹)</td>
<td>Lag (h)</td>
<td>A_max (mAU)</td>
<td>μ_max (mAU h⁻¹)</td>
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<tr>
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<td>1081</td>
<td>182</td>
<td>3.78</td>
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<tr>
<td>Gln</td>
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<td>4.5</td>
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<td>Arg</td>
<td>842</td>
<td>87</td>
<td>6.1</td>
<td>855</td>
<td>87</td>
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</table>

**Fig. 3.** Kinetics of Glu and Asp uptake by *P. putida* KT2440 wild-type (○) and mutants KtaatJ (▲) and KtaatP (▼) at low and high amino acid concentrations. (a) 14C-labelled amino acids were added at zero time to a final concentration of 5 μM. 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 ± 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 \( \mu \)M for Glu and 1.5–2 \( \mu \)M 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 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 \(^{33}\)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

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**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).

**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 ± 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d$ (µmol $\text{l}^{-1}$)</th>
<th>Estimated from:</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>0.3 ± 0.1</td>
<td>ED</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>1.5 ± 0.2</td>
<td>ED</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>$C_{\text{Asp}}$</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>&gt;100</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>18</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>$C_{\text{Asp}}$</td>
</tr>
<tr>
<td>Succinate</td>
<td>&gt;100</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
<td>$C_{\text{Asp}}$</td>
</tr>
<tr>
<td>Fumarate</td>
<td>&gt;100</td>
<td>$C_{\text{Glu}}$</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
<td>$C_{\text{Asp}}$</td>
</tr>
</tbody>
</table>

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 TTCGGNNNNCCGAA. 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 $\sigma^{54}$ to the initial transcription complex, was found in the aat promoter.

In order to identify further aat-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 $^{32}$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.](image-url)
role in the maturation of extracellular enzymes (Urban et al., 2001), while ppsA (PP2082) encodes phosphoenolpyruvate synthase (PpsA), a key enzyme in gluconeogenesis from \( \alpha \)-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 (TTCGGNNNNCCGAA) 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 \( \text{NH}_4^+ \) 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 GltJKLM (Linton & Higgins,
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 $\mu M$ for Glu and 1.2 $\mu M$ 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 $\alpha$-methylglutamate (Kalman et al., 1991). Bacterial uptake systems with a strict selectivity for Asp seem to be uncommon. However, many organisms express $C_4$-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 $C_4$-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 AatJMPQ 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 NH$_4$/glucose, i.e. under conditions where the Aat system was not upregulated by amino acids. Thus, even at its basal level of activity, AatJMPQ appears to account for about 85% of the capacity for high-affinity Glu transport. Cells lacking a functional Aat or a functional AatP 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 $P. putida$

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**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.
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 (DscB) 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 gluconeogenetic 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.

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

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

<table>
<thead>
<tr>
<th>Uptake system</th>
<th>E. coli K12 Identity (%)</th>
<th>Similarity (%)</th>
<th>P. putida KT2440 Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC transporter (Glu/Asp)</td>
<td>GltI 55</td>
<td>69</td>
<td>AatJ (PP1071) Solute-binding protein</td>
</tr>
<tr>
<td></td>
<td>GltJ 49</td>
<td>68</td>
<td>AatM (PP1070) Permease subunit</td>
</tr>
<tr>
<td></td>
<td>GltK 52</td>
<td>66</td>
<td>AatQ (PP1069) Permease subunit</td>
</tr>
<tr>
<td></td>
<td>GltL 70</td>
<td>79</td>
<td>AatP (PP1068) ATP-binding domain</td>
</tr>
<tr>
<td>Glu/Asp: H⁺ symporter</td>
<td>GltP 62</td>
<td>73</td>
<td>GltP (PP0137) Permease</td>
</tr>
<tr>
<td>Glu: Na⁺ symporter</td>
<td>GltS 50</td>
<td>60</td>
<td>GltS (PP0996) Permease</td>
</tr>
<tr>
<td>C₄-dicarboxylate/Asp: H⁺ symporter</td>
<td>DctA 73</td>
<td>86</td>
<td>DctA (PP1188) Permease</td>
</tr>
</tbody>
</table>

### Fig. 8. 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 transaminase; 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 AatJMPQ, at least under our experimental conditions. The ABC transporters BraCDEFG as well as the related AapJQMP were shown to catalyse 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.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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