

A periplasmic, pyridoxal-5'-phosphate-dependent amino acid racemase in *Pseudomonas taetrolens*

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Abstract The pyridoxal-5'-phosphate (PLP)-dependent amino acid racemases occur in almost every bacterium but may differ considerably with respect to substrate specificity. We here isolated the cloned broad substrate specificity racemase ArgR of *Pseudomonas taetrolens* from *Escherichia coli* by classical procedures. The racemase was biochemically characterized and amongst other aspects it was confirmed that it is mostly active with lysine, arginine and ornithine, but merely weakly active with alanine, whereas the alanine racemase of the same organism studied in comparison acts on alanine only. Unexpectedly, sequencing the amino-terminal end of ArgR revealed processing of the protein, with a signal peptide cleaved off. Subsequent localization studies demonstrated that in both *P. taetrolens* and *E. coli* ArgR activity was almost exclusively present in the periplasm, a feature so far unknown for any amino acid racemase. An ArgR-derivative carrying a carboxy-terminal His-tag was made and this was demonstrated to localize even in an *E. coli* mutant devoid of the twin-arginine translocation (Tat) pathway in the periplasm. These data indicate that ArgR is synthesized as a prepeptide and translocated in a Tat-independent manner. We therefore propose that ArgR translocation depends on the Sec system and a post-translocational insertion of PLP occurs. As

further experiments showed, ArgR is necessary for the catabolism of D-arginine and D-lysine by *P. taetrolens*.

Keywords Racemase specificity · Sec pathway · Alanine racemase · Lysine racemase · Tat-translocation · Periplasmic localization

Introduction

The pyridoxal-5'-phosphate (PLP)-dependent amino acid racemases catalyze the racemization of their substrate by removal of an α -hydrogen bound from a chiral carbon followed by the nonspecific return of a hydrogen to the same carbon. A number of different amino acid racemases of this type has been studied, and the racemases differ remarkably with respect to their substrate specificity. An example for a highly specific amino acid racemase is the alanine racemase, like that of *Bacillus psychrosaccharolyticus* (Okubo et al. 1999) or that of *Corynebacterium glutamicum* (Oikawa et al. 2006) which is active with alanine only, but not with the structurally related serine, aminobutyrate, not to mention aspartate, glutamate, methionine, or lysine. These enzymes, encoded by *alr*, act on L-alanine to provide D-alanine for cell wall synthesis and they are therefore widespread among bacteria to ensure provision of D-alanine as a cell wall building block. In addition to this anabolic alanine racemase, a catabolic alanine racemase is also known. This latter enzyme is less frequently distributed and encoded by *dadX* (but also annotated as *alr*). Thus, *E. coli* has both *alr* and *dadX* (Wild et al. 1985). Another example of a highly specific amino acid racemase is the catabolic ornithine racemase of *Clostridium sticklandii*. The latter is active with ornithine only, but not with L-lysine, L-arginine, L-methionine, or other amino acids (Chen et al. 2000). In contrast to these specific

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racemases, the broad substrate specificity racemases are less frequent. An example for such a racemase with broad substrate specificity is an arginine racemase isolated from *Pseudomonas taetrolens* as early as 1971 (Yorifuji and Ogata 1971; Yorifuji et al. 1971). Its analysis revealed activity with the basic amino acids D-arginine, L-lysine, and L-ornithine, and further amino acids, but almost no activity with hydrophobic, acidic or aromatic amino acids. It was designated ArgR, but proteins with similar enzymological features and termed BsRC (for broad specificity racemase) are also present in *Pseudomonas putida* ATCC 17642 (Lim et al. 1993; Roise et al. 1984), or *Aeromonas punctata* (Inagaki et al. 1984), suggesting a close relationship of the proteins. Recently, the gene sequence of *P. putida* ATCC 17642 became available, and the rather weak racemase activity of wild-type BsRC with L-tryptophan as substrate was evolved to increase its activity with this specific substrate about 20-fold (Kino et al. 2007). Although there is some information on the enzymological features of broad substrate specificity racemases, which is in part due to their relaxed substrate specificity for preparing D-amino acids, there is only limited information on the physiological function of these enzymes. We therefore started to clone the gene encoding the broad substrate specificity racemase of *P. taetrolens* but noticed that the open reading frame was apparently larger than the peptide sequence of *P. putida* ATCC 17642 found in the literature (Kino et al. 2007). Moreover, we found distracting that in *P. putida* KT2440 (ATCC 47054) which is one of the fully sequenced *P. putida* strains currently available, there are two genes annotated alanine racemases (Nelson et al. 2002). Based on this information, we started to analyze in detail the corresponding genes and enzymes, respectively, of *P. taetrolens* leading to the first identification of a PLP-containing amino acid racemase located in the periplasm.

Materials and methods

Strains and growth

The *P. taetrolens* strain used was obtained from the NITE Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan as strain NBRC 3460. Aliases of this strain are *P. graveolens* IFO 3460, and *P. taetrolens* ATCC 4683. The further strains and plasmids used are given in Table 1.

The medium used for cultivation of *P. taetrolens* was usually LB. For carbon source utilization studies, minimal medium No. 154 was used (Stalon et al. 1967). Cells were grown in 50 ml medium in a 500-ml baffled Erlenmeyer flask at 30 °C and 160 rpm on a rotary shaker. Growth was followed by measuring the optical density at 600 nm.

Gene isolation and cloning

Chromosomal DNA was isolated using the FastPure kit of TaKaRa (TaKaRa, Kyoto, Japan). Primers A and B, 0.2 pmol of each, were used together with 1.4 µg chromosomal DNA from *P. taetrolens* as a template to generate an amplification product at an annealing temperature of 40 °C. This was used as the template in a second PCR at an annealing temperature of 52 °C with primers C and D. The product was purified, cloned in pT7 Blue T, and sequenced using the DNA sequencer SQ5500E (Hitachi High-Technologies Corp., Tokyo, Japan). Based on the partial sequence obtained, new primers were designed to perform genome-walking PCR with the LA PCR™ in vitro cloning kit supplying HindIII and EcoRI cassettes (TaKaRa, Kyoto, Japan). Based on the 5'- and 3'- ends of *argR* obtained, primers E, F were designed to amplify the entire *argR* gene from genomic DNA of *P. taetrolens*. The product obtained was digested with NdeI and BamHI, and ligated into NdeI/BamHI digested pET11b to form pET11b-*argR*. Cloning of *alr* was similarly achieved, involving a final amplification step with the primer pair G and H, digestion of the amplification product with NdeI and BamHI, and ligation with the accordingly treated vector to yield pET21b-*alr*. To generate the inactivation vector pK18mob-*argR*-int, an internal HincII fragment of *argR* derived by restriction was prepared from pET11b-*argR*, which was ligated with SmaI-cleaved pK18mob. Subsequently, pK18mob-*argR*-int was used to transform *P. taetrolens* NBRC 3460 by electroporation to kanamycin resistance. One colony was isolated and PCR analyses used to confirm disruption of *argR*.

Isolation of ArgR

Cells of *E. coli* BL21 (DE3) (pET11b-*argR*) were harvested from 1.2 l LB medium and washed twice with cold 0.85% (w/v) NaCl, followed by resuspension in 74 mM Tris-HCl (pH 8.0) buffer containing 0.5 M sucrose and 1 mM EDTA (buffer A). After centrifugation at 8,000×g for 20 min, the cells were resuspended in ice-chilled water, and after brief incubation centrifuged at 5,500×g for 10 min. The supernatant was collected and used as a cell-free extract. This was dialyzed against a 10 mM potassium phosphate buffer (pH 7.3) containing 0.01% 2-mercaptoethanol and 20 µM pyridoxal-5'-phosphate (PLP; buffer B). The enzyme solution was applied to a column of DEAE-Toyopearl 650 M (2.5×30 cm) equilibrated with buffer B, and washed with the same buffer containing 40 mM KCl. Proteins were eluted with a linear gradient of KCl at a concentration of 40 to 80 mM in buffer B, and active fractions combined and dialyzed against buffer B containing 1.2 M (NH₄)₂SO₄. The resulting solution was applied to a column of Butyl-Toyopearl 650 M (1.25×15 cm) equilibrated with the same

Table 1 Strains, plasmids, and primers used

	Comments
Strains	
<i>E. coli</i> BL21 (DE3)	Expression host
<i>E. coli</i> GSJ101	Derivative of GSJ100 deleted of Tat apparatus (Blaudeck et al. 2003)
<i>E. coli</i> GSJ100	Control strain (Blaudeck et al. 2003)
<i>P. taetrolens</i>	Wild type
<i>P. taetrolens</i> ::argR	Wild type with <i>argR</i> disrupted
Plasmids	
pET11b- <i>argR</i>	Expression vector, contains full-length <i>argR</i>
pET21b- <i>alr</i>	Expression vector, contains C-terminal His-tagged <i>alr</i>
pK18mob- <i>argR</i> -int	Non-replicative vector for disruption of <i>argR</i>
pBBR1MCS2	Low copy number vector (Kovach et al. 1995)
pBBR1MCS2ArgR-His	Vector with C-terminal His-tagged full-length <i>argR</i>
pBBR1MCS2ArgR	Vector with full-length <i>argR</i>
Primers	
A	GTGCTCAA(A/G)GC(C/G)GA(C/T). First amplification of <i>argR</i>
B	GGTGCGGTC(A/G)TAGCC(C/G)AC. First amplification of <i>argR</i>
C	GACGCCA(C/T)GG(A/T/C)CA(C/T)GGT. Second amplification of <i>argR</i>
D	GGTGCGGTC(A/G)TAGCC(C/G)AC. Second amplification of <i>argR</i>
E	TCAGCATATGCCCTTCTCCCGTAC. Amplification of <i>argR</i>
F	GACGGATCCTGGTTCAATATACGG. Amplification of <i>argR</i>
G	TATCCATATGCGTCCCGCCCGCGCC. Amplification of <i>alr</i>
H	ATAGGATCCCGTTCGCCGATGTA. Amplification of <i>alr</i>

buffer, and the column washed with buffer B containing 0.9 M (NH₄)₂SO₄. Proteins were eluted with a linear gradient of (NH₄)₂SO₄ at a concentration of 0.9 to 0.7 M in buffer B, and active fractions combined and dialyzed against buffer B. Finally, the enzyme solution obtained was applied a second time to DEAE-Toyopearl 650 M chromatography under identical conditions as described to yield pure ArgR which was dialyzed and stored frozen in buffer B.

Alr was prepared from *E. coli* BL21 (DE3) harboring pET21b-*alr* which was disrupted by sonification. The cell-free extract was dialyzed against a 10 mM potassium phosphate buffer (pH 7.5) containing 300 mM NaCl and 10 mM imidazole (buffer C), and the protein solution applied to a Ni-NTA agarose column (1.0×10 cm) equilibrated with the same buffer. The column was washed with buffer C but containing 50 mM imidazole, and eluted by increasing the imidazole concentration to 250 mM. Alr was finally dialyzed and stored in buffer B.

Analysis of phosphopyridoxyl peptide

ArgR was reduced with sodium borohydride, digested with trypsin and the peptides were separated by HPLC on a Wakosil-II 5C-18 AR column (Wako, Osaka, Japan). Fluorescence detection was at 330 nm excitation and 390 nm emission. The fluorescent phosphopyridoxyl peptide was isolated and subjected to Edman degradation using an

automated protein sequencer (PPSQ-21, Shimadzu Co., Kyoto, Japan).

Enzyme assays

Amino acid racemase activities with arginine or lysine as a substrate were in part measured in coupled assays with L-arginase (Yorifuji and Ogata 1971; Yorifuji et al. 1971) or lysine 6-aminotransferase (Yagi et al. 1980). However, generally, amino acid racemase activities were measured via HPLC quantification of the respective enantiomer formed (Ono et al. 2006). The assay mixtures contained a 0.6 M CHES–NaOH buffer, pH 10.0 (130 μl), 0.1 M L-amino acid (80 μl), 0.1 mM PLP (200 μl), and enzyme. Reactions were carried out at 37 °C for different time intervals, stopped by heat treatment at 100 °C for 2 min, and subjected to HPLC analysis. Protein was determined with bovine serum albumin as standard, with one unit being defined as the amount of enzyme producing one μmol of enantiomer per min.

Localization studies

Fractionation of *P. taetrolens* and *E. coli* BL21 (DE3) harboring pET11b-*argR* was essentially as described (Morita et al. 2006). Both bacteria were pre-cultured at 30 °C in 5 ml LB and 100 μl of this pre-culture transferred into four Sakaguchi flasks (500 ml) containing 100 ml LB

and cultivated at 30 °C aerobically. Per data point, cells of one flask were harvested by centrifugation at 14,000×g for 20 min. The supernatant was collected as the extracellular fraction. The pelleted cells were washed twice with 0.85% NaCl. Washed cells were subsequently resuspended in buffer (40 ml per 1 g wet weight) containing 500 mM sucrose and 1 mM EDTA in a 30 mM Tris–HCl buffer (pH 8.0) for disruption by osmotic pressure. After centrifugation at 14,000×g for 20 min, the cell debris was resuspended in deionized water and centrifuged again. The supernatant obtained was collected as periplasmic fraction. The residual cell debris was disrupted by ultrasonication and the supernatant obtained after centrifugation used as cytosolic fraction. Alkaline phosphatase and β -galactosidase activities were quantified as marker enzymes present in the periplasm and cytosol, respectively, as described (Craven et al. 1965; Schlesinger and Olsen 1968).

Translocation studies

Fractionation of *E. coli* into a fraction containing the cytoplasm together with membranes (CP/M) and a periplasmic fraction (PP) was done by using an EDTA-lysozyme spheroplasting method as described earlier (Kreutzenbeck et al. 2007). Also SDS-PAGE and Western blotting of fractions which correspond to an identical number of cells is described in the reference. For the detection of the His-tagged ArgR protein, a penta-His-antibody (Qiagen, Hilden, Germany) was used as the primary antibody in a dilution of 1:1,000. The detection of transaldolase B protein (TalB) was carried out using an anti-transaldolase B antibody in a dilution of 1:1,000. Further processing was done by use of the ECL Western blotting detection kit (GE Healthcare, Munich, Germany) according to the manufacturer's instructions. The chemiluminescent protein bands were recorded using the Fujifilm LAS-3000 Mini CCD camera and image analyzing system together with the software AIDA 4.15 (raytest Inc., Willmington, USA).

Accession numbers

The nucleotide sequence for *P. taetrolens* NBRC 3436 *argR* is deposited at DDBJ/EMBL/GenBank as AB096176, that for *alr* as AB296103.

Results

Cloning of *argR* and *alr*

Sequences of the broad specificity amino acid racemases of *Pseudomonas striata* (Roise et al. 1984) and *Pseudomonas putida* (Kino et al. 2007) were used as the basis to enable

isolation of *argR* from *P. taetrolens*. Primers A and B (Table 1) close to the conserved PLP binding site were designed and used to amplify *argR* by PCR applying chromosomal DNA of *P. taetrolens* as the template. Two subsequent amplifications were made with increasing annealing temperature, the resulting fragments cloned, and one of them was shown by sequencing to possess the expected PLP motif. This fragment was used for isolation of the entire *argR* by genome-walking which was done with the LA PCRTM in vitro cloning kit to result in pET11b-*argR* containing a 3,160-bp genomic fragment of *P. taetrolens*, with the open reading frame termed *argR*. The *argR* gene of *P. taetrolens* consists of 1,227 bp and encodes a protein of 408 amino acids.

In order to study also the alanine racemase gene of *P. taetrolens* for comparison, we used the genome sequence of *P. putida* KT2440 (Nelson et al. 2002) to design primers G and H within the gene annotated *alr*. Using PCR amplification and genome-walking in a procedure similar to that described above the *alr* gene from *P. taetrolens* was obtained in pET21b-*alr*. The gene consists of 1,074 bp and encodes a protein of 357 amino acids.

Isolation of ArgR and Alr proteins

ArgR was isolated from *E. coli* BL21 (DE3) harboring pET11b-*argR* enabling expression of the native enzyme without any tag. As shown in Table 2, the isolation procedure based on ion exchange and hydrophobic interaction chromatography yielded 16-fold enriched enzyme with a high specific activity of 1,120 $\mu\text{mol}^{-1} \text{min}^{-1} \text{mg}^{-1}$. After dialysis of the protein against a 10 mM potassium phosphate buffer (pH 7.3) containing 10 mM hydroxylamine for 12 h activity was lost. However, the activity was restored to 80% of its initial value upon incubation of the apoenzyme with 10 mM PLP, indicating reconstitution of the protein by PLP addition. The apoenzyme and the PLP-enzyme were reduced with sodium borohydride, and digested with trypsin. Separation of the peptides by reversed-phase liquid column chromatography yielded one strong fluorescent peak in the PLP-enzyme preparation, which was absent in the apoenzyme treated identically. This phosphopyridoxyl peptide was isolated and sequenced, yielding the sequence SQIX₁AVLX₂ADAYGHGIGL corresponding to amino acyl residues 73–83 of the protein as derived from the nucleotide sequence. The X-residues could not be determined. According to the DNA sequence X₁ is a cysteine and X₂ a lysine, the latter lysine apparently serving to establish the aldimine linkage between its epsilon-amino group and the carbonyl group of PLP. The isolated ArgR was furthermore subjected to Edman degradation and the amino-terminal sequence APPLSMTDGV determined. This is at variance with the peptide sequence derived from the

Table 2 Isolation of ArgR

Step	Total activity (U)	Total protein (mg)	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Yield (%)	Fold enrichment (–)
Cell-free extract	127,000	1,810.0	70	100.0	1.0
DEAE-Toyopearl	118,000	140.0	840	92.9	12.0
Butyl-Toyopearl	78,800	72.3	1,090	62.0	15.5
DEAE-Toyopearl	56,000	50.0	1,120	44.0	16.0

argR open reading frame used and as present in the *P. taetrolens* genome. It corresponds to amino acids 24 to 33 of the deduced peptide sequence, suggesting that native ArgR is made as a preprotein processed by cleavage (see below).

Alr was isolated from *E. coli* BL21 (DE3) pET21b-*alr* as a carboxy-terminal His6-tagged protein. The homogeneous protein was dialyzed against buffer B and stored at -20°C , as was the ArgR protein.

Enzyme properties of ArgR and Alr proteins

Using gel filtration on a calibrated column a mass of 84 ± 11 kDa was determined for ArgR, and one of 79 ± 16 kDa for Alr (data not shown). Since the calculated monomer mass of mature ArgR is 41.5 kDa and of Alr 38.8 kDa, both native enzymes adopt a dimeric structure. The optimum activity of the Alr was at pH 8.0 and this was similar the case for ArgR (Yorifuji and Ogata 1971). Whereas Alr showed maximal activity at 35°C , this was the case for ArgR only at 65°C (data not shown). The activation energy as calculated from Arrhenius plots was 126 kJ mol^{-1} for Alr with L-Ala and 61.8 kJ mol^{-1} for ArgR with L-Arg as a substrate.

The specificity of both racemases was determined with L-amino acids as substrates by quantification of the formed enantiomers via HPLC (Ono et al. 2006). ArgR was most active with Lys and Arg, whereas with Ala only a minor activity was obtained (Table 3), and these values largely agree with that described (Yorifuji and Ogata 1971). With Phe as substrate only a trace activity was detectable, and

with Glu the enzyme was inactive. Interestingly, the BsRC of *P. putida* exhibits subtle but significant differences in its specificity (Kino et al. 2007). In contrast to ArgR of *P. taetrolens* with its broad substrate specificity, the alanine racemase Alr of this organism exhibited activity with Ala only and acted neither on Arg nor on any other of the amino acids tested (Table 3).

Localization of ArgR in *P. taetrolens*

Following the observation that ArgR was 23 amino acids shorter than expected from the gene sequence, and that SignalP predicted a cleavage site for the full-length protein (Bendtsen et al. 2004), we studied the localization of the protein in *P. taetrolens*. Supernatants of cell suspensions yielded the extracellular enzyme fraction, whereas osmotically shocked cells yielded periplasmic and cytoplasmic fractions (Morita et al. 2006). As shown in Fig. 1, the greatest racemase activities of cultures grown to different optical densities were always localized in the periplasm, whereas significantly lower activities were present in the cytosol and the supernatant.

Localization of ArgR in *E. coli*

In addition, localization in *E. coli* BL21 (DE3) harboring pET11b-*argR* used for protein isolation was studied. For this purpose, *E. coli* was grown in 100 ml volumes to four different time points, fractionated, and the ArgR activity together with that of alkaline phosphatase and β -galactosidase

Table 3 Specificity of ArgR and Alr

Substrate	Arginine racemase (%)	Alanine racemase (%)
L-Alanine	12.4	100
L-Lysine	100.0	0
L-Arginine	95.2	0
L-Ornithine	42.0	n.d.
L-Methionine	12.4	0
L-Ethionine	11.8	n.d.
L-Homoarginine	6.2	n.d.
L-Norvaline	6.5	n.d.
L-Leucine	1.7	0
L-2-Aminobutyrate	1.4	n.d.
L-Phenylalanine	0.02	0

n.d. Not determined

Amino acids were used in a concentration of 40 mM

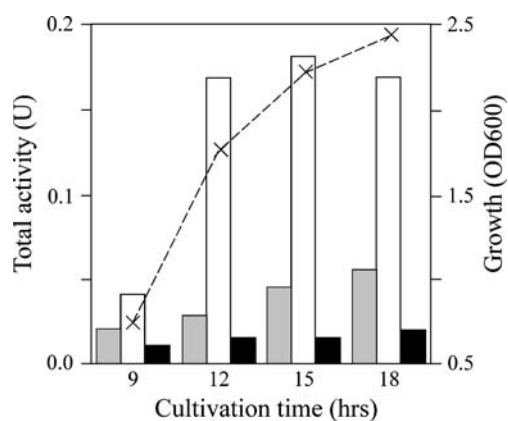


Fig. 1 Localization of ArgR activity in *P. taetrolens*. Cells were grown on complex medium containing L-arginine with the line showing the growth curve. Samples were removed at the 4 different time points indicated and processed to derive cytoplasmic (gray bar), periplasmic (empty bar), and extracellular fractions (black bar), with the height of the bars giving the specific activities determined

determined, the latter enzymes serving as lead enzymes for either periplasmic or cytoplasmic localizations. As shown in Table 4, the largest fractions of ArgR activity were present in the periplasmic fraction, and the contribution of the cytoplasmic fraction plus the medium fraction to the total activity was at best 16%. The periplasmic alkaline phosphatase and the cytosolic β -galactosidase quantified as control localized as expected. This confirms that the broad substrate specificity racemase ArgR also localizes in *E. coli* in the periplasm and means processing of ArgR in the heterologous host.

Transport of ArgR in *E. coli*

As shown above, enzymatically active ArgR can be detected in the periplasm when *argR* is expressed in *E. coli*. The ArgR signal peptide lacks a typical Tat-consensus motif and possesses only one arginine at the boundary between the n- and the h-region of the deduced signal peptide, suggesting

that ArgR might be exported into the *E. coli* periplasm separately from its PLP cofactor via the Sec pathway. However, since it is possible that ArgR may be a Tat-substrate with an atypical Tat-consensus motif, the subcellular localization of a C-terminal His-tagged version of ArgR (ArgR-His) was determined in an *E. coli* wild-type strain and its isogenic Tat-defective mutant derivative, in which all known *tat* genes were deleted (Blaudeck et al. 2003).

E. coli (GSJ100) and *E. coli* Δ *tat* (GSJ101) cells expressing either untagged ArgR or ArgR-His were fractionated into a cytoplasm/membrane (CP/M) fraction and a periplasmic (PP) fraction by EDTA-lysozyme spheroplasting. The fractions were subsequently analyzed by SDS-PAGE and Western blotting, using anti-5His antibody for the detection of ArgR (Fig. 2a). As a control for the quality of the fractionation experiments, the subcellular distribution of the cytoplasmic enzyme transaldolase B was analyzed in parallel (Blaudeck et al. 2003). As expected, transaldolase B was found exclusively in the CP/M fraction of all cells examined (Fig. 2b). As shown in Fig. 2a, various polypeptides were detected with the anti-5His antibody in the subcellular fractions of the strains expressing the ArgR-His (lanes 1 to 4), but not in the strain expressing the untagged ArgR (lanes 5 and 6). In the CP/M fraction of both the *E. coli* wild-type (lane 1) and the Δ *tat* strain (lane 3) expressing ArgR-His, a band (p) is detected by the anti-5His antibody that corresponds in molecular weight to the unprocessed precursor of ArgR. In addition, a smaller migrating form (*) of ArgR-His is detected in the same fraction of both strains. Most likely, this ArgR-His-derived polypeptide corresponds to a cytosolic degradation product. Importantly, however, in both the *E. coli* wild-type strain (lane 2) and the *E. coli* Δ *tat* strain (lane 4), a band (m) of identical intensity is detected by the anti-5His antibody in the periplasmic fraction. This ArgR-His-derived polypeptide corresponds in size to the correctly processed mature form of ArgR-His. Since no difference in the subcellular distribution of ArgR-His-derived

Table 4 Localisation of ArgR in *E. coli**

Culture time (h)	Localization	Alkaline phosphatase	β -Galactosidase	Arginine racemase
5.0	Extracellular	11	0	6
	Periplasm	84	20	92
	Cytoplasm	5	80	2
6.5	Extracellular	3	1	4
	Periplasm	85	7	89
	Cytoplasm	12	92	7
8.0	Extracellular	1	1	5
	Periplasm	93	6	89
	Cytoplasm	6	93	6
9.5	Extracellular	6	1	8
	Periplasm	88	10	84
	Cytoplasm	6	89	8

*Numbers are percentages of the respective enzyme at the respective time point. Total ArgR activity at 5.0, 6.5, 8.0, and 9.5 h was 7, 22, 59, and 75 U mg(protein)⁻¹, and the respective cell culture density (OD) 0.6, 0.9, 2.4, and 3.3, respectively

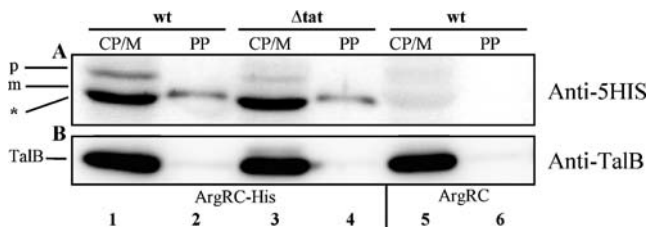


Fig. 2 Subcellular localization of His-Tagged ArgR polypeptides. Cells of the *tat* wild-type (*wt*) strain GSJ100 and the *tat* deletion mutant (Δ *tat*) GSJ101 containing pBBR1MCS2ArgR-His or pBBR1MCS2ArgR were fractionated into a CP/M fraction and a PP fraction by EDTA-lysozyme spheroplasting as described in “Materials and methods” section. Samples were subjected to SDS-PAGE and Western blotting using anti-5His antibodies (A) or, as a control, using antibodies directed against the cytoplasmic protein TalB (B). *p* ArgR-His precursor, *m* exported mature form of ArgR-His in the PP fraction, *asterisk* position of the ArgR-His degradation product in the CP/M fraction

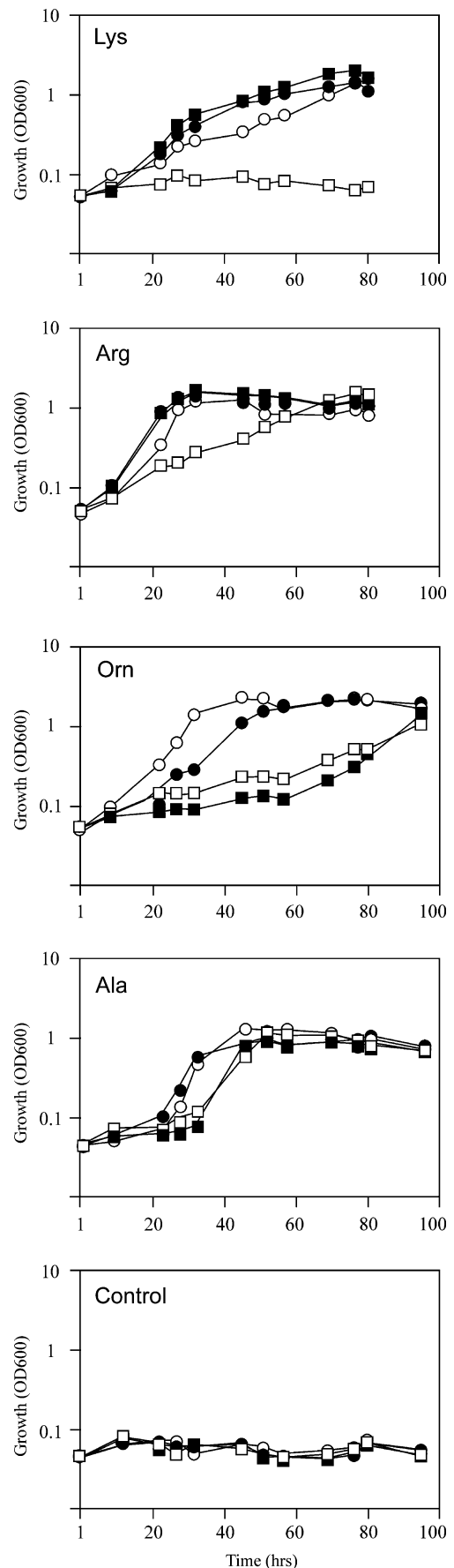
polypeptides (nor in the enzymatic activities of the corresponding subcellular fractions, data not shown) is detected between the Tat-proficient wild-type strain and the Tat-defective Δ *tat* mutant strain, our results clearly demonstrate that membrane translocation of ArgR occurs independently of a functional Tat system in *E. coli*.

Utilization of amino acids due to ArgR

To permit studies to be made on the physiological function of ArgR, the non-replicative vector pK18mob-*argR*-int was constructed to enable disruption of *argR* by homologous recombination (see “Materials and methods” section). The vector was used to transform *P. taetrolens* to kanamycin resistance and the correct integration was verified by PCR (data not shown). The resulting strain was named *P. taetrolens*::*argR*. An enzyme assay using L-Lys as a substrate yielded an activity below 0.01 $\mu\text{mol min}^{-1} \text{mg (protein)}^{-1}$, whereas it was 0.43 $\mu\text{mol min}^{-1} \text{mg (protein)}^{-1}$ for the wild type, thus confirming absence of racemase activity in the mutant.

In minimal medium (Stalon et al. 1967), we studied the utilization of 10 mM L-, and D-amino acids as a carbon source. Figure 3 shows that the basic amino acids D-Lys, L-Lys, D-Arg, L-Arg, and L-Orn were used to roughly the same extent by *P. taetrolens* wild type, whereas D-Orn enabled rather slow growth. Also, the enantiomers of Ala could be utilized as carbon source, whereas this was not the case with Met (data not shown). In contrast to this, *P. taetrolens*::*argR* was no longer able to utilize D-Lys, and

Fig. 3 Utilization of D- and L-amino acids as a carbon source by *P. taetrolens*. The amino acids used are as indicated. The square indicates D-amino acid, the circle, L-amino acid, wild-type, closed symbols, and mutant with inactivated *argR*, open symbols. Single representative data of two different experiments are shown



also the utilization of D-Arg was strongly reduced, showing that ArgR plays a key role in the metabolization of these basic D-amino acids. The slightly influenced L-Orn utilization also observed remains unexplained but could indicate, for instance, regulatory interactions of the unknown amino acid degradation pathways. In addition to growth on the synthetic medium, also LB was assayed. It was observed that growth of *P. taetrolens::argR* was slightly retarded to a growth rate of 0.36 h^{-1} as compared to 0.59 h^{-1} for the wild type, which might indicate that ArgR is also generally involved in amino acid degradation. The addition of D-Ala did not influence the growth of the wild type or that of the mutant.

Discussion

The fractionation data obtained with the *P. taetrolens* arginine racemase clearly show that ArgR is located in the periplasm. Also, in *E. coli* transport of the ArgR protein into the periplasm and processing into its mature form takes place. The signal sequence present shows the typical tripartite structure characteristic of signal peptides, consisting of a positively-charged ‘n-region’, a central hydrophobic ‘h region’, and a polar ‘c-region that contains the signal peptidase cleavage site’. However, the sequence itself does not allow translocation to be attributed either via the twin-arginine translocation (Tat) pathway, or the general secretion (Sec) pathway (Palmer et al. 2005). We first suggested that ArgR is translocated via the Tat pathway, in particular since it requires pyridoxal-5'-phosphate (PLP) for activity and the Tat pathway translocates its substrates in their fully folded form, including integrated cofactors. One feature of Tat substrates is the eponymous twin-arginine motif at the boundary between the n- and the h-region. However, the ArgR signal peptide possesses only one arginine, suggesting that ArgR might be exported via the Sec pathway. Indeed, we experimentally confirmed that ArgR is translocated into the periplasm of *E. coli* independent of the Tat pathway and, therefore, must have been translocated via the Sec route. Generally, this pathway is used to transport proteins devoid of an attached cofactor through the membrane in an unfolded manner, whereas the Tat pathway is mainly used by cofactor-containing proteins. However, it has been demonstrated that some heme-cofactor-containing proteins are in fact translocated into the periplasm via the Sec pathway, followed by further post-translocational modifications, including heme-cofactor insertion. For example, the cytochromes MtrC and OmcA of the Gram-negative bacterium *Shewanella oneidensis* are first translocated across the cytoplasmic membrane using the Sec pathway, and undergo an extensive post-translocational maturation process inside the periplasm to become the heme-containing

lipoproteins (Shi et al. 2008). Due to the Tat-independence of ArgR export and the incompatibility of the Sec pathway with transport of folded structures, our findings imply that the PLP cofactor of ArgR must have been added to the apoprotein in the periplasm. Otherwise, also the high activity in the periplasm is difficult to explain. Therefore, ArgR represents a new class of enzymes utilizing the Sec pathway, where protein and cofactor use different pathways to arrive in the periplasm, but details on these particular events are completely unknown yet.

As the growth experiments with *P. taetrolens* demonstrate, ArgR is a catabolic enzyme. It is necessary for efficient utilization of the two basic D-amino acids D-Lys, and D-Arg, and for D-Lys utilization it is even essential. For D-Arg utilization an additional activity might be present to compensate for the absence of ArgR, but this is not Alr. Alr is very specific, just utilizing D,L-Ala as a substrate. The localization of ArgR within the periplasm of *P. taetrolens* is surprising; but this could be related to its catabolic function. It might give the cell an advantage for substrate utilization. Since racemization occurs in the periplasm, the bacterium would then be equipped with fewer specific import systems. However, little is known about the import and catabolism of basic amino acids in *Pseudomonas* (Revelles et al. 2005; Yamanishi et al. 2007), and it should also be mentioned that according to genome information *argR* sequences exist which do not have a signal sequence and could be arginine racemases. For instance Q9KSE5 of *Vibrio cholerae* might be a cytosolic ArgR (Heidelberg et al. 2000). In *P. taetrolens* the genes adjacent to *argR* exhibit similarities to an *N*-acyl amino acid amidohydrolase and a protease (pfam: PF01546), respectively, which could indicate a relation of the entire gene cluster with catabolic activities.

As mentioned, the alanine racemase Alr of *P. taetrolens* is highly specific and acts on Ala only, as do all the other alanine racemases studied (Strych et al. 2000). Interestingly, Alr of *P. taetrolens* has slightly greater identity to the catabolic alanine racemase Alr2 of *E. coli*, also termed DadX, than to the biosynthetic alanine racemase Alr1. Due to this fact and the fact that in *P. taetrolens* *alr* is located adjacent to *dadA*, the latter gene predicted to encode the small subunit of a D-amino acid dehydrogenase, we initially thought that Alr served only catabolic purposes. However, in an attempt to inactivate *alr* of *P. taetrolens* we obtained only very poor growing colonies (data not shown). The possibility exists that the single alanine racemase in *Pseudomonas* serves both anabolic and catabolic purposes, as we found growth of *P. taetrolens* on D-alanine. The interaction of Alr with catabolism and anabolism deserves further study.

Due to the apparent similarity of the alanine racemases Alr and DadX and the fact that also ArgR sequences are

annotated as alanine racemases, we inspected selected genomes of gammaproteobacteria for the occurrence and similarities of such racemases. We found only two genes in *P. putida* KT2440, one corresponding to *alr* of *P. taetrolens*, and the other to *argR*. A similar situation prevails in the *P. putida* strains GB-1, F1, W619, and further *Pseudomonas* species like *P. aeruginosa* PAO1. From these genomic data and our experimental analysis, it appears that the *Pseudomonas* strains mentioned have the arginine racemase with broad substrate specificity and just one alanine racemase. Whereas, a similar situation exists for *V. cholerae* O1 biovar eltor str. N16961 and *Yersinia pestis* CO92, the situation in *Aeromonas salmonicida* subsp. *salmonicida* A449 is different. This bacterium has the arginine racemase, together with two alanine racemases. These two alanine racemases have 67% identity, with that encoded by ASA_3286 (gi: 4995230) probably serving anabolic purposes due to its genomic context. Thus, with respect to the two paralogous alanine racemases, the situation in *A. salmonicida* resembles that in *E. coli* which has the two alanine racemases *dadX* (*alr2*) and *alr* (*alr1*) (Yamanishi et al. 2007), both specific for alanine, and one for anabolism and one for catabolism, with *alr2* probably expressed on demand. A distinction between the catabolic and anabolic alanine racemases based on sequence identities only is less evident, and this is probably not surprising due to the identical substrate and mechanism they use.

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