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Metabolic Function of *Corynebacterium glutamicum* Aminotransferases AlaT and AvtA and Impact on L-Valine Production▽

Jan Marienhagen and Lothar Eggeling*

Institute of Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany

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Aminotransferases (ATs) interacting with L-alanine are the least studied bacterial ATs. Whereas AlaT converts pyruvate to L-alanine in a glutamate-dependent reaction, AvtA is able to convert pyruvate to L-alanine in an L-valine-dependent manner. We show here that the wild type of *Corynebacterium glutamicum* with a deletion of either of the corresponding genes does not exhibit an explicit growth deficiency. However, a double mutant was auxotrophic for L-alanine, showing that both ATs can provide L-alanine and that they are the only ATs involved. Kinetic studies with isolated enzymes demonstrate that the catalytic efficiency, k_{cat}/K_m, of AlaT is higher than 1 order of magnitude in the direction of L-alanine formation (3.5×10^4 M^{-1} s^{-1}), but no preference was apparent for AvtA, suggesting that AlaT is the principal L-alanine-supplying enzyme. This is in line with the cytosolic L-alanine concentration, which is reduced in the exponential growth phase from 95 mM to 18 mM by a deletion of *alaT*, whereas *avtA* deletion decreases the L-alanine concentration only to 76 mM. The combined data show that the presence of both ATs has subtle but obvious consequences on balancing intracellular amino acid pools in the wild type. The consequences are more obvious in an L-valine production strain where a high intracellular drain-off of the L-alanine precursor pyruvate prevails. We therefore used deletion of *alaT* to successfully reduce the contaminating L-alanine in extracellular accumulated L-valine by 80%.

Among the numerous transformations of amino acids performed by pyridoxal-5′-phosphate (PLP)-dependent enzymes, the reversible transfer of amino groups from the amino acids to 2-oxo-acids is predominant. Such reactions are catalyzed by aminotransferases (ATs), which play a vital role in the synthesis of amino acids as well as in amino acid interconversions (5).

A functional study of ATs in a single organism may encounter problems due to the large number of ATs usually present, their closely related structure, and overlapping substrate specificities (10, 19). This is evident, for instance, for *Escherichia coli* and its three ATs encoded by *tyrB*, *aspC*, and *ilvE*, which are involved in the synthesis of aromatic amino acids. Here, the in vivo function could be studied only when the other two genes were inactivated (8). Nonetheless, we succeeded in identifying the function of 11 ATs of 20 putative PLP-dependent proteins encoded in the *Corynebacterium glutamicum* genome, thus gaining a global view of the transamination activities in this bacterium (16, 17). This bacterium is of particular interest due to its excellent amino acid production properties. Currently, most of the 2×10^6 tons of amino acids produced per year are synthesized by fermentation with *C. glutamicum* (13), and a recent monograph discusses a number of features of this useful bacterium (6).

Among the amino acids made with *C. glutamicum* are L-isoleucine and L-valine, which are mostly used for pharmaceutical purposes and therefore are required to have the highest purity. We succeeded in developing strains suitable for the production of both amino acids (20, 22). In this regard, we noted that the branched-chain AT IlvE is essential for the synthesis of L-isoleucine and L-leucine, whereas an additional overlapping activity still achieves L-valine formation. The corresponding activity was attributed to AvtA, known from *E. coli* and *Salmonella enterica* serovar Typhimurium as transaminase C (2, 28). Together with *C. glutamicum*, these are the only organisms for which enzymological and physiological studies of this enzyme have been performed (14). AvtA occupies an exceptional position since it is an AT which does not use L-glutamate as an amino donor but employs L-alanine instead. A second AT interacting with L-alanine is the alanine aminotransferase AlaT, which is again unusual in that it has rather broad substrate specificities utilizing L-glutamate and also L-aspartate as amino donors and pyruvate or 2-oxobutyrate as amino acceptors (16). Although orthologs of *alaT* are widely distributed, knowledge of the corresponding enzymes is surprisingly scarce. Besides *C. glutamicum*, only one bacterial alanine AT has been isolated and characterized, the alanine AT of *Pyrococcus furiosus* (27). An AlaT-like activity has been described in *E. coli* by complementation analyses but could not be linked to a specific gene (26). In fact, AlaT together with AvtA, both interfering with L-alanine metabolism (Fig. 1), belong to the least-well-characterized bacterial ATs. In particular, knowledge of their in vivo function and interaction with intracellular amino acid pools is limited.

Driven by the awareness that the role and interaction of AlaT and AvtA in the metabolism is not yet clear, together with the fact that even traces of L-alanine in L-valine produced with *C. glutamicum* are disadvantageous, we here study AlaT

* Corresponding author. Mailing address: Institute of Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany. Phone: 49 2461 61 5132. Fax: 49 2461 61 2710. E-mail: l.eggeling@fz-juelich.de.
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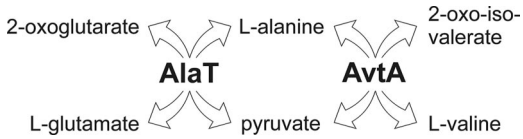

FIG. 1. Schematic representation of overlapping substrate specificities of AlaT and AvtA of *C. glutamicum*.

and AvtA at the enzymological and cellular levels, together with their influence on product formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *C. glutamicum* was cultured at 30°C in the complex medium CGIII or in the salt medium CGXII (11). *E. coli* was grown at 30 or 37°C in Luria-Bertani medium. When appropriate, chloramphenicol (25 mg liter^{-1}) or kanamycin (15, 25, or 50 mg liter^{-1}) was added. In cases where threonine dehydratase (*ilvA*), ketopantoatehydroxymethyl transferase (*panB*), and pantothenate synthetase (*panC*) were deleted, growth medium was supplemented with L-isoleucine and D-pantothenate (22, 23).

For bioreactor cultivations of *C. glutamicum*, the first preculture was grown for 10 h in 500-ml Erlenmeyer flasks without baffles containing 50 ml of CGIII medium. One milliliter of this culture was transferred to another 500-ml flask with 50 ml of CGXII medium. After 15 h, the second preculture was transferred to 1.5 liters of CGXII medium containing 40 g/liter glucose in a 3.6-liter bioreactor (Infors, Bottmingen, Switzerland). The experiment was done with a Sixfors instrument, allowing us to operate six fermentors in parallel, and thus a duplicate of each of the three strains was compared in this study. During fermentation the sample was maintained at a temperature of 30°C and a pH of 7.2 by the addition of either ammonia or hydrochloric acid. Oxygen saturation was kept constant at 30% at least by controlling the stirrer speed. Samples for the determination of extracellular amino acid concentrations via high-pressure liquid chromatography (HPLC) were collected at several time points. Each biological experiment (intracellular metabolite preparation and product formation) was done at least twice, with the deviation of metabolites quantified as below 16%.

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference or source[a]
Strains		
E. coli DH5αMCR	F$^-$ *endA*1 *supE*44 *thi-1* λ- *recA1 gyrA*96 *relA1 deoR* Δ(*lacZYA-argF*) *U169*ϕ80d*lacZ*ΔM15 *mcrA* Δ(*mrr-hsdRMS-mcrBC*)	8
C. glutamicum		
ATCC 13032	WT	ATCC
Δ*alaT* strain	WT with *alaT* deleted	15
Δ*avtA* strain	WT with *avtA* deleted	15
Δ*alaT* Δ*avtA* strain	WT with *alaT* and *avtA* deleted	This work
Δ*ilvA* Δ*panBC* strain	WT with *ilvA* and *panBC* deleted	20
Δ*ilvA* Δ*panBC* Δ*alaT* strain	WT with *alaT*, *ilvA*, and *panBC* deleted	This work
Δ*ilvA* Δ*panBC* Δ*avtA* strain	WT with *avtA*, *ilvA*, and *panBC* deleted	This work
Plasmids		
pJC1*ilvBNCD*	pKK5 with 2.6-kb XbaI fragment encompassing *ilvD*	21
pJM*alaT*	pASK-IBA-3C with *alaT*	15
pJM*avtA*	pASK-IBA-3C with *avtA*	15
pK19mobsacBΔ*alaT*	Plasmid to delete *alaT* in the *C. glutamicum* chromosome	15
pK19mobsacBΔ*avtA*	Plasmid to delete *avtA* in the *C. glutamicum* chromosome	15

[a] ATCC, American Type Culture Collection.

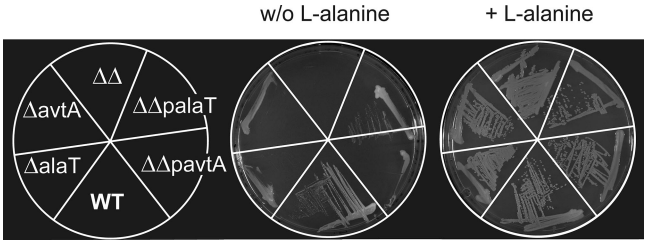

FIG. 2. Growth of *C. glutamicum* WT and its isogenic mutants on CGXII salt medium (middle) and supplemented with 1 mM L-alanine (right). On the left is shown the mutant code, which is as follows: ΔalaT, WT Δ*alaT*; ΔavtA, WT Δ*avtA*; ΔΔ, WT Δ*alaT* Δ*avtA*; ΔΔpalaT, WT Δ*alaT* Δ*avtA* pEKEx2alaT; ΔΔpavtA, WT Δ*alaT* Δ*avtA* pEKEx2avtA.

Construction of strains. *C. glutamicum* was transformed by electroporation (25), and *E. coli* was transformed by chemical transformation (9). The AT deletion mutants were constructed by using plasmids pK19mobsacBΔ*avtA* and pK19mobsacBΔ*alaT* (16). Clones were selected for kanamycin resistance to establish integration of the plasmid in the chromosome. In a second round of positive selection using sucrose resistance, clones were selected for deletion of the vector (24). The deletions in the chromosomes were verified by PCR analysis using primers hybridizing approximately 500 bp upstream and 500 bp downstream of the open reading frames in question.

Enzyme assays and determination of kinetic parameters. Heterologous gene expression, protein purification, and crude extract preparation to assay AT activities were performed as described previously (16). The AT assay contained 200 mM Tris-HCl (pH 8), 0.25 mM PLP, 4 mM oxo-acid, and 50 mM L-amino acid. The reaction was started by the addition of purified protein or crude extract and performed at 30°C. At least six 50-μl samples were collected over 20 min. The reaction was terminated by mixing each sample with 30 μl of 5% perchloric acid and 38% ethanol. After the sample had been neutralized by the addition of 20 μl of 20 mM Tris-HCl (pH 8) buffer with 23 mM K_2CO_3, the precipitated salts were removed by centrifugation (for 10 min at 13,000 rpm). Subsequently, amino acids were quantified by HPLC using automatic precolumn derivatization with *o*-phthaldialdehyde and reverse-phase HPLC, as described previously (15). Assays were linear over time and proportional to the protein concentration used. Kinetic parameters, such as the Michaelis-Menten constant (K_m), the turnover number (k_{cat}), and the catalytic efficiency (k_{cat}/K_m), were determined with 10 different 2-oxo-acid concentrations ranging from 50 to 0.05 mM for each amino donor-amino acceptor combination. The amino donors L-glutamate, L-alanine, and L-valine were kept constant at 0.5 M. To derive the Michaelis-Menten constant, Origin 7G software was used (Additive, Friedrichsdorf, Germany).

Determination of cytoplasmatic amino acid concentrations. For the determination of internal L-alanine and L-valine concentrations, *C. glutamicum* cells were grown on CGXII medium in shake flask cultures. After 10 h and 14 h, cells were separated from the medium and inactivated by silica oil centrifugation, with further preparation as described previously (20). Briefly, aliquots (100 to 200 μl) of the cultures were transferred to a 400-μl microcentrifuge tube containing a layer of silicone oil floating on a layer of 20% perchloric acid. The tube was immediately centrifuged in a Beckman Microfuge E for 30 s, and the tip of the tube containing the sedimented cells in perchloric acid was cut at the silicon layer. After homogenization of the sediment by sonication and removal of the denatured protein by centrifugation, the extracted amino acid concentrations were determined by HPLC.

RESULTS

The ATs AlaT and AvtA of *C. glutamicum* have substrate specificity for L-alanine in common (Fig. 1). Both the Δ*avtA* and the Δ*alaT* mutants of *C. glutamicum* exhibit reduced and slightly variable growth on minimal medium-containing plates which can be fully restored by the addition of 1 mM

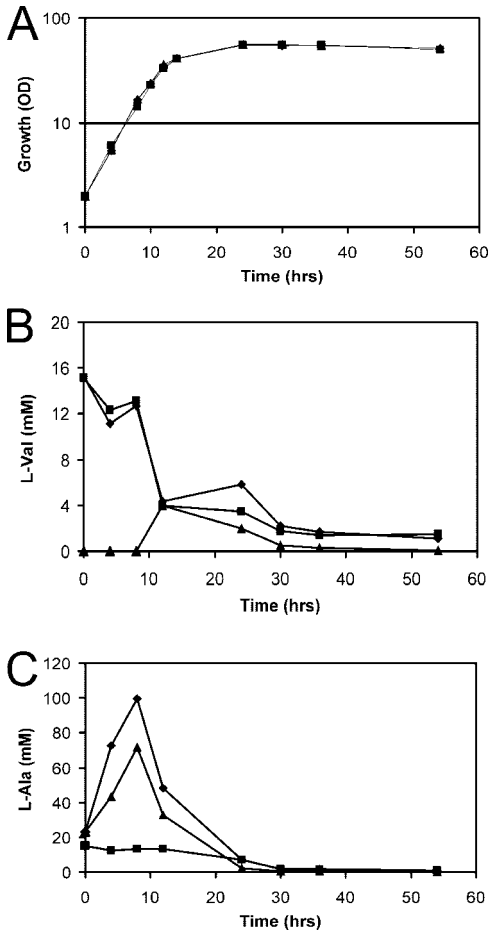

FIG. 3. (A) Growth of *C. glutamicum* strains. (B and C) Cytoplasmic concentrations of L-valine (B) and L-alanine (C) in *C. glutamicum* strains. ◆, *C. glutamicum* WT; ■, *C. glutamicum* Δ*alaT*; ▲ *C. glutamicum* Δ*avtA*. All strains were grown in CGXII salt medium.

L-alanine (Fig. 2). In liquid medium this phenotype was not visible with any of the mutants (Fig. 3), possibly due to altered regulation. Based on a wild-type (WT) strain carrying a deletion of Δ*alaT* (WT Δ*alaT*) we constructed the double mutant WT Δ*alaT* Δ*avtA*. When this mutant was assayed on minimal medium-containing plates it was explicitly unable to grow, but growth could be fully restored by the addition of L-alanine (Fig. 2). Therefore, it can be concluded that AlaT and AvtA are the only ATs that contribute to L-alanine synthesis in *C. glutamicum*, and the two activities may, in part, replace each other.

For a further characterization of these enzymes we determined selected kinetic parameters of the isolated AlaT and AvtA proteins. This was done by direct chemical quantification following the time-dependent formation of products via HPLC. We first assayed amination of pyruvate to L-alanine (Table 2). AvtA uses specifically L-valine and AlaT uses L-glutamate as amino donors (14, 16). As a result of the assay, comparable K_m values of 1.5 (AvtA) and 2.9 mM (AlaT) were obtained (Table 2). Therefore, both ATs are in principle able to contribute to L-alanine formation, as we observed in vivo (Fig. 2). However, determination of the k_{cat}/K_m value for both ATs shows that the AlaT protein, compared to AvtA, has a 10-fold increased catalytic efficiency for L-alanine formation at saturating amino donor concentrations. In the reverse direction of pyruvate formation from L-alanine, the catalytic efficiency of AlaT is 1.4×10^3 M^{-1} s^{-1}, which is 1 order of magnitude lower than the pyruvate-aminating reaction. These data imply that AlaT is probably most relevant for L-alanine formation. The data for AvtA show that this AT exhibits comparable V_{max}, K_m, and k_{cat}/K_m values for the substrate pair L-alanine–2-oxo-isovalerate or pyruvate–L-valine.

These enzymological data reflect activities of the isolated proteins. In an attempt to obtain additional information on these ATs within the cell, we determined their specific activities and potential regulation by L-alanine. For this purpose, cells of WT, WT Δ*avtA*, WT Δ*alaT*, and WT Δ*avtA* Δ*alaT* were grown on CGXII minimal medium with and without supplementation of 50 mM L-alanine. As a result, no L-alanine-dependent transamination reaction was detected in the crude extract of the double mutant (data not shown), confirming that AvtA and AlaT are the only L-alanine-dependent ATs in *C. glutamicum*. Furthermore, no AvtA activity could be observed in the *avtA* mutant, and no AlaT activity was detected in the *alaT* mutant. In response to added L-alanine, AlaT activity was reduced to a maximum of 58% in WT and WT Δ*avtA* (Table

TABLE 3. Specific activity of AlaT and AvtA in crude extracts of *C. glutamicum* WT, *C. glutamicum* Δ*alaT*, and *C. glutamicum* Δ*avtA*

Protein	Time (h)	Activity (nmol min^{-1} mg^{-1}) in the indicated strain (treatment)[a]					
		WT	WT (with Ala)	Δ*avtA*	Δ*avtA* (with Ala)	Δ*alaT*	Δ*alaT* (with Ala)
AlaT	10	95 ± 12	65 ± 10	89 ± 12	52 ± 5		
	14	48 ± 3	38 ± 2	61 ± 7	35 ± 2		
AvtA	10	52 ± 4	32 ± 3			45 ± 2	32 ± 1
	14	42 ± 1	31 ± 4			50 ± 1	35 ± 4

[a] Cells were grown with or without supplementation of 50 mM L-alanine, as indicated.

TABLE 2. Kinetic parameters of AlaT and AvtA with the substrates pyruvate, 2-oxoglutarate, and 2-oxoisovalerate

Amino acceptor	Amino donor		V_{max} (μmol min^{-1} mg^{-1})		K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})	
	AlaT	AvtA	AlaT	AvtA	AlaT	AvtA	AlaT	AvtA	AlaT	AvtA
Pyruvate	Glu	Val	124	7.8	2.9	1.5	101	5	3.5×10^4	3.5×10^3
2-Oxo-glutarate	Ala	Ala/Val	18.6	ND[a]	10.8	ND	15	ND	1.4×10^3	ND
2-Oxo-isovalerate	Ala/Glu	Ala	ND	20.1	ND	2.5	ND	14	ND	5.6×10^3

[a] ND, not detected.

3). In addition, AlaT showed a weak response to the growth state since in the exponential phase (10 h) the specific activity was generally higher than at the beginning of the stationary phase (14 h). For AvtA in WT and WT Δ*alaT*, there was again a significant reduction in the specific activity to a maximum of 61%, but a growth state-dependent effect was not apparent. Altogether, high L-alanine concentrations in the culture medium led to a weak but significant reduction of AlaT and AvtA activity, probably due to decreased gene expression.

As another means of obtaining information on the in vivo situation, we determined the cytosolic L-alanine and L-valine concentrations by silica oil centrifugation during growth on CGXII minimal medium. As already mentioned, no difference between the three strains was detected concerning growth (Fig. 3A). The initial cytosolic L-valine concentration in the WT was 15 mM, which decreased during cultivation to 2 mM (Fig. 3B). This was not affected in WT Δ*alaT*, in line with the inability of AlaT to use L-valine–2-oxo-isovalerate as a substrate. However, in the WT Δ*avtA* strain, L-valine was almost undetectable during the first 8 h of cultivation but later increased, possibly due to a surplus activity of the biosynthetic pathway filling the cytosolic pool.

The cytosolic L-alanine concentrations behaved differently (Fig. 3C). They were in general higher than that of L-valine and peaked at a concentration of about 100 mM during exponential growth. In the WT Δ*avtA* strain, only a slight effect on L-alanine was apparent, and the characteristics of the trajectory were retained. In contrast, in WT Δ*alaT* a strong decrease in the cytosolic L-alanine concentration was present. Directly after inoculation, the level was still comparable to that of the other two strains, but the concentration did not increase and decreased continuously during cultivation. These data suggest that L-alanine synthesis from pyruvate in vivo is mainly carried out by AlaT. However, even without any accumulation of L-alanine, the amount of this amino acid produced by AvtA in WT Δ*alaT* still seems to be sufficient since growth of this mutant is not impaired on the CGXII salt medium.

We were also interested in studying the consequences of the deletion of AvtA and AlaT in an L-valine producer of *C. glutamicum*, where a different flux situation prevails. The strain chosen was WT Δ*ilvA* Δ*panBC* pJC1*ilvBNCD* (strain Val-1), in which the genes of L-isoleucine and pantothenate synthesis are deleted and those of L-valine synthesis are overexpressed (22). Strain WT Δ*ilvA* Δ*panBC* was used to delete either *alaT* or *avtA*, and both resulting strains were transformed with plasmid pJC1*ilvBNCD* to give Val-1-Δ*alaT* and Val-1-Δ*avtA*, respectively. These strains together with Val-1 were analyzed in 1.5-liter fermentations with oxygen and pH control to compare growth and product formation (Fig. 4). A clear reduction in the growth rate of Val-1-Δ*alaT* of 0.15 h^{-1} compared to 0.3 h^{-1} or 0.31 h^{-1} for Val-1 or Val-1-Δ*avtA*, respectively, was detectable. The final L-valine concentrations were hardly influenced, and a replicate did not yield any differences with respect to L-valine accumulation (data not shown). However, L-alanine was reduced in Val-1-Δ*alaT* in marked contrast to Val-1 and Val-1-Δ*avtA*. Although the absolute L-alanine concentration is low, the strong decrease from 1.2 mM, obtained with the original strain, to 0.16 mM is a great improvement with respect to the high purity of L-valine required for pharmaceutical purposes, thereby facilitating the cost-intensive downstream processing.

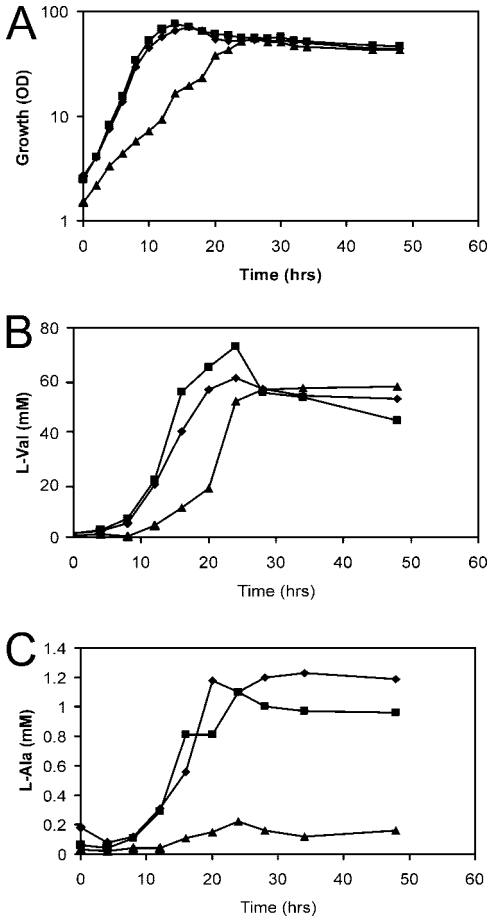

FIG. 4. (A) Growth of *C. glutamicum* strains. (B and C) Extracellular concentrations of L-valine (B) and L-alanine (C). All strains were grown in CGXII salt medium in batch in pH- and oxygen-controlled 1.5-liter reactors. ◆, L-valine-producing strain; ▲, Δ*alaT*; and ■, Δ*avtA* (both Δ*alaT* and Δ*avtA* were based on strain Val-1).

DISCUSSION

Bacterial ATs interacting with L-alanine are among the least studied ATs. Essentially, only the AlaT enzymes of *P. furiosus* (27) and *C. glutamicum* have been investigated as yet (16). In the case of the *P. furiosus* enzyme, the k_{cat}/K_m values for alanine and pyruvate formation are 41 and 33 s^{-1} mM^{-1}, respectively, suggesting that the enzyme is not biased toward the formation of either pyruvate or alanine. Due to the observed coregulated expression with the glutamate dehydrogenase in *P. furiosus*, the physiological role for this particular enzyme is assumed to be to help form an electron sink during anaerobiosis in the absence of elemental sulfur (12). The reduced expression of the corresponding *aat* gene (AlaT) upon pyruvate addition is in agreement with this view (27). For the *C. glutamicum* enzyme we determined k_{cat}/K_m values in the same range, although the catalytic efficiency is 1 order of magnitude greater for the L-alanine-forming reaction (Table 2). This indicates that AlaT in *C.*

glutamicum primarily serves to supply L-alanine for anabolism. A further indication is the L-alanine requirement in the absence of AlaT under selected growth conditions and, in particular, the clear consequences of AlaT deletion for the intracellular L-alanine level. Moreover, the reduction of the specific AlaT activity upon the addition of L-alanine fully agrees with the view that AlaT serves anabolic processes. In *C. glutamicum* the *alaT* gene appears to be monocistronic, but in a number of *Proteobacteria* such as *Gluconobacter oxydans*, *Burkholderia pseudomallei*, and *Nitrosomonas europea*, *alaT* is apparently located within a transcriptional unit of *hom* and *thrB*, thus providing a genomic link to enzymes of the aspartate family of amino acid synthesis (21).

Whereas the function of AlaT is fairly clear, this is not the case for AvtA. The catalytic properties do not favor a specific direction, and a phenotype is not apparent when *avtA* is deleted in the WT background. The same was observed in *E. coli* and *S. enterica* serovar Typhimurium, where *avtA* deletion did not cause a phenotype (2, 18). Only when a second transaminase activity was absent in *C. glutamicum* was growth dependence observed, which was L-alanine auxotrophy in the case of the *avtA alaT* double mutant (this work) or L-valine auxotrophy in the case of the *avtA ilvE* double mutant (16). The fact that the *avtA alaT* double mutant is strictly dependent on L-alanine supplementation shows that AvtA and AlaT are the key players in establishing suitable intracellular concentrations of L-alanine-enabling growth. Together with the slightly reduced cytosolic L-alanine concentration in the *avtA* mutant, we take this as an indication that AvtA serves to balance the intracellular L-alanine pool. Reduced AvtA activity due to L-alanine present in the medium also links AvtA to L-alanine metabolism and suggests repression of *avtA*. This would be similar to the situation in *E. coli* and *S. enterica* serovar Typhimurium, where *avtA* expression is repressed by L-alanine and also L-leucine but not by any other amino acid (2, 7, 28).

In summary, it appears that the presence of both ATs confers a certain degree of flexibility on the cell. Thus, in a more natural situation with fluctuating nutrient supply or under different flux conditions the role of AvtA or AlaT might become even more apparent. This is indeed the case for the L-valine-accumulating strain studied in our work, whose growth rate is reduced from 0.30 h^{-1} to 0.15 h^{-1} upon *alaT* deletion. L-Valine derives from two pyruvate molecules. Therefore, the strain Val-1 was originally engineered to have reduced drain-off of pyruvate via the pyruvate dehydrogenase (1), and significantly more elaborated strains using this target are available now (3, 4). Overexpression of L-valine biosynthesis genes in Val-1 results in the desired massive flux of pyruvate toward L-valine, which may lead to a drain-off of pyruvate. As a consequence, AvtA activity still present in the AlaT mutant of Val-1 is not sufficient to sustain synthesis of enough L-alanine for normal growth. This fits the reduced extracellular L-alanine accumulation, thereby reducing its content in the final product and improving the purification and recovery of L-valine from the fermentation broth. A similar relation between increased L-valine flux and reduced L-alanine accumulation could indeed be shown to be due to decreased intracellular pyruvate availability. In the L-valine producer without pyruvate dehydrogenase activity and with increased valine formation due to overexpression of the L-valine biosynthesis genes, the intracellular pyruvate concentration decreased from 25.9 mM to 2.3 mM (3).

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