Appl Microbiol Biotechnol (2004) 65: 576–582
DOI 10.1007/s00253-004-1636-0

Frank Schneider · Reinhard Krämer · Andreas Burkovski

Identification and characterization of the main β-alanine uptake system in *Escherichia coli*

Received: 1 March 2004 / Revised: 13 April 2004 / Accepted: 16 April 2004 / Published online: 24 June 2004

Abstract In *Escherichia coli*, β-alanine is a direct precursor in the biosynthesis of pantothenic acid (vitamin B_5). Although a sufficient β-alanine supply is crucial for biotechnological vitamin B_5 production, nothing was known about β-alanine transport in *E. coli* until now. The aim of this work was the characterization of β-alanine transport by *E. coli* and the identification and overexpression of the corresponding carrier-encoding gene for the rational improvement of pantothenic acid-producing strains. β-Alanine uptake was found to be an active process catalyzed by the amino acid carrier CycA. The corresponding gene was cloned and overexpressed, resulting in an increase in the uptake rate, compared with the wild type. In all tested strains, this overexpression led to a strong sensitivity to β-alanine, but not to the other CycA substrates, such as L-alanine, D-alanine, and glycine. This prevented a direct application for the improvement of pantothenic acid-producing strains by an enhanced precursor supply.

Introduction

As one of the B-complex vitamins, pantothenic acid (vitamin B_5) is an essential component of mammalian nutrition. It is the precursor for phosphopantotheine and thereby participates in over 100 metabolic reactions, as part of coenzyme A and acyl carrier protein (Jackowski 1996). The pantoate-β-alanine ligase (PanC)-catalyzed condensation of β-alanine and pantoic acid is the final step in pantothenic acid biosynthesis. Pantoic acid is synthesized from α-ketoisovalerate by ketopantoate hydroxymethyltransferase (PanB) and ketopantoate reductase (PanE). Additionally, the second reaction can be carried out by the *ilvC* gene product, acetohydroxyacid isomeroreductase (Primerano and Burns 1983).

In *Escherichia coli*, the vast majority of β-alanine is synthesized de novo by the L-aspartate-1-decarboxylase (EC 4.1.1.15; PanD)-catalyzed α-decarboxylation of L-aspartic acid (Begley et al. 2001). While β-alanine formation from aspartic acid seems to be the main pathway in K12 strains (Cronan 1980), *E. coli* B strains are also capable of degrading uracil to β-alanine (Patel and West 1987), a metabolic pathway usually found in eukaryotes (Gojkovic et al. 2001). Furthermore, the hydrolysis of carnosine by peptidase D (Klein et al. 1986) and possibly the degradation of spermidine (Tabor and Kellogg 1970) might be alternative pathways, but their relevance for pantothenate biosynthesis is unclear.

In recent years, efficient *E. coli* production strains for pantothenic acid have been developed. Good producers, obtained by random mutagenesis, are further improved by overexpressing the *panBCD* genes (Hikichi et al. 1996) or additionally *ilvGM* coding for pyruvate acetohydroxyacid synthase (Moriya et al. 1999). Hikichi and coworkers pointed out that the yield of pantothenic acid is raised dramatically by the addition of high β-alanine concentrations (2% or more) to the medium (Hikichi et al. 1996), indicating that β-alanine synthesis is the rate-limiting step, even in *panD*-overexpressing strains. Since the currently used fermentation processes are strictly dependent on β-alanine uptake, this transport process may in principle be a bottleneck.

The aim of this work was the characterization of the transport of this building block into *E. coli* MG1655 and the identification of the responsible carrier-protein for a possible application in an improvement of pantothenic acid-producing strains.

F. Schneider · R. Krämer · A. Burkovski (✉)
Institut für Biochemie, Universität Köln,
Zülpicher-Strasse 47,
50674 Cologne, Germany
e-mail: a.burkovski@uni-koeln.de
Tel.: +49-221-4706472
Fax: +49-221-4705091

Materials and methods

Bacterial strains and growth The strains and plasmids used in this study are shown in Table 1. *E. coli* cells were routinely grown at 37°C in Luria–Bertani (LB) medium

supplemented with antibiotics (100 μg ml^{-1} carbenicillin, 10 μg ml^{-1} tetracycline, or 20 μg ml^{-1} chloramphenicol) as appropriate. For uptake measurements, cells were grown in M9 minimal medium (Sambrook et al. 1989) supplemented with 0.5% glucose, 1 mM $MgSO_4$, 100 μM $CaCl_2$, and 0.01% of a trace elements solution containing (per liter): 10 g $FeSO_4 \cdot 7H_2O$, 10 g $MnSO_4 \cdot H_2O$, 10 g $CaCl_2 \cdot 2H_2O$, 1 g $ZnSO_4 \cdot 7H_2O$, 0.2 g $CuSO_4$, and 0.02 g $NiCl_2$. For pantothenic acid production with strain FV5069/pFV31/pACYC-*cycA*, M9 minimal medium was supplemented with 5% glucose and 0.5 mg l^{-1} thiamine. *Corynebacterium glutamicum* strain R127 (*panC*::*pK18-mob'panC'*; Sahm and Eggeling 1999) was grown in CGXII minimal medium (Keilhauer et al. 1993) supplemented with 20 μg ml^{-1} kanamycin and 4% glucose as a carbon source. If necessary, 50 μg l^{-1} Ca^{2+}-D-pantothenate was added.

Determination of β-alanine uptake by [1-^{14}C]β-alanine Cells were grown for 17 h at 37°C in M9 minimal medium, washed, suspended in fresh M9 medium to an optical density at 600 nm (OD_{600}) of 5.0, and stored on ice until used. Aliquots (2.7 ml) were transferred to test tubes and prewarmed in a water bath for 3 min at 37°C with continuous stirring. Uptake was started by the addition of [1-^{14}C]β-alanine (specific activity 250 cpm $nmol^{-1}$; Biotrend, Germany). If appropriate, inhibitors were added 30 s prior to the substrate. Samples (200 μl) were taken, vacuum-filtrated, and washed twice with 2.5 ml of 100 mM LiCl. Radioactivity incorporated in the cells was measured using a Beckman LS6500 scintillation counter.

Nonradioactive determination of amino acid uptake Cultivation and treatment of the cells was the same as for radioactive uptake measurements. Experiments were started by the addition of substrate and samples (200 μl) were taken at intervals. The cells were immediately centrifuged through a silicon oil layer into 30 μl 20% perchloric acid (Klingenberg and Pfaff 1967) to separate them from the medium. After the addition of 45 μl H_20, the cells were lyzed by ultrasonic treatment. Cell debris was precipitated by adding 5 M KOH/1 M triethanolamine (25 μl), incubating on ice for 30 min, and centrifuging for 10 min at 20,000 *g*. The aqueous phase was collected for HPLC analysis.

HPLC analysis of β-alanine and other amino acids Amino acids (including β-alanine) in the samples were pre-column-derivatized with *o*-phthalaldehyde (Lindroth and Mopper 1979) and separated on a Hewlett Packard HP1100 HPLC system, using a Hypersil ODS5 125.0×4.6 mm column (CS-Chromatographie-Service, Germany). A gradient ranging from 97% buffer A (20 mM sodium acetate, 0.036% triethylamine, 0.3% tetrahydrofurane) to 100% buffer B (20% 100 mM sodium acetate, 40% methanol, 40% acetonitrile) was used for elution and the derivatives were detected by their fluorescence at 230 nm.

Microbiological determination of pantothenic acid Pantothenic acid concentrations in samples were determined by monitoring the growth of the pantothenic acid auxotrophic strain *C. glutamicum* R127 (*panC*::*pK18mob'panC'*). The final cell density of this strain in CGXII minimal medium is strictly dependent on the pantothenic acid content and can thereby be used for quantification. For details, see Sahm and Eggeling (1999).

Calculations Intracellular solute concentrations were calculated assuming a cytoplasmic volume of 0.69 μl and 1.82 μl of extracytoplasmic bound medium per OD_{600} unit and per milliliter, respectively (Stock et al. 1977;

Table 1 Bacterial strains and plasmids used

Strain or plasmid	Characteristics	Reference
E. coli		
MG1655	K12 wild type (λ$^-$ F$^-$*rph-1*)	Blattner et al. (1997), Jensen (1993)
CAG12073	MG1655 (*cycA30*::Tn*10*)	Singer et al. (1989), kindly provided by M. Berlyn (*E. coli* stock center)
FV5069	D-Pantothenic acid production strain	Hikichi et al. (1996)
FV5069/pFV31	FV5069 containing plasmid pFV31 for *panBCD*-overexpression	Hikichi et al. (1996)
C. glutamicum		
R127 (*panC*::pK18*mob'panC'*)	R127 (*panC*::pK18*mob'panC'*)	Sahm and Eggeling (1999)
Plasmids		
pDRIVE	PCR cloning vector	Qiagen, Germany
pTrc99a	Ap^r*lacI*^{q} ColE1 P_{trc}	Amann and Abel (1989)
pTrc-cycA	Derivative of pTrc99a containing the gene *cycA* of *E. coli* MG1655	This study
pACYC184	Cm^r Tc^r p15A	Chang and Cohen (1978)
pACYC-cycA	Derivative of pACYC184 containing *cycA* of *E. coli* MG1655 with its natural promoter	This study

Cayley et al. 1991). For transport rate calculations, 1 ml of cell suspension at OD_{600}=1.0 was assumed to contain 0.34 mg dry weight of bacteria.

Cloning of cycA-expressing plasmids For the cloning of pTrc-*cycA*, the 1,413 bp *cycA* gene of MG1655 was amplified from genomic DNA by PCR, using the primers 5′-AGA*GAATTC*CATGGTAGATCAGGTAAAAGTCG-3′ and 5′-GCAG*AAGCTT*ATTTCCGCAGTTCAGCAGC-3′. After digestion with *Eco*RI and *Hin*dIII (restriction sites shown in italics), this product was inserted into the *Eco*RI/*Hin*dIII sites of vector pTrc99a. For the generation of pACYC-*cycA*, first the *cycA* promoter of MG1655 was amplified by PCR, using the primers 5′-TCGTTG*GTATA-C*AATATTAACTGCTGGAAATCCTCTAAGC-3′ and 5′-GATCTA*CCATGG*TTTTTTCTTCCTG-3′. Then, product was ligated to the pDRIVE vector (Qiagen, Hilden, Germany) by A-T cloning. *lacI*^{q} and the *trc* promoter were cut out of pTrc-*cycA* using *Acc*I and *Nco*I and the remaining plasmid was purified. With *Acc*I and *Nco*I (sites shown in italics) the *cycA* promoter was cut out of pDRIVE and ligated with the pTrc-*cycA* fragment. This plasmid was then cut with *Ssp*I and a 2.3-kb fragment (consisting of the *cycA* promoter and *cycA*) was isolated. This fragment was ligated with *Xmn*I-cut pACYC184, leading to the low-copy expression vector pACYC-*cycA*.

Results

Biochemical characterization of β-alanine uptake For the biotechnological production of pantothenic acid, the uptake of β-alanine is crucial (Hikichi et al. 1996). Therefore, we first characterized this transport process in *E. coli* MG1655. Uptake measurements with [1-^{14}C]-labeled substrate showed an intracellular accumulation of β-alanine at 1 mM external substrate (Fig. 1). Using the H^+-specific ionophore carbonylcyanide-*m*-chlorophenyl-hydrazone (CCCP), the energetics of the transport process was investigated. As a potent uncoupler, CCCP causes the dissipation of the electrochemical H^+ potential, a driving force for energy-dependent transport systems. When CCCP was added 30 s prior to [1-^{14}C]β-alanine, the uptake rate dropped dramatically and the intracellular concentration did not exceed 1 mM during the experiment. Transport experiments with varying substrate concentrations revealed Michaelis–Menten kinetics, with a K_m of 2.4 mM and a maximal transport rate (V_{max}) of 46 nmol mg^{-1} dry weight min^{-1} under our experimental conditions (data not shown).

Identification of the β-alanine uptake system During the transport measurements, a strong inhibition of β-alanine uptake by traces of LB medium was observed. To test whether this might result from competitive inhibition of β-alanine transport by an amino acid in the LB medium, two cultures of strain MG1655 were incubated in M9 minimal medium containing a final concentration of 1% LB medium and β-alanine was added to one of the cultures,

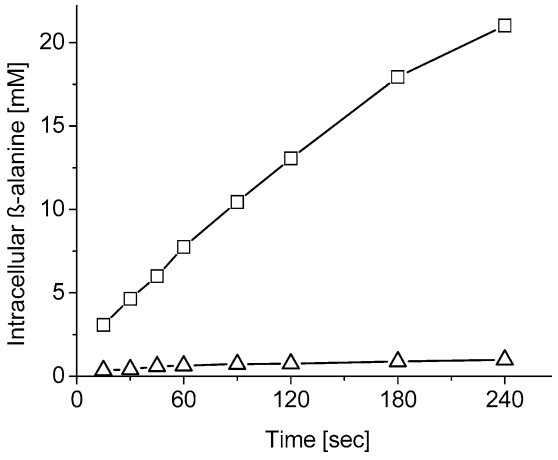

Fig. 1 Dependency of β-alanine transport on the electrochemical H^+ potential as measured by [1-^{14}C]β-alanine. β-Alanine concentration was 1 mM and uptake was examined with (*open triangles*) and without (*open squares*) the addition of CCCP to a final concentration of 50 μM. Independent uptake experiments were carried out in duplicate with identical results

to a final concentration of 10 mM. After 1 min of incubation, cells of both cultures were separated from the medium by silicon oil centrifugation and the cytoplasmic amino acids pools were determined by reversed-phase HPLC. In the presence of β-alanine, significantly less L-alanine was transported into the cells (data not shown). From this result, it was suggested that β-alanine uptake is mediated by an L-alanine uptake carrier. In *E. coli*, two different transport systems were described for L-alanine uptake, the *cycA* gene product and the LIV-I system (Robbins and Oxender 1973), which differ in their energetics and substrate specificities. Besides L-alanine, the CycA permease mediates uptake of glycine, D-alanine, D-serine, and D-cycloserine, whereas LIV-I is a primary active ABC transporter for the import of the branched-chain amino acids L-leucine, L-isoleucine, and L-valine. These properties were used to differentiate between CycA and LIV-I. Uptake of 1 mM β-alanine was strongly inhibited by an equimolar amount of the CycA substrate D-cycloserine, whereas L-isoleucine had no effect (Fig. 2). In an additional experiment, glycine revealed a strong inhibitory effect but the LIV-I substrate L-leucine did not (data not shown). These data suggest CycA may be the β-alanine carrier of *E. coli*. To confirm this assumption of β-alanine transport in strain CAG12073 (Singer et al. 1989), a MG1655 derivative carrying a Tn*10* insertion in *cycA* was measured. Here, the uptake was reduced to 12.6% of the wild type activity at 1 mM of external substrate (1.4 nmol mg^{-1} dry weight min^{-1} vs 11.1 nmol mg^{-1} dry weight min^{-1}) and could not be further inhibited by glycine or L-leucine (data not shown). Efficient β-alanine uptake obviously depends on a functionally active CycA protein.

For the overexpression of *cycA*, the plasmid pTrc-*cycA* was generated. This high copy number vector is a derivative of pTrc99a (Pharmacia Biotech) and contains the 1,413-bp *cycA* gene of MG1655. Although the strong

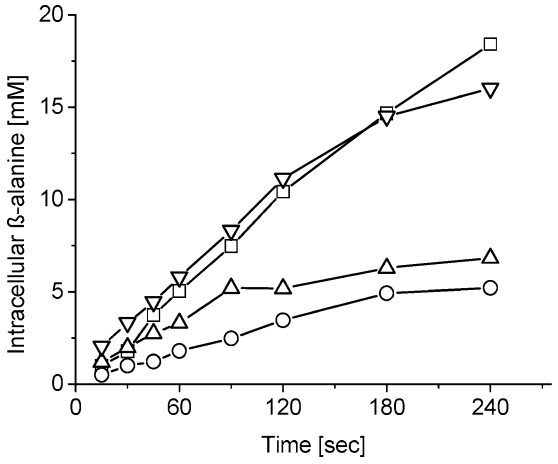

Fig. 2 Uptake of β-alanine by *E. coli* MG1655. The substrate concentration was 1 mM and transport was measured with [1-^{14}C]β-alanine in the presence of 1 mM L-alanine (*open circles*), D-cycloserine (*open upright triangles*), L-isoleucine (*open downward triangles*), or without the addition of a competitor (*open squares*)

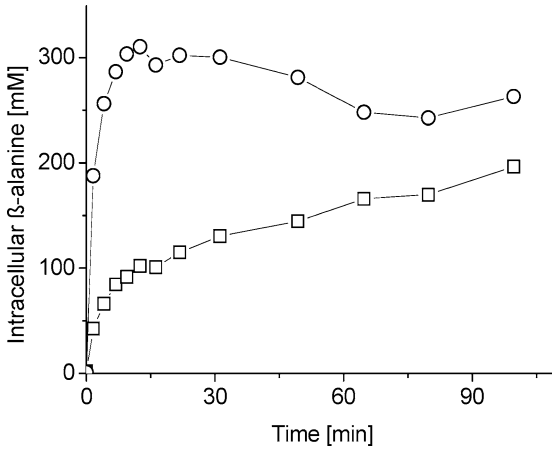

Fig. 3 β-Alanine uptake of MG1655 carrying either plasmid pTrc99a (*open squares*) or pTrc-*cycA* (*open circles*) at a substrate concentration of 10 mM. Cells were separated by silicon oil centrifugation and cytoplasmic β-alanine was determined by HPLC analysis

trc promoter is repressed by the plasmid-encoded LacI repressor, the leakiness of the system allowed sufficient gene expression even without induction. As expected, transformation of strain CAG12073 by pTrc-*cycA* restored its β-alanine uptake activity, while vector pTrc99a without insert had no effect (data not shown).

Enhancement of β-alanine uptake by cycA-overexpression
For an enhancement of β-alanine uptake, strain MG1655 was transformed with pTrc-*cycA* and with pTrc99a as a control. Figure 3 shows β-alanine uptake upon the addition of 10 mM β-alanine (data obtained by HPLC analysis). In MG1655/pTrc-*cycA*, β-alanine uptake was much faster than in the control strain; and the internal concentration rose up to 300 mM in 10 min. Interestingly, in the *cycA*-overexpressing strain, net import stopped at about 300 mM and declined only slightly. Without β-alanine feeding, endogenously synthesized β-alanine was not detectable in any strain, indicating a concentration below 0.5 mM, which is the detection limit of the HPLC method used. From these results, we conclude that the overexpression of *cycA* is an effective tool for the enhancement of β-alanine uptake in *E. coli*.

CycA overexpressing strains become sensitive to β-alanine
During the investigation of strain MG1655/pTrc-*cycA*, a severe growth inhibition by β-alanine was observed. When cells were cultivated to OD_{600}=1.3 in minimal medium, the addition of β-alanine to 1 mg l^{-1} significantly delayed further growth (Fig. 4); and at 10 mg l^{-1} it stopped growth completely. As seen in other experiments, 891 mg l^{-1} β-alanine (equivalent to 10 mM) even caused cell lysis (data not shown). In contrast, strains MG1655/pTrc99a and MG1655 grew normally at these concentrations and tolerated more than 18 g l^{-1} β-alanine. As seen in additional experiments, the CycA substrates glycine, D-alanine, and L-alanine did not inhibit the growth of MG1655/pTrc-*cycA* (data not shown). Since β-alanine per se is not toxic (Fig. 3) and growth inhibition occurred only in pTrc-*cycA* strains, we assumed that a high level of CycA protein or its activity might cause sensitivity.

To decrease *cycA* expression, we first replaced the strong *trc* promoter of pTrc-*cycA* with the native *cycA* promoter of *E. coli* MG1655. The promoter-*cycA* construct was cut-out of the high-copy vector and ligated into the low-copy plasmid pACYC184, leading to the expression plasmid pACYC-*cycA*. Subsequently, *E. coli* MG1655 was transformed with pACYC-*cycA* and tested for β-alanine susceptibility. In the resulting strain, MG1655/pACYC-*cycA*, the addition of 10 mM β-alanine alone slowed down growth drastically, while in the presence of β-alanine other CycA substrates such as glycine, L-alanine, or D-alanine restored growth (data not shown)—

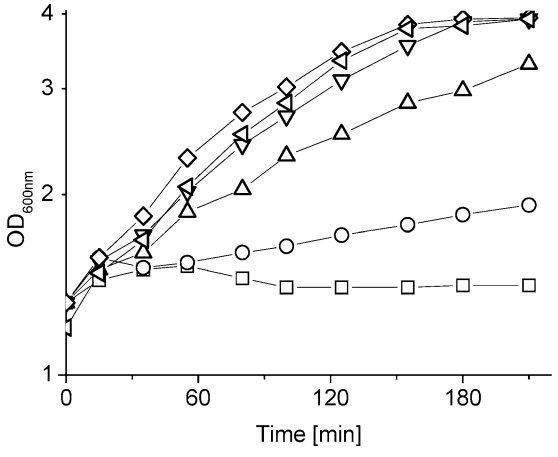

Fig. 4 Growth inhibition of *E. coli* MG1655/pTrc-*cycA* by β-alanine. Cells were grown in M9 minimal medium to OD_{600}=1.3 and then subjected to the following concentrations of β-alanine: 10 mg l^{-1} (*open squares*), 3 mg l^{-1} (*open circles*), 1 mg l^{-1} (*open upright triangles*) and 0.3 mg l^{-1} (*open downward triangles*). As a control, MG1655/pTrc-*cycA* (*open diamonds*) and MG1655/pTrc99a (*open left-pointing triangles*) were grown without the addition of β-alanine

probably by a competitive displacement of β-alanine from the transporter. Interestingly, D-alanyl–D-alanine, a precursor in cell wall biosynthesis, was also able to suppress the inhibitory effect of β-alanine at least partially (data not shown). This hints at an interference in β-alanine transport and cell wall biosynthesis.

Consequence of cycA overexpression in E. coli FV5069 derivatives for pantothenic acid production *E. coli* FV5069 and FV5069/pVF31 (containing a *panBCD* expression plasmid) are efficient pantothenic acid-producing strains (Hikichi et al. 1996). To investigate the consequence of *cycA* overexpression, these strains were transformed by the *cycA* expression plasmids and subsequently tested for pantothenate production. As FV5069/pTrc-*cycA* turned out to be even more sensitive to β-alanine than strain MG1655, we used only 25 μM β-alanine for the production assay in M9 minimal medium (Fig. 5a). Compared with the control, FV5069/pTrc-*cycA* showed a prolonged lag phase and slower growth during the exponential phase. Nevertheless, because of the similar amount of pantothenic acid produced, its specific productivity (units of product per unit biomass) was significantly higher (Fig. 5a).

The transformation of strain FV5096/pFV31 with the low-copy expression vector pACYC-*cycA* also led to β-alanine sensitivity, but cell lysis was not observed (data not shown). For the production assay, cells were initially grown in M9 minimal medium until they reached the stationary phase (Fig. 5b). Then, β-alanine was added to a final concentration of 2% (224 mM). After the addition, FV5069/pFV31/pACYC-*cycA* and FV5069/pFV31/pACYC184 both started to produce pantothenic acid, but the amount in the FV5069/pFV31/pACYC-*cycA* culture was even lower than in the control.

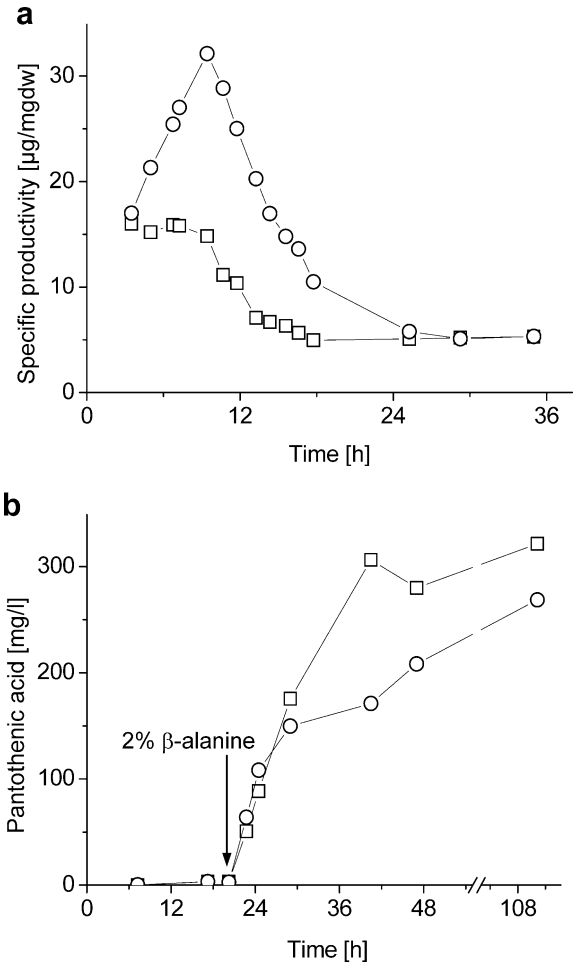

Fig. 5 a,b Effect of *cycA* overexpression on pantothenic acid production. **a** Specific productivity (units of pantothenic acid per unit biomass) of FV5069/pTrc99a (*open squares*) and FV5069/pTrc-*cycA* (*open circles*). The experiment was carried out in duplicate. **b** Pantothenic acid production by FV5069/pFV31/pACYC184 (*open squares*) and FV5069/pFV31/pACYC-*cycA* (*open circles*) in M9 minimal medium after the addition of 2% (224 mM) β-alanine

Discussion

E. coli MG1655 is able to accumulate β-alanine more than 20-fold in the cytoplasm compared with the external medium. In combination with the observed transport inhibition by CCCP, this demonstrates the presence of an active β-alanine uptake system. While the determined V_{max} of 46 nmol mg^{-1} dry weight min^{-1} is in the range of other amino acid transporters, the K_m of 2.4 mM is rather high. This indicates that β-alanine is presumably not the preferred substrate of this uptake system.

The β-alanine transporter was identified as CycA. The CycA protein is a secondary transporter which mediates the uptake of L-alanine, D-alanine, glycine, D-serine, and D-cycloserine (Wargel et al. 1970; Cosloy 1973). It belongs to the amino acid transporter family (Jack et al. 2000), a subgroup within the amino acid/polyamine/organocation superfamily of transporters. The 470-amino-acid transmembrane protein is predicted to comprise 12 transmembrane helices and to utilize H^+ co-transport as the energy source (Swiss-Prot data base; http://www.expasy.org/sprot; accession no. P39312). Together with *fklB*, *cycA* is thought to form the *fklB-cycA* operon, whose expression is regulated by the nitrogen assimilation control (Nac) protein (Zimmer et al. 2000). Additionally, the obvious functionality of the expression plasmid pACYC-*cycA* implicates the activity of a predicted promoter located directly upstream of *cycA*.

Uptake measurements using the *cycA* mutant CAG12073 revealed a strongly reduced uptake rate of only 12.6%, compared with the wild type. Since this residual activity could not be further inhibited by glycine or L-leucine, it is not caused by CycA or LIV-I and may rather be due to the unspecific action of other amino acid transport systems or simply diffusion. The restored uptake activity of CAG12073/pTrc-*cycA* shows that CycA in fact is the main β-alanine carrier of MG1655. Moreover, the drastically increased transport rates in strain MG1655/pTrc-*cycA* suggest that *cycA* overexpression is a suitable tool for the improvement of β-alanine uptake.

However, this basically positive effect was counteracted by the observed β-alanine sensitivity caused by *cycA*

overexpression. β-Alanine concentrations above 1 mg l^{-1} (11.2 μM) inhibited the growth of MG1655/pTrc-*cycA* significantly and 10 mM caused cell lysis. The following calculation illustrates that the observed growth inhibition is unlikely to be caused by high intracellular β-alanine concentrations. In the experiment shown in Fig. 4, β-alanine was added to the MG1655/pTrc-*cycA* culture at OD_{600}=1.3, corresponding to approximately 0.9 μl cytoplasm ml^{-1} cell suspension. If the cells took up the entire substrate at a concentration of 1 mg l^{-1} (11.2 μM), which is already growth-inhibitory, the intracellular β-alanine pool would have been 12.5 mM. As can be seen in Fig. 3, the control strain MG1655/pTrc99a reached ten-fold higher intracellular concentrations without any growth inhibition when exposed to 10 mM β-alanine.

Although the reason for this effect is not yet clear, some results indicate an impairment of cell wall biosynthesis. In *E. coli*, the antibiotic D-cycloserine is taken up by CycA (Wargel et al. 1971). It acts by competitive inhibition of the D-alanyl–D-alanine ligase and thereby blocks cell wall biosynthesis (Lugtenberg 1972). β-Alanine could have a similar effect in the overexpressing strains, if a direct channeling of substrates from CycA to the D-alanyl–D-alanine ligase could be assumed. If, as in the overexpressing strains, the ratio of CycA to D-alanyl–D-alanine ligase was too high, this might result in a block of all dipeptide-forming enzymes, while a low transporter:ligase ratio in the wild type would prevent this detrimental effect. This assumption is supported by the finding that addition of the dipeptide D-alanyl–D-alanine was found to partially restore the growth of MG1655/pACYC-*cycA* in the presence of β-alanine.

As observed for the MG1655 derivatives, the *cycA*-overexpressing pantothenic acid production strains also turned out to be sensitive to β-alanine. Since high β-alanine concentrations in the medium are a prerequisite for pantothenic acid production (Hikichi et al. 1996), this fact excludes the direct application of *cycA* for strain improvement. Although the increase of specific productivity in FV5069/pTrc-*cycA* proves that the concept of *cycA* overexpression is in principle valid, the applicable substrate concentrations are too low to obtain commercially interesting yields. Since the yield of FV5069/pFV31/pACYC-*cycA* (Fig. 5b) was even lower than the control, we conclude that a benefit of *cycA* overexpression for pantothenic acid production is rather doubtful.

Acknowledgement This work was supported by Degussa AG (Hanau).

References

Amann E, Abel KJ (1989) Expression vectors for the production of non-fusion proteins in microorganisms. European patent EP0345615

Begley TP, Kinsland C, Strauss E (2001) The biosynthesis of coenzyme A in bacteria. Vitam Horm (Leipzig) 61:157–171

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1474

Cayley S, Lewis BA, Guttman HJ, Record MT Jr (1991) Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein–DNA interactions in vivo. J Mol Biol 222:281–300

Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134:1141–1156

Cosloy SD (1973) D-Serine transport system in *Escherichia coli* K-12. J Bacteriol 114:679–684

Cronan JE Jr (1980) Beta-alanine synthesis in *Escherichia coli*. J Bacteriol 141:1291–1297

Gojkovic Z, Sandrini MP, Piskur J (2001) Eukaryotic β-alanine synthases are functionally related but have a high degree of structural diversity. Genetics 158:999–1011

Hikichi Y, Moriya T, Miki H, Yamaguchi T, Nogami I (1996) Production of D-pantoic acid and D-pantothenic acid. US patent 5,518,906

Jack DL, Paulsen IT, Saier MH (2000) The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microbiology 146:1797–1814

Jackowski S (1996) Biosynthesis of pantothenic acid and coenzyme A. In: Neidhardt FC, Curtiss R III, Ingraham J, Lin ECC, Low KB, Magasanik B, Reznikoff W, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd edn. ASM, Washington, D.C., pp 687–694

Jensen KF (1993) The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. J Bacteriol 175:3401–3407

Keilhauer C, Eggeling L, Sahm H (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J Bacteriol 175:5595–5603

Klein J, Henrich B, Plapp R (1986) Cloning and expression of the *pepD* gene of *Escherichia coli*. J Gen Microbiol 132:2337–2343

Klingenberg M, Pfaff E (1967) Means of terminating reactions. Methods Enzymol 10:680–684

Lindroth P, Mopper K (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phtalaldehyde. Anal Chem 51:1667–1674

Lugtenberg EJ (1972) Studies on *Escherichia coli* enzymes involved in the synthesis of uridine diphosphate-*N*-acetyl-muramyl-pentapeptide. J Bacteriol 110:26–34

Moriya T, Hikichi Y, Moriya Y, Yamaguchi T (1999) Process for producing D-pantoic acid and D-pantothenic acid or salts thereof. US patent 5,932,457

Patel BN, West TP (1987) Degradation of the pyrimidine bases uracil and thymine by *Escherichia coli* B. Microbios 49:107–113

Primerano DA, Burns RO (1983) Role of acetohydroxy acid isomeroreductase in biosynthesis of pantothenic acid in *Salmonella typhimurium*. J Bacteriol 153:259–269

Robbins JC, Oxender DL (1973) Transport systems for alanine, serine, and glycine in *Escherichia coli* K-12. J Bacteriol 116:12–18

Sahm H, Eggeling L (1999) D-Pantothenate synthesis in *Corynebacterium glutamicum* and use of *panBC* and genes encoding L-valine synthesis for D-pantothenate overproduction. Appl Environ Microbiol 65:1973–1979

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Singer M, Baker TA, Schnitzler G, Deischel SM, Goel M, Dove W, Jaacks KJ, Grossman AD, Erickson JW, Gross CA (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol Rev 53:1–24

Stock JB, Rauch B, Roseman S (1977) Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J Biol Chem 252:7850–7861

Tabor CW, Kellogg PD (1970) Identification of flavin adenine and heme in a homogeneous spermidine dehydrogenase from *Serratia marcescens*. J Biol Chem 245:5424–5433

Wargel RJ, Shadur CA, Neuhaus FC (1970) Mechanism of D-cycloserine action: transport systems for D-alanine, D-cycloserine, L-alanine, and glycine. J Bacteriol 103:778–788

Wargel RJ, Shadur CA, Neuhaus FC (1971) Mechanism of D-cycloserine action: transport mutants for D-alanine, D-cycloserine, and glycine. J Bacteriol 105:1028–1035

Zimmer DP, Soupene E, Lee HL, Wendisch VF, Khodursky AB, Peter BJ, Bender RA, Kustu S (2000) Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc Natl Acad Sci USA 97:14674–14679