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Identification of *glyA* (Encoding Serine Hydroxymethyltransferase) and Its Use Together with the Exporter ThrE To Increase L-Threonine Accumulation by *Corynebacterium glutamicum*

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L-Threonine can be made by the amino acid-producing bacterium *Corynebacterium glutamicum*. However, in the course of this process, some of the L-threonine is degraded to glycine. We detected an aldole cleavage activity of L-threonine in crude extracts with an activity of 2.2 nmol min^{-1} (mg of protein)$^{-1}$. In order to discover the molecular reason for this activity, we cloned *glyA*, encoding serine hydroxymethyltransferase (SHMT). By using affinity-tagged *glyA*, SHMT was isolated and its substrate specificity was determined. The aldole cleavage activity of purified SHMT with L-threonine as the substrate was 1.3 μmol min^{-1} (mg of protein)$^{-1}$, which was 4% of that with L-serine as substrate. Reduction of SHMT activity in vivo was obtained by placing the essential *glyA* gene in the chromosome under the control of P_{tac}, making *glyA* expression isopropylthiogalactopyranoside dependent. In this way, the SHMT activity in an L-threonine producer was reduced to 8% of the initial activity, which led to a 41% reduction in glycine, while L-threonine was simultaneously increased by 49%. The intracellular availability of L-threonine to aldole cleavage was also reduced by overexpressing the L-threonine exporter *thrE*. In *C. glutamicum* DR-17, which overexpresses *thrE*, accumulation of 67 mM instead of 49 mM L-threonine was obtained. This shows that the potential for amino acid formation can be considerably improved by reducing its intracellular degradation and increasing its export.

Amino acids are of great economic interest (17), and *Corynebacterium glutamicum* is particularly well suited for producing these cellular metabolites. For instance, the synthesis pathways and the biochemical conditions for the production of L-glutamate (12), L-lysine (8), L-tryptophan (18), and L-isoleucine (11) have been studied in detail in this organism. However, in high cellular synthesis, competing reactions may lead to the degradation of the desired amino acid (11, 35) and thus limit product formation. In the same way, export from inside the cell to the outside into the medium may place a considerable limitation on product formation (4, 35, 39, 44). While the synthesis pathways are well studied and the general principles of increasing the flux via these pathways are established (7), this is only true to a lesser extent of the degradation reactions and the translocation of the intracellularly formed amino acids from the cell into the medium (9, 10).

In the present study we investigated these aspects in L-threonine formation with *C. glutamicum*. L-Threonine synthesis proceeds in three steps, starting from aspartate semialdehyde. The corresponding biosynthesis genes *hom* and *thrB*, which form an operon, were cloned (14), as was *thrC* (16). The *hom* gene codes for homoserine dehydrogenase, and alleles of this gene, such as HomG378E [*hom*(Fbr)], that code for a dehydrogenase which is no longer feedback-inhibited by L-threonine have been identified (34). The overexpression of *hom* and *thrB* with high-copy-number plasmids is possible (13, 29), whereas *hom*(Fbr) *thrB* can only be expressed at low levels (35). This is due to the resulting high internal L-threonine concentration of up to 100 mM, versus less than 1 mM in the wild type. Increased internal L-threonine concentration is associated with increased glycine formation (5, 35). Furthermore, the very high internal concentration of L-threonine indicates that its export is limited. This export is catalyzed by the recently identified ThrE carrier (39), which is present not only in *C. glutamicum*, but also in bacteria, archaea, and fungi (46).

The purpose of our present work was to determine how L-threonine is degraded to glycine in *C. glutamicum*. There are indications that a threonine 3-dehydrogenase may be responsible for such reaction in *Corynebacteriaceae* (3). In *Corynebacterium* sp. strain B6, an activity of as much as 2.04 μmol min^{-1} (mg of protein)$^{-1}$ was detected (2). Furthermore, we wanted to reduce an identified activity in *C. glutamicum* and to examine whether improved L-threonine accumulation occurs. A further goal was to increase the ThrE-catalyzed export of L-threonine and again to assay for improved L-threonine accumulation.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. Luria-Bertani (LB) was used as the standard medium for *Escherichia coli*, while *C. glutamicum* was precultivated on brain heart infusion medium (BHI; Difco). The minimal medium used for product accumulation and enzyme assays was CGXII (19). Cultures of strain DR-17 were additionally supplied with 300 mg of L-leucine $liter^{-1}$. When appropriate, *E. coli* strains received carbenicillin or kanamycin (each at 50 μg ml^{-1}) or tetracycline (10 μg ml^{-1}). *C. glutamicum* received kanamycin (50 μg ml^{-1}) or tetracycline (5 μg ml^{-1}). A reduced concentration of kanamycin, 15 μg ml^{-1}, was used to obtain recombinant cells of *C. glutamicum* after transformation (42). Strains with a

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5αMCR	Cloning strain	15
M15/pREP4	K-12 derivative suitable for protein isolation, containing vector supplying *lac* repressor, Km^r	Qiagen
S17-1	Mobilizable donor strain	40
C. glutamicum		
ATCC 13032	Wild type	
DR-17	Threonine producer derived from lysine producer MH20-22B	28, 35
DM368-2	Threonine producer with feedback-resistant aspartate kinase and homoserine dehydrogenase	Degussa
13032::pK18mob*glyA′*	Wild type with *glyA* under control of *tac* promoter	This work
DM368-2::pK18mob*glyA′*	DM368-2 with *glyA* under control of *tac* promoter	This work
Plasmids		
pK18mob	Mobilizable vector, nonreplicative in *C. glutamicum*, Km^r	37
pQE30	Expression vector supplying 6× His tag coding sequence, Ap^r	Qiagen
pVWEx2	*C. glutamicum* expression vector with *tac* promoter, Tet^r	45
pUC18*glyA*	pUC18 with 1.7-kb PCR fragment containing *glyA*	This work
pUC18*glyA*-2	pUC18 with 1.3-kb PCR fragment containing promoterless *glyA*	This work
pVWEx2*glyA*	pVWEx2 with 1.4-kb *Tfi*I-*Eco*RI insert from pUC18*glyA*	This work
pK18mob*glyA′*	pK18mob with 2.0-kb PCR fragment from pVWEx2*glyA*, containing $lacI^q$ and P*tac* fused to 5′-terminal fragment of *glyA*	This work
pQE30*glyA*	pQE30 with 1.3-kb PCR fragment containing *glyA*	This work
pEC-T18mob2	*E. coli-C. glutamicum* shuttle vector, Tet^r	AF445081
pUC18*thrE*	pUC18 with *thrE* of *C. glutamicum*	39
pEC-T18mob2*thrE*	pEC-T18mob2 with *Sac*I-*Xba*I fragment of pUC18*thrE*	This work

chromosomally integrated vector received a concentration of 25 μg of kanamycin ml^{-1}. *E. coli* was grown at 37°C, and *C. glutamicum* was grown at 30°C.

Construction of plasmids. The degenerate primers given in the Results section were used to amplify and clone an internal fragment of *glyA.* This fragment, together with genome information, was used to amplify the entire *glyA* gene as a 1,722-bp fragment with the primers 5′-GCTTGCAGCGTTTTGCTCTGCC-3′ and 5′-ACCCGTAACCTCTTCCACATAGG-3′. The amplified fragment was blunted and ligated with *Sma*I-cleaved pUC18 to give pUC18*glyA*. This served as a template to amplify promoterless *glyA* with the primers 5′-GCGGATCCATG ACCGATGCCCACCAAG-3′ and 5′-CCGTCGACTTAGACGATGGTCCAG TCTTC-3′, where a *Bam*HI and a *Sal*I site, respectively, were attached. The resulting structural gene together with the added restriction sites was ligated with *Sma*I-cleaved pUC18 to give pUC18*glyA*-2. The *glyA* gene was excised from this vector as a *Bam*HI-*Sal*I fragment and ligated with *Bam*HI- and *Sal*I-cleaved pQE30 to yield pQE30*glyA*, containing *glyA* with six His codons attached to its 5′ end. The entire construct was verified by sequencing.

To place *glyA* under the control of the *tac* promoter, a 1,418-bp *Eco*RI-*Tfi*I fragment of *glyA* was isolated which contained *glyA* without its own promoter. The fragment was blunted and ligated with *Bam*HI-cleaved pVWEx2, made by V. Wendisch (45), which is a derivative of pKW0 (20), enabling isopropylthiogalactopyranoside (IPTG)-dependent expression. In the resulting construct, pVWEx2*glyA,* the *glyA* gene is fused with P_{tac}. With pVWEx2*glyA* as a template and the primer pair 5′-CCGGAATTCTCACTGCCCGCTTTCCAGTC-3′ and 5′-CGGGATCCCAGCTTTCCGGAGAAGTTCAAC-3′, a 2-kb fragment was amplified, containing a 437-bp fragment of the 5′ end of *glyA* together with the fused *tac* promoter and in addition the *lac* repressor $lacI^q$, which is also present on pVWEx2*glyA*. By use of the attached *Bam*HI and *Eco*RI sites, this fragment was ligated with the mobilizable and nonreplicative vector pK18mob to yield pK18mob*glyA′* (see Fig. 3).

Plasmid pEC-T18mob2 was used to overexpress plasmid-encoded *thrE*. This plasmid confers tetracycline resistance to *C. glutamicum* and is based on pGA1 (41, 43). To insert *thrE* into this vector, the exporter gene was excised from pUC18*thrE* as a *Sac*I-*Xba*I fragment and ligated with the correspondingly digested vector.

Construction of strains. Intergeneric gene transfer was used to place *glyA* in the chromosome of *C. glutamicum* under the control of the IPTG-inducible *tac* promoter (37). For this purpose, *E. coli* S17-1 was transformed with the nonreplicative plasmid pK18mob*glyA′* to kanamycin resistance. Conjugation and selection for kanamycin resistance were done in the presence of 0.1 mM IPTG. The correct integration into the chromosome via *glyA* sequences was verified with appropriate primer pairs and controls. The integration mutants DM368-2::pK18mob*glyA′* and ATCC13032::pK18mob*glyA′* were made by this procedure. These mutants carry one intact copy of *glyA* under the control of the inducible *tac* promoter and one incomplete copy under the natural promoter (see Fig. 3).

Isolation of SHMT. Serine hydroxymethyltransferase (SHMT) was isolated from *E. coli* M15/pREP4 containing pQE30*glyA*. LB cultures containing 50 μg of carbenicillin and 25 μg of kanamycin per ml were grown for 2 h to give an optical density at 600 nm (OD_{600}) of 0.6. Then 1 mM IPTG was added for the induction of His_6-*glyA* expression, and incubation was continued for a further 3 h up to an OD_{600} of 2.5. Cells were harvested and lysed by sonication. The resulting extract was centrifuged, and the enzyme was isolated via Ni^{2+}-nitrilotriacetic acid affinity chromatography.

Enzyme assays. The threonine 3-dehydrogenase activity (EC 1.1.1.103) coupled to amino-ketobutyrate lyase (Fig. 1A) was assayed in a system containing (per 0.7 ml) 300 μl of Tris-acetate-EDTA–potassium phosphate buffer (200 mM Tris-acetate-EDTA, 25 mM potassium phosphate, pH 8.6), 100 μl of KCl (400 mM), 70 μl of NAD^+ (100 mM), 70 μl of coenzyme A (CoA, 50 mM), 17.5 μl of L-threonine (200 mM), 42.5 μl of water, and 100 μl of crude extract. The reaction was started by the addition of substrate. Aliquots were removed after 0, 30, 60, and 90 min of incubation at 30°C, treated with 15% (wt/vol) trichloroacetic acid, and, after neutralization, used for glycine analysis by high-pressure liquid chromatography (HPLC).

An independent second assay made use of the fact that in the absence of CoA, amino-ketobutyrate spontaneously decarboxylates to aminoacetone, which can be quantified with Ehrlich's reagent (26). The system contained (per 1.6 ml) 800 μl of Tris-HCl (250 mM, pH 8.4), 50 μl of NAD^+ (100 mM), 250 μl of L-threonine (500 mM, pH 8.4), 400 μl of water, and 100 μl of crude extract. The reaction was started by the addition of NAD^+, stopped after incubation for 30 min (37°C) with 400 μl of 25% (wt/vol) trichloroacetic acid, and, after centrifugation, used for aminoacetone quantification. This was done by mixing 500 μl of sample, 500 μl of sodium acetate buffer (2 M, pH 4.6), 50 μl of NaOH (2.5 M), and 25 μl of acetylacetone. Assay mixtures were boiled for 10 min, and after cooling, 1 ml of Ehrlich's reagent was added (0.2 g of dimethylamino benzaldehyde dissolved in 8.4 ml of acetic acid) plus 1.6 ml of 70% (vol/vol) perchloric acid. After incubation for 15 min at room temperature, absorption readings were taken at 553 nm and compared with those of controls and standards.

The threonine aldolase activity generating glycine plus acetaldehyde (EC 4.1.2.5) (Fig. 1B) was assayed in the following system: 400 μl of equilibrated crude extract was passed over a PD10 column (Amersham Pharmacia) with Tris-acetate-EDTA–potassium phosphate buffer (200 mM Tris-acetate-EDTA, 25 mM potassium phosphate, pH 8.6), 100 μl of pyridoxal 5′-phosphate (2 mM), 100 μl of L-threonine (200 mM), 100 μl of L-isoleucine (10 mM), and 300 μl of water. Reaction mixtures were incubated for up to 120 min. The protein was precipitated with trichloroacetic acid and, after neutralization, used for glycine quantification via HPLC.

The same assay was also used to assay for formation of acetaldehyde. After termination of the reaction with trichloroacetic acid, 800 μl of supernatant was mixed with 200 μl of *N*-methylbenzothiazolon hydrazone (1%, wt/vol) and the pH was adjusted to 3 to 4 with 45 μl of neutralization buffer (31.8 g of K_2CO_3 in

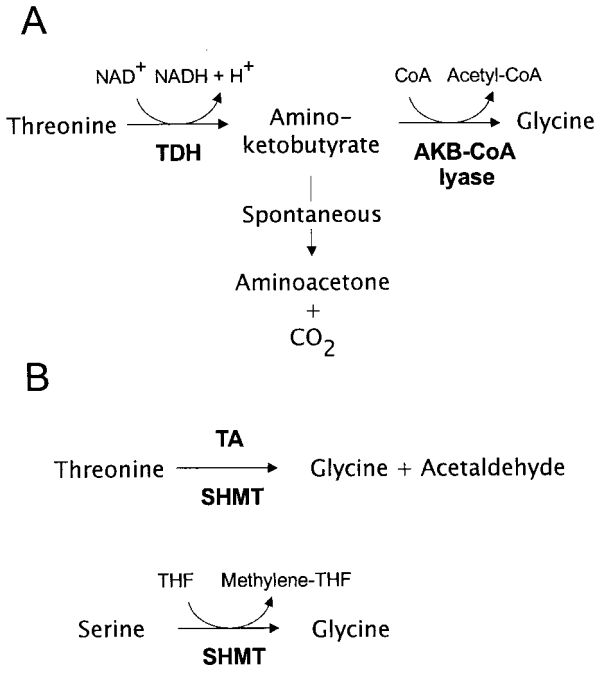

FIG. 1. Pathways for L-threonine degradation. (A) Pathway initiated by threonine-3-dehydrogenase (TDH) activity, with subsequent CoA-dependent conversion of 2-amino-3-ketobutyrate by amino-ketobutyrate lyase (AKB-CoA lyase) or spontaneous decarboxylation of 2-amino-3-ketobutyrate. (B) L-Threonine cleavage by transaldolase (TA) or SHMT activity and the main activity of SHMT generating 5,10-methylene tetrahydrofolate (THF).

100 ml of 20 mM Tris-HCl, pH 8.0). Assay mixes were boiled for 3 min, and after cooling, 2.5 ml of 0.2% (wt/vol) $FeCl_3$ was added. After 5 min at room temperature they were mixed with 6.5 ml of acetone, and absorptions were read at 670 nm.

The SHMT (EC 2.1.2.1) was assayed with 300 μl of HEPES-NaOH (200 mM, pH 7.0), 200 μl of crude extract (equilibrated via passage through PD-10 columns with the same buffer), 100 μl of pyridoxal-5′-phosphate (2 mM), 50 μl of 5,10-methylene tetrahydrofolate (18 mM in 0.1% [wt/vol] dithiothreitol), 200 μl of substrate (100 mM, either L-threonine or L-serine), and 150 μl of water. Reaction mixes were incubated for up to 15 min, and 500-μl samples were mixed with 125 μl of 25% (wt/vol) trichloroacetic acid, placed on ice, and centrifuged in the cold. Then 480 μl of the resulting supernatant was neutralized with buffer (31.8 g of K_2CO_3 in 100 ml of 20 mM Tris-HCl, pH 8.0), and glycine was quantified via HPLC.

All enzyme activities are given in nanomoles per minute per milligram of protein, with protein determined with the biuret reaction.

Amino acid quantification. Amino acids were quantified by automated precolumn derivatization with *ortho*-phthaldialdehyde (21), followed by reversed-phase chromatography with fluorometric detection as previously described (39).

Nucleotide sequence accession number. The sequence data have been submitted to GenBank under accession number AF327063.

RESULTS

L-Threonine-degrading enzyme activities. The L-threonine accumulation of *C. glutamicum* is accompanied by an undesirable glycine accumulation (35). Since it was reported that *Corynebacterium* species possess threonine dehydrogenase activity (3), yielding, together with 2-amino-3-ketobutyrate, CoA ligase glycine as one end product (Fig. 1A), we assayed for this activity in *C. glutamicum*. In extracts of the type strain ATCC 13032 and the L-threonine producer DR-17, neither NAD^+ nor CoA-dependent glycine formation could be detected. Furthermore, no specific amino acetone formation occurred due to spontaneous decarboxylation of the intermediate 2-amino-3-ketobutyrate (26).

A second possible glycine-yielding reaction is the pyridoxal-5′-phosphate-dependent aldole cleavage of L-threonine. This can be catalyzed by an aldolase (22) or as a side reaction of SHMT (38) (Fig. 1B). In extracts of the same strains used previously, glycine formation with a specific activity of 2.2 nmol min^{-1} (mg of protein)$^{-1}$ was detected. Also, the expected increase in formation of the second end product, acetaldehyde, could be detected. Therefore, a specific threonine aldolase or an aldolase activity due to SHMT is present in *C. glutamicum*. However, it is not possible to differentiate between these two threonine-degrading possibilities without genetic or biochemical work. Attempts to complement the aldolase mutant *E. coli* GS245 with gene banks used successfully for complementation of other *E. coli* mutations (19) failed, although *E. coli* GS245 was successfully used to isolate *lta* from a *Pseudomonas* strain (23). We therefore focused on *glyA*, the gene encoding SHMT.

Cloning of *glyA* and purification of SHMT. The degenerate primers 5′-GAYATGGCNCACTTCGCNGG-3′ and 5′-AC NARGTGNACRTCNGTNCC-3′, designed to match conserved regions within known *glyA* genes, were used to clone a 372-bp PCR product. Based on this fragment, *glyA* of *C. glutamicum* was eventually cloned as a 1.7-kb fragment to give pUC18glyA. Sequence analysis identified *glyA* (1,305 nucleotides), which is preceded by two inverted repeats together with two possible promoter regions. This might indicate highly regulated transcriptional control of the gene, as is the case in *E. coli* (24). The deduced polypeptide has an M_r of 46,539 and exhibits the highest identities over its whole length with homologues from *Mycobacterium tuberculosis* (73%), *Bacillus subtilis* (53%), and *E. coli* (48%).

To further characterize the gene product, *glyA* was amplified together with attached *Bam*HI and *Sal*I sites to enable cloning in pQE30. This resulted in a fusion of *glyA* with $(His)_6$-Gly-Thr codons attached to its 5′ end. Plasmid pQE30*glyA* was used to transform *E. coli* M15, and a culture of the recombinant strain was induced to enable isolation of the His-tagged SHMT. Figure 2 demonstrates the successful isolation of the polypeptide. Its molecular weight is fully consistent with that deduced from the *glyA* sequence. The protein (fraction E3) was used to assay L-serine- and L-threonine-dependent glycine formation. With L-serine as the substrate (and 5,10-methylene tetrahydrofolate [THF]), the specific activity was 31.0 μmol min^{-1} (mg of protein)$^{-1}$, and with L-threonine it was 1.3 μmol min^{-1} (mg of protein)$^{-1}$. This shows that *glyA* encodes functionally active SHMT and that this enzyme also converts L-threonine at a rate of about 1/25th that of L-serine.

Construction of a GlyA depletion strain. In order to study the consequences of reduced SHMT activity on product formation, we tried to inactivate *glyA* by use of an internal fragment in a mobilizable inactivation vector (37). However, even on complex medium supplemented with glycine, an appropriate integration mutant could not be obtained, indicating that *glyA* is essential (see below). This resembles the situation with *E. coli*, in which the *glyA* gene product is the major enzyme generating one-carbon units (24). Therefore, a strain in which the chromosomal wild-type *glyA* gene is controlled by the IPTG-inducible *tac* promoter was constructed.

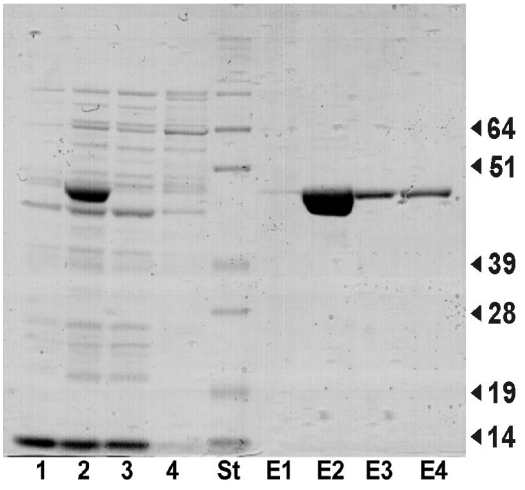

FIG. 2. Purification of SHMT. A sodium dodecyl sulfate gel is shown, containing an extract of *E. coli* without induced *glyA* expression (lane 1), with induced expression (lane 2), or the flowthrough (lanes 3 and 4). Lane St, size standards, with sizes indicated on the right (in kilodaltons). Lanes E1 to E4, eluted SHMT protein.

For this purpose, promoterless *glyA* obtained as a 1,418-bp *Tfi*I-*Bam*HI fragment was cloned into pVWEx2, supplying the *tac* promoter as well as its repressor *lacI*^{q} (Fig. 3). From this vector, together with the appropriate primers, *lacI*^{q}, P*tac*, and a 5′-*glyA* part of 437 bp was amplified and subsequently cloned into the nonreplicative vector pK18mob (37). By using conjugative transfer, *C. glutamicum* DM368-2 was transformed to kanamycin resistance in the presence of IPTG. One resulting transformant was designated DM368-2::pK18mob*glyA′* and verified by PCR to have the full chromosomal copy of *glyA* under the control of P*tac* (Fig. 3).

Growth and SHMT activity of the GlyA depletion strain.

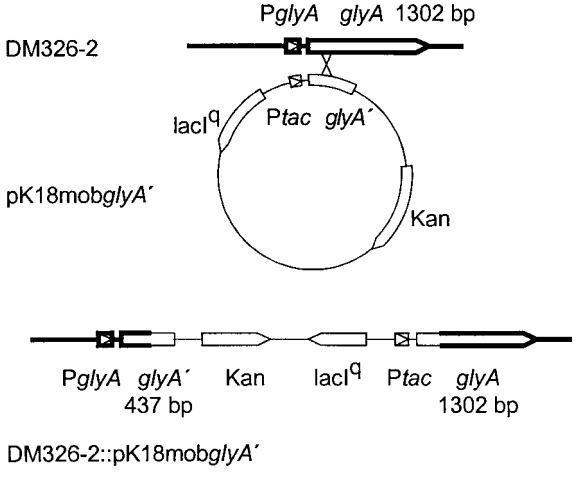

FIG. 3. Depletion construct pK18mob*glyA′* to reduce expression of SHMT. Shown is the construct itself with the inducible *tac* promoter and the 3′ part of *glyA*. Furthermore, the recombination of pK18mob*glyA′* with the chromosomal *glyA* sequences of strain DM326-2 is shown, and at the bottom is shown the resulting genomic organization in the strain enabling IPTG-dependent P_{tac}-driven expression of *glyA*.

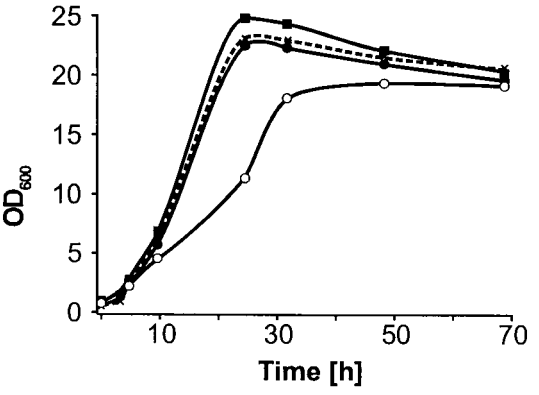

FIG. 4. Growth of strain DM326-2::pK18mob*glyA′* on minimal medium without IPTG (○) or with 10 μM (×) or 100 μM (■) IPTG and the control strain DM326-2 pZ1 without IPTG (●).

As shown in Fig. 4, growth of the constructed strain DM368-2::pK18mob*glyA′* was reduced without IPTG addition, whereas the addition of 10 μM IPTG restored growth comparable to that of the control. This is in accord with a reduced and limiting SHMT activity without IPTG being present. The remaining growth is probably due to SHMT activity in the cells used as the inoculum. This was made from washed cells grown in complex medium plus 100 μM IPTG. When we used cells as grown in Fig. 4 without IPTG addition and then used them as an inoculum for a new culture, growth was completely abolished in the absence of IPTG (not shown). This indicates that SHMT is essential for *C. glutamicum* and *glyA* is the sole relevant gene encoding this enzyme activity.

To confirm this view, cells were grown as before on the minimal medium and harvested after 10 h of cultivation, and SHMT activity was determined with L-serine or L-threonine as the substrate. There was a clear relation of the IPTG concentration to SHMT activity (Table 2). The roughly 10% activity in the absence of IPTG is apparently too low to sustain normal growth of *C. glutamicum.* Identical experiments made with a depletion mutant of the type strain ATCC 13032 yielded almost identical results (not shown). The analysis confirms the P_{tac}-driven *glyA* expression and the fact that the cassette is suitable for assaying the consequences of reduced SHMT activity in an L-threonine producer strain.

L-Threonine accumulation at reduced *glyA* expression. In order to quantify the effects of reduced *glyA* expression on product accumulation, cells of DM368-2::pK18mob*glyA′* were grown as described above on minimal medium, and L-threo-

TABLE 2. IPTG-dependent SHMT activity[a]

C. glutamicum strain	IPTG concn (μM)	SHMT sp act [nmol min^{-1} (mg of protein)$^{-1}$]	
		L-Serine	L-Threonine
DM368-2 pZ1 (control)	0	25.8	1.6
DM368-2::pK18mob*glyA′*	0	2.1	<0.2
DM368-2::pK18mob*glyA′*	10	5.1	0.8
DM368-2::pK18mob*glyA′*	100	21.8	1.7

[a] Assays were done with the respective amino acid at 20 mM as the substrate and are based on glycine formation quantified by HPLC.

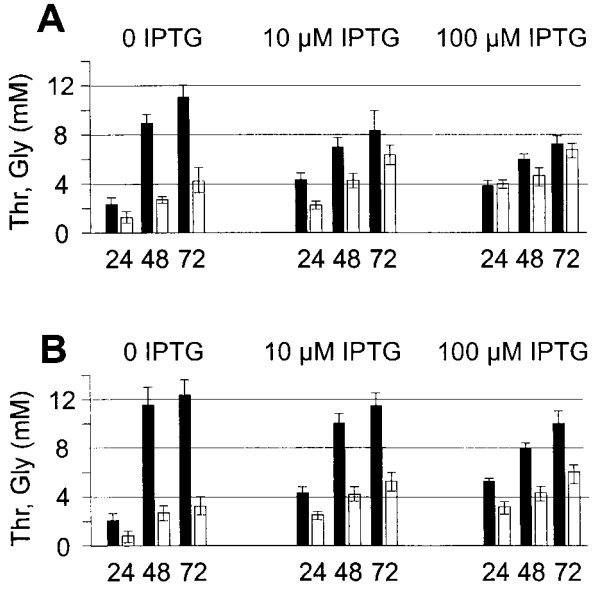

FIG. 5. L-Threonine (solid bars) and glycine accumulation (open bars) with IPTG concentrations as indicated at 24, 48, and 72 h. (A) Product accumulation with DM326-2::pK18mob*glyA′*. (B) Product accumulation with DM326-2::pK18mob*glyA′*/pEC-T18mob2*thrE*, overexpressing the exporter gene. Mean values and standard deviations for three independent cultures are presented.

nine and glycine accumulation was determined. As can be seen in Fig. 5A, the absence or a reduced concentration of IPTG resulted in increased L-threonine accumulation. At high *glyA* expression (100 μM IPTG) 7.5 mM L-threonine had accumulated after 72 h, whereas at low expression (no IPTG), an 11.2 mM concentration of this amino acid was present. This was inversely related to the glycine concentration, which was 6.8 and 4.2 mM, respectively, indicating the in vivo formation of glycine from L-threonine.

L-Threonine accumulation at increased *thrE* and reduced *glyA* expression. We tried to combine the positive effect of reduced *glyA* expression with *thrE* overexpression; the latter gene encodes the L-threonine exporter of *C. glutamicum* (39). For this purpose, plasmid pECT18mob2*thrE* was made (see Materials and Methods) and introduced into the same strain as used before to give DM368-2::pK18mob*glyA′* pECT18mob2*thrE*. This strain was cultivated again with the three different IPTG concentrations, and the amino acid accumulation was quantified. As shown in Fig. 5B, at all time points and all IPTG concentrations, elevated levels of L-threonine were obtained. Together with reduced *glyA* expression (0 mM IPTG), the L-threonine accumulation increased from 11.2 to 12.5 mM. This indicates that due to the observed increased export rate of L-threonine (39), the limiting export for this amino acid in *C. glutamicum* (32, 35) was in part overcome. At the same time, the glycine concentration was further reduced.

The effect of *thrE* expression on amino acid accumulation was also studied in strain DR-17 (35). This strain has three copies of the biosynthesis genes *hom*(Fbr) *thrB* integrated in its chromosome and is kanamycin resistant, preventing the use of pK18mob*glyA′*. Strain DR-17 was transformed with pECT18mob2*thrE* to tetracycline resistance, and cells were

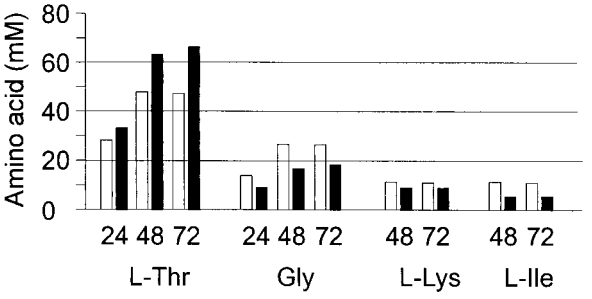

FIG. 6. Amino acid accumulation at 24, 48, and 72 h as a function of *thrE* expression with strain DR-17. Solid bars, strain DR-17/pEC-T18mob2*thrE*; open bars, control DR-17/pEC-T18mob2. Each value represents the average of two independent experiments.

grown in minimal medium to follow product accumulation (Fig. 6). Without *thrE*, 48.6 mM L-threonine had accumulated after 72 h, whereas in its presence a 67.6 mM concentration was formed. In addition to glycine, strain DR-17 also accumulated L-isoleucine and L-lysine (35). Upon *thrE* overexpression, the concentration of all three accompanying amino acids was reduced (Fig. 6). The strongest relative reduction was found for L-isoleucine (11.6 to 6.0 mM), but not L-lysine (12.1 to 10.7 mM). This suggests a direct intracellular competition of threonine dehydratase (*ilvA*) and carrier (*thrE*), together with SHMT (*glyA*), for their common substrate L-threonine.

DISCUSSION

SHMT is essential in *C. glutamicum* and may thus be the only enzyme which makes activated C-1 units available in the form of 5,10-methylene tetrahydrofolate. Because a large fraction of about 15% of the glycolytic flux is diverted to satisfy this C-1 requirement during growth on glucose, *glyA* expression is strictly regulated. Thus, for example, at least two regulators, MetR and PurR, are involved in *glyA* expression control in *E. coli* in accordance with this central role of SHMT (25). It is therefore not surprising that in the promoter region of *glyA* in *C. glutamicum*, two pairs of inverted repeats are present which are candidates for the interaction of regulator proteins (not shown).

In general, the L-threonine-degrading enzymes are often difficult to differentiate. Among other reasons, this is due in part to overlapping enzyme activities or identical products (31). An L-threonine 3-dehydrogenase activity (EC 1.1.1.103) (*tdh* in *E. coli*) is considered responsible for the degradation of L-threonine in *Corynebacterium* sp. strain B6 (2). However, this enzyme activity is not detectable in *C. glutamicum* ATCC 13032. Furthermore, the degradation of L-threonine by L-threonine aldolase is possible, generating glycine plus acetaldehyde (EC 4.1.2.5). *E. coli*, for example, and also *Pseudomonas* spp. (23) have the corresponding *lta* gene. In *E. coli*, threonine aldolase can partially replace SHMT activity (22). In principle, there is also L-allo-threonine aldolase activity (EC 4.1.2.6), which has lower activity with L-threonine as the substrate. However, this enzyme is not very widespread.

We conclude that *C. glutamicum* does not have an aldolase because our genetic attempt to isolate this gene by complementation was unsuccessful. Analysis of the genome did not

result in a corresponding gene either (not shown). A third argument is that the SHMT in *C. glutamicum* is essential, which would not be expected in a situation analogous to that of *E. coli*. We have no indications of a catabolic threonine dehydratase (EC 4.2.1.16) comparable to *tdh* in *E. coli*. All experimental findings were obtained not only with the wild type of *C. glutamicum* but also with strain DR-17, which originated from it. This also rules out specific degrading enzymes being expressed in *C. glutamicum* only at a high intracellular L-threonine concentration.

For the reasons discussed, we ascribe the L-threonine degradation in *C. glutamicum* to SHMT (EC 2.1.2.1). As shown with the isolated enzyme, the enzyme from *C. glutamicum* catalyzes the aldole cleavage of L-threonine with 4% of the activity of the cleavage of L-serine. It is thus similar to the rabbit enzyme, which also cleaves L-threonine with 6% of the activity of the cleavage of L-serine (39). However, this L-threonine cleavage is not a general property of SHMTs, since the rat enzyme is without detectable cleavage activity (27). This may be why there is such an intensive discussion of the in vivo participation of the enzyme in L-threonine degradation (31). Our experiments with L-threonine-producing strains on the product spectrum as a function of *glyA* expression, however, show that the enzyme can also cleave L-threonine in vivo.

An interesting question is, of course, how L-threonine cleavage by this essential enzyme can be further avoided in the case of L-threonine production by *C. glutamicum*. An attractive possibility would be the specific reduction of the enzyme's side activity. The three-dimensional structure of the protein is known (36). An essential structural element of the active site of the *E. coli* enzyme is 222-VVTTTTHKT-230, pyridoxal 5′-phosphate being bound to the ε-amino group of the lysine residue in position 229 as an internal aldimine (36). The motif with the string of T residues is strongly conserved and is present in an almost identical form in *B. subtilis*, *Haemophilus influenzae*, and *Homo sapiens*. As found in the present work, it is perceptibly modified in *C. glutamicum*, which has 230-VVSSTVHKT-239. Nevertheless, within the original string of T residues, the residue corresponding to T226 in the *E. coli* enzyme is retained, probably because this residue, as in the *E. coli* enzyme, is directly involved in the catalytic cycle.

As shown by systematic replacement of threonine residues with alanine, the *E. coli* enzyme tolerates substitutions of all the T residues except T226 (1). Most interestingly, the T230A substitution strongly reduces the activity with L-threonine as the substrate to less than 5% of the original activity with L-threonine, whereas more than 50% of that with L-serine is retained. This is an indication of the potential for altering the specificity of the enzyme by engineering residues within this catalytic site. In this connection, the question of how this problem of L-threonine cleavage might have been solved in the L-threonine producer of *E. coli* used industrially is naturally also of interest. This strain originated from undirected mutagenesis (12) and could therefore, for example, be mutated in the region of the SHMT discussed. Promoter mutations are, of course, also possible, and such mutations have also already been used successfully in *C. glutamicum* to improve L-lysine formation (6, 33).

In any case, reduced SHMT activity can reduce the undesirable in vivo aldole cleavage of L-threonine, so that less glycine is formed and more L-threonine is available for export. Even *thrE* overexpression alone boosts L-threonine accumulation. In this case, less glycine is also formed, i.e., glycine formation can be reduced via increased L-threonine export, probably due to reduced availability of this substrate for SHMT. The joint reduction of the internal aldole cleavage of L-threonine and the simultaneously increased export raises L-threonine accumulation from 7.5 to 11.2 mM. In strain DR-17, the L-threonine level was increased from 48.6 to 67.6 mM by *thrE* overexpression. The great increase obtained with this strain could mean that in DR-17, in which very high internal L-threonine concentrations of about 100 mM are present (35), the exporter is saturated with its substrate L-threonine. Alternatively, it is also conceivable that the permeability of the cell wall (9, 30) in strain DR-17 is different from that in the wild type, since, in addition to active export, another route contributing about 40% to total L-threonine efflux is diffusion (32, 39).

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REFERENCES

1. **Angelaccio, S., S. Pascarella, E. Fattori, F. Bossa, W. Strong, and V. Schirch.** 1992. Serine hydroxymethyltransferase: origin of substrate specificity. Biochemistry **31:**155–162.
2. **Bell, S. C., and J. M. Turner.** 1976. Bacterial catabolism of threonine. Threonine degradation initiated by L-threonine-NAD^+ oxidoreductase. Biochem. J. **156:**449–458.
3. **Bell, S. C., and J. M. Turner.** 1977. Bacterial catabolism of L-threonine. Threonine degradation initiated by L-threonine hydrolyase (deaminating) in a species of *Corynebacterium*. Biochem. J. **164:**579–587.
4. **Bellmann, A., M. Vrljic, M. Pátek, H. Sahm, R. Krämer, and L. Eggeling.** 2001. Regulation and specificity of the LysE-mediated export of amino acids by *Corynebacterium glutamicum*. Microbiology **147:**1765–1774.
5. **Colon, G. E., M. S. Jetten, T. T. Nguyen, M. E. Gubler, M. T. Follettie, A. J. Sinskey, and G. Stephanopoulos.** 1995. Effect of inducible *thrB* expression on amino acid production in *Corynebacterium lactofermentum* ATCC 21799. Appl. Environ. Microbiol. **61:**74–78.
6. **de Graaf, A. A., L. Eggeling, and H. Sahm.** 2001. Metabolic engineering for L-lysine production by *Corynebacterium glutamicum*. Adv. Biochem. Eng. Biotechnol **73:**9–29.
7. **Eggeling, L., and H. Sahm.** 1999. Amino acid production: principles of metabolic engineering, p. 153–176. *In* S. Y. Lee and E. T. Papoutsakis (ed.), Metabolic engineering. Marcel Dekker, Inc., New York, N.Y.
8. **Eggeling, L., and H. Sahm.** 1999. L-Glutamate and L-lysine: traditional products with impetuous developments. Appl. Microbiol. Biotechnol. **52:**146–153.
9. **Eggeling, L., and H. Sahm.** 2001. The cell wall barrier of *Corynebacterium glutamicum* and amino acid efflux. J. Biosci. Bioeng. **92:**201–213.
10. **Eggeling, L., K. Krumbach, and H. Sahm.** 1999. L-Glutamate efflux with *Corynebacterium glutamicum*: why is penicillin treatment or Tween addition doing the same? J. Mol. Microbiol. Biotechnol. **3:**67–68.
11. **Eggeling, L., S. Morbach, and H. Sahm.** 1997. The fruits of molecular physiology: engineering the L-isoleucine biosynthesis pathway in *Corynebacterium glutamicum*. J. Biotechnol. **56:**167–182.
12. **Eggeling, L., W. Pfefferle, and H. Sahm.** 2001. Amino acids, p. 281–303. *In* C. Radledge and B. Kristiansen (ed.), Basic biotechnology. Cambridge University Press, Cambridge, England.
13. **Eikmanns, B. J., M. Metzger, D. Reinscheid, M. Kircher, and H. Sahm.** 1991. Amplification of three biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl. Microbiol. Biotechnol. **34:**617–622.
14. **Follettie, M. T., H. K. Shin, and A. J. Sinskey.** 1988. Organization and regulation of the *Corynebacterium glutamicum hom-thrB* and *thrC* loci. Mol. Microbiol. **2:**53–62.
15. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
16. **Han, K., J. A. Archer, and A. J. Sinskey.** 1990. The molecular structure of the *Corynebacterium glutamicum* threonine synthase gene. Mol. Microbiol. **4:**1693–1702.

17. **Hodgson, J.** 1994. Bulk amino-acid fermentation: technology and commodity trading. Bio/Technology **12:**152–155.
18. **Ikeda, M., and R. Katsumata.** 1999. Hyperproduction of tryptophan by *Corynebacterium glutamicum* with the modified pentose phosphate pathway. Appl. Environ. Microbiol. **65:**2497–2502.
19. **Keilhauer, C., L. Eggeling, and H. Sahm.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. **175:**5595–5603.
20. **Kronemeyer, W., N. Peekhaus, R. Krämer, H. Sahm, and L. Eggeling.** 1995. Structure of the *gluABCD* cluster encoding the glutamate uptake system of *Corynebacterium glutamicum*. J. Bacteriol. **177:**1152–1158.
21. **Lindroth, P., and K. Mopper.** 1979. High performance liquid chromatographic detection of subpicomole amounts of amino acids by pecolumn fluorescence derivatization with *o*-phthdialdehyde. Anal. Chem. **51:**1167–11174.
22. **Liu, J. Q., T. Dairi, N. Itoh, M. Kataoka, S. Shimizu, and H. Yamada.** 1998. Gene cloning, biochemical characterization and physiological characterization of a thermostable low-specificity L-threonine aldolase from *Escherichia coli.* Eur. J. Biochem. **255:**220–226.
23. **Liu, J. Q., S. Ito, T. Dairi, N. Itoh, M. Kataoka, S. Shimizu, and H. Yamada.** 1998. Gene cloning, nucleotide sequencing, and purification of the low-specificity L-threonine aldolase from *Pseudomonas* sp. strain NCIMB 10558. Appl. Environ. Microbiol. **64:**549–554.
24. **Lorenz, E., and G. V. Stauffer.** 1995. Characterization of the MetR binding sites for the *glyA* gene of *Escherichia coli*. J. Bacteriol. **177:**4113–4120.
25. **Lorenz, E., M. D. Plamann, and G. V. Stauffer.** 1996. *Escherichia coli* cis- and trans-acting mutations that increase *glyA* gene expression. Mol. Gen. Genet. **250:**81–88.
26. **Marcus, J. P., and E. E. Dekker.** 1993. pH-dependent decarboxylation of 2-amino-3-ketobutyrate, the unstable intermediate in the threonine dehydrogenase-initiated pathway for threonine utilization. Biochem. Biophys. Res. Commun. **190:**1066–1072.
27. **Masuda, T., M. Sakamoto, I. Nishizaki, H. Hayashi, M. Yamamoto, and H. Wada.** 1987. Affinity purification and characterization of serine hydroxymethyltransferases from rat liver. J. Biochem. (Tokyo) **101:**643–652.
28. **Menkel, E., G. Thierbach, L. Eggeling, and H. Sahm.** 1989. Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. Appl. Environ. Microbiol. **55:**684–688.
29. **Morinaga, Y., H. Takagi, M. Ishida, K. Miwa, T. Sato, S. Nakamori, and K. Sano.** 1987. Threonine production by coexistence of cloned genes encoding homoserine dehydrogenase and homoserine kinase in *Brevibacterium lactofermentum*. Agric. Biol. Chem. **51:**93–100.
30. **Nampoothiri, K. M., C. Hoischen, B. Bathe, B. Möckel, W. Pfefferle, K. Krumbach, H. Sahm, and L. Eggeling.** 2002. Expression of genes of lipid synthesis and altered lipid composition modulates L-glutamate efflux of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **58:**89–96.
31. **Ogawa, H., T. Gomi, and M. Fujioka.** 2000. Serine hydroxymethyltransferase and threonine aldolase: are they identical? Int. J. Biochem. Cell. Biol. **32:** 289–301.
32. **Palmieri, L., D. Berns, R. Krämer, and M. Eikmanns.** 1996. Threonine diffusion and threonine transport in *Corynebacterium glutamicum* and their role in threonine production. Arch. Microbiol. **165:**48–54.
33. **Pátek, M., B. J. Eikmanns, J. Pátek, and H. Sahm.** 1996. Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif. Microbiology **142:**1297–1309.
34. **Reinscheid, D. J., B. J. Eikmanns, and H. Sahm.** 1991. Analysis of a *Corynebacterium glutamicum hom* gene coding for a feedback-resistant homoserine dehydrogenase. J. Bacteriol. **173:**3228–3230.
35. **Reinscheid, D. J., W. Kronemeyer, L. Eggeling, B. J. Eikmanns, and H. Sahm.** 1994. Stable expression of *hom1-thrB* in *Corynebacterium glutamicum* and its effect on the carbon flux to threonine and related amino acids. Appl. Environ. Microbiol. **60:**126–132.
36. **Scarsdale, J. N., S. Radaev, G. Kazanina, V. Schirch, and H. T. Wright.** 1999. Crystal structure at 2.8 Å resolution of *E. coli* serine hydroxymethyltransferase in complex with glycine substrate and 5-formyl tetrahydrofolate. J. Mol. Biol. **296:**155–168.
37. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
38. **Schirch, V., and T. Gross.** 1968. Serine transhydroxymethylase: Identification as the threonine and allothreonine aldolases. J. Biol. Chem. **243:**5651–5668.
39. **Simic, P., L. Eggeling, and H. Sahm.** 2001. L-threonine export: use of peptides to identify a new translocator from *Corynebacterium glutamicum*. J. Bacteriol. **183:**5317–5324.
40. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **11:**784–791.
41. **Tauch, A., A. Pühler, J. Kalinowski, and G. Thierbach.** 2000. TetZ, a new tetracycline resistance determinant discovered in gram-positive bacteria, shows high homology to gram-negative regulated efflux systems. Plasmid **44:**285–291.
42. **van der Rest, M. E., C. Lange, and D. Molenaar.** 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52:**541–545.
43. **Vašicová, P., Z. Abrhámová, J. Nešvera, M. Pátek, H. Sahm, and B. Eikmanns.** 1998. Integrative and autonomously replicating vectors for analysis of promoters in *Corynebacterium glutamicum*. Biotechnol. Techniques **12:** 743–746.
44. **Vrljić, M., H. Sahm, and L. Eggeling.** 1996. A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol. Microbiol. **22:**815–826.
45. **Wendisch, V. F.** 1997. Ph.D. thesis. Heinrich-Heine-Universität, Düsseldorf, Germany.
46. **Yen, M., Y. Tseng, P. Simic, H. Sahm, L. Eggeling, and M. H. Saier, Jr.** 2002. The ubiquitous ThrE family of putative transmembrane amino acid efflux transporters. Res. Microbiol. **153:**19–25.