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L-Threonine Export: Use of Peptides To Identify a New Translocator from *Corynebacterium glutamicum*

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Bacterial mechanisms for the uptake of peptides and their hydrolysis to amino acids are known in great detail, whereas much less is known about the fates of the peptide-derived amino acids. We show that the addition of L-threonine-containing di- or tripeptides results in reduction of the growth of *Corynebacterium glutamicum*, with concomitant high intracellular accumulation of L-threonine to up to 130 mM. Using transposon mutagenesis and isolation of mutants with increased Thr peptide sensitivity, nine open reading frames (ORFs) were identified, almost all encoding hypothetical proteins of unknown function. Three ORFs encode membrane proteins. Their individual functional characterizations in the wild-type background led to the identification of *thrE*. Upon *thrE* overexpression, growth is no longer sensitive to the presence of the Thr peptide, and L-threonine is exported at a rate of 3.8 nmol min^{-1} mg of dry $weight^{-1}$, whereas the rate of export of a *thrE* inactivation mutant is reduced to 1.1 nmol min^{-1} mg of dry $weight^{-1}$. In addition to L-threonine, L-serine is also a substrate for the exporter. The exporter exhibits nine predicted transmembrane-spanning helices with long charged C and N termini and with an amphipathic helix present within the N terminus. All these data suggest that the carrier encoded by *thrE* serves to export small molecules such as L-threonine and that the carrier is a prototype of a new translocator family. Homologues of ThrE are present in *Mycobacterium tuberculosis* and *Streptomyces coelicolor*.

As is evident from current genome analyses, a substantial number of bacterial genes encode membrane transport proteins. These proteins enable the controlled solute exchange between the cell and its environment. For instance, in *Mycobacterium tuberculosis*, about 120 gene products might encode transporters (9), and in *Escherichia coli*, about 300 candidates are present (5). However, at least half of these putative transport proteins are functionally undefined. Of course, many of the transport proteins are necessary to import nutrients such as carbohydrates, ions, amino acids, or peptides. However, in addition, some carriers are known to act as exporters. In most situations these export carriers catalyze the extrusion of noxious substances. Examples are the very well known multidrug resistance carriers (40), the metal resistance carriers (27), and the substrate-product exchange carriers (37). In addition, recent studies have shown that there are also export carriers with rather unexpected substrates, such as sugars (6, 24) and amino acids (1, 11, 49). Although in many situations the primary function of these latter carriers is still unknown, there is at least good evidence that one of the amino acid exporters naturally serves for the export of basic amino acids. This new exporter is LysE of *Corynebacterium glutamicum* (49), which is necessary during growth on complex medium or in the presence of peptides rich in L-lysine or L-arginine (4). Under such special growth conditions, L-lysine or L-arginine might accumulate to toxic levels, a situation which is prevented by their export. Homologues of the protein are widespread and occur in bacteria and archaea (50).

Studies on the peptide uptake systems of *Staphylococcus aureus* (29), *Streptococcus faecalis*, and *E. coli* (35) indicate that in these bacteria, peptide use can be accompanied by the efflux of their constituent amino acids. It thus appears that more exporters exist which accept amino acids as substrates. The efflux of selected amino acids also occurs with *Lactococcus lactis* grown on milk (18) or in the presence of milk-derived peptides (21). With *C. glutamicum* there is evidence that the efflux of L-glutamate (16), L-isoleucine (52), and L-threonine (32) is at least in part actively driven.

Studies with *C. glutamicum* are significant because of its enormous economic impact, since it is used worldwide for the production of L-glutamate and L-lysine. Together with *Mycobacterium* and *Nocardia* spp., they comprise the *Corynebacterium-Mycobacterium-Nocardia* (CMN) bacteria. These bacteria possess a mycolic acid layer which is thought to contribute significantly to the flux properties of the cell envelope and which is a major barrier to antibiotic access to *Mycobacterium* (28). We here describe the identification of a carrier exporting L-threonine from *C. glutamicum*; this exporter represents a previously unknown family of membrane transport proteins. The approach that we used to identify this exporter is based on the well-known peptide utilization of bacteria. It might therefore be suited to the isolation of further exporters with amino acids or amino-acid-related compounds as substrates, thus reducing the gap between putative and identified membrane transport proteins.

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MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. As the standard medium for *E. coli*, Luria broth was used. *C. glutamicum* was precultivated on brain heart infusion (BHI) medium

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference(s) or source[b]
Strains		
E. coli		
DH5αMCR	*endA1 supE44 recA1 gyrA96 relA1 U169*Δ(*mrr-hsdRMS-mcrBC*)	14
GM2929	*dam-13*::Tn*9 dcm-6 hsdR2 recF143 mcrA mcrB*	31
C. glutamicum		
R127	Restriction deficient	23
ATCC 14752	Wild type	ATCC
13032::*thrE*	Wild type with *thrE* (ORF53) disrupted by pK18*mob*	This work
13032::ORF22	Wild type with ORF22 disrupted by pK18*mob*	This work
13032::ORF81	Wild type with ORF81 disrupted by pK18*mob*	This work
14752 Δ*ilvA*	Wild type with a deletion of a 241-nt *Bgl*II fragment of *ilvA*	This work
R127 Δ*ilvA*	R127 with a deletion of a 241-nt *Bgl*II fragment of *ilvA*	This work
13032 Δ*thrE*	Wild type with a deletion of a 968-nt *Sac*II-*Eco*RV fragment of *thrE*	This work
Plasmids		
pCGL0040	Donor of Tn*5531* (IS*1207* Km^r); Ap^r $oriV_{E.c.}$	U53587
pET2	Promoter-probe vector	46, 48
pZ1	Shuttle vector; Km^r $oriV_{E.c.}$ $oriV_{C.g.}$	25
pK18*mob*	Integration vector; Km^r $oriV_{E.c.}$ *oriT*	44
pK19*mobsacB*	Integration vector; Km^r $oriV_{E.c.}$ *oriT sacB*	44
pK19*mobsacB*Δ*ilvA*	pK19*mobsacB* with *ilvA* with a deletion of a 242-bp internal fragment	42
pK19*mobsacB*Δ*thrE*	pK19*mobsacB* with *thrE* with a deletion of a 968-bp internal fragment	This work
pK18*mobthrE*$_{int}$	pK18*mob* with a 411-bp internal fragment of *thrE*	This work
pK18*mob*ORF22$_{int}$	pK18*mob* with a 274-bp internal fragment of ORF22	This work
pK18*mob*ORF81$_{int}$	pK18*mob* with a 368-bp internal fragment of ORF81	This work
pUC18*thrE*	pUC18 with a 1.9-kb PCR fragment containing *thrE*	This work
pZ1*thrE*	pZ1 with a 1.9-kb *SacI-XbaI* insert from pUC18*thrE*	This work
pZ1ORF22	pZ1 with a 1.0-kb PCR fragment containing ORF22	This work
pZ1ORF81	pZ1 with a 1.5-kb PCR fragment containing ORF81	This work
pET2*thrE*	pET2 with a 267-bp *Bam*HI-*Kpn*I PCR fragment containing the promoter region of *thrE*	This work

[a] Km^r, kanamycin resistant; Ap^r, ampicillin resistant. *E.c., E. coli; C.g., C. glutamicum*.
[b] ATCC, American Type Culture Collection.

(Difco). The minimal medium used for *C. glutamicum* was CGXII (19). To induce amino acid export, cells were cultivated in CGXII containing 1 mM tripeptide Thr-Thr-Thr, 1 mM tripeptide Ser-Ser-Ser, or 2 mM dipeptide Lys-Ala (13). When appropriate, ampicillin (50 μg ml^{-1}) or kanamycin (15, 25, or 50 μg ml^{-1}) was added to the medium. A *C. glutamicum* Δ*ilvA* strain received 300 mg of L-isoleucine $liter^{-1}$. *E. coli* was grown at 37°C, and *C. glutamicum* was grown at 30°C.

Transposon mutagenesis, screening for threonine-sensitive mutants, and localization of transposon insertion sites. The Tn*5531*-containing plasmid pCGL0040 was isolated from *E. coli* GM2929, and *C. glutamicum* ATCC 14752 Δ*ilvA* was transformed with the plasmid by electroporation. Transposon insertion mutants were selected by plating on LBHIS (Luria broth with brain heart infusion) containing 15 μg of kanamycin ml^{-1} (23). The resulting colonies were transferred to CGXII agar plates containing 300 mg of L-isoleucine $liter^{-1}$, 25 μg of kanamycin ml^{-1}, and either 2 mM Thr-Thr-Thr or no peptide. Mutants that were able to grow normally on CGXII minimal medium without any addition of Thr-Thr-Thr but that exhibited retarded growth in the presence of peptide were retrieved from the master plate and retested in liquid CGXII medium. For that purpose, mutant strains were precultivated on BHI medium containing 25 μg of kanamycin ml^{-1}. CGXII minimal medium (containing 300 mg of L-isoleucine $liter^{-1}$ and 25 μg of kanamycin ml^{-1}) was inoculated to an initial optical density at 600 nm (OD_{600}) of 0.1. Growth was monitored in parallel in liquid CGXII medium without any addition of peptide or with 2 mM Thr-Thr-Thr. Mutants that grew more slowly in the presence of Thr-Thr-Thr were stored in glycerol at −70°C for further studies.

For the localization of the transposon insertion locus in the *C. glutamicum* ATCC 14752 Δ*ilvA* chromosome, genomic DNA from the transposon mutants was isolated as described previously (12). The insertion loci of Tn*5531* were identified by cloning of transposon-chromosome junctions into pUC18 and subsequent DNA sequencing with oligonucleotides Tn5531-Eco (5′-CGGGTCTA CACCGCTAGCCCAGG-3′) and Tn5531-Xba (5′-CGGTGCCTTATCCATTC AGG-3′) as primers as described by Ankri et al. (3).

Construction of plasmids. All plasmid constructions were made in *E. coli* DH5αMCR. Open reading frames (ORFs) ORF22, ORF81, and ORF53 (*thrE*) were cloned from strain ATCC 13032 by PCR. Plasmids pZ1ORF22 and pZ1ORF81 were obtained by ligating the corresponding PCR fragments into the *Sca*I site of pZ1. To construct pZ1*thrE*, the PCR fragment was first cloned into the *Sma*I site of pUC18. The resulting plasmid, pUC18*thrE*, was digested with *Sac*I and *Xba*I, and the *thrE*-containing insert obtained was blunted and ligated into the *Sca*I site of pZ1. Plasmids pK18*mob*ORF22$_{int}$ and pK18*mob*ORF81$_{int}$ were obtained by ligating the corresponding internal fragments made by PCR into the *Sma*I site of pK18*mob*. To construct pK18*mobthrE*$_{int,}$ pUC18*thrE* was digested with *Cla*I and *Eco*RV (see Fig. 3) to yield a 411-bp internal fragment of *thrE*. This fragment was blunted and cloned into the *Sma*I site of pK18*mob*. The promoter region of *thrE* was cloned into vector pET2 via its *Bam*HI and *Kpn*I sites.

Construction of strains. *C. glutamicum* ATCC 13032 was transformed by electroporation (47). To obtain *thrE*, ORF22, and ORF81 insertion mutants of *C. glutamicum* ATCC 13032, nonreplicating plasmids pK18*mobthrE*$_{int}$, pK18*mob* ORF22$_{int}$, and pK18*mob*ORF81$_{int}$, respectively, were transferred to *C. glutamicum* ATCC 13032. The correct integration of the vector into the chromosome of the obtained insertion mutants, 13032::*thrE*, 13032::ORF22, and 13032::ORF81, was verified by PCR analysis. Deletion mutant *C. glutamicum* ATCC 13032 Δ*thrE* was constructed as follows. Vector pUC18*thrE* was restricted with *Eco*RV and *Ksp*I, blunted, and religated. From this vector, a fragment with a deletion of 968 nucleotides (nt) of the *thrE* coding region was excised as a *Sac*I-*Xba*I fragment; the latter was subsequently blunted and ligated with *Sma*I-digested pK19*mobsacB. C. glutamicum* ATCC 13032 was transformed with the resulting vector, pK19*mobsacB*Δ*thrE*, and chromosomal deletion was carried out using the method described by Schäfer et al. (44) and verified by PCR analysis. Construction of *ilvA* deletion mutants of *C. glutamicum* ATCC 14752 and R127 was performed as described previously for strain ATCC 13032 (42) and verified by PCR analysis.

Primer extension. Total RNA was isolated from *C. glutamicum* using extraction with hot acidic phenol (12). The transcription start site of *thrE* was determined by primer extension using SuperScript II reverse transcriptase (Gibco BRL) and primers labeled with [^{32}P]ATP. In parallel, the respective DNA (pET2p*thrE*) was sequenced using ^{32}P-labeled primers and a Thermosequenase (Amersham Pharmacia Biotech, Uppsala, Sweden) kit. The sequencing reaction mixtures and primer extension products were heated at 95°C for 4 min, and 2-μl samples were loaded onto a polyacrylamide gel.

Assay of amino acid export. For the determination of amino acid export rates, pregrown cells (BHI medium) were washed once with 0.9% NaCl, transferred into prewarmed CGXII minimal medium containing 1 mM Thr-Thr-Thr or 1 mM Ser-Ser-Ser at an initial OD_{600} of 2.0, and cultivated for 2 h at 30°C. The cells were harvested by centrifugation (5,000 × *g*, 10 min) and washed once with ice-cold CGXII minimal medium. Amino acid excretion was initiated by resuspending the cells in prewarmed CGXII minimal medium (30°C) containing the appropriate peptide at the concentration given above. The resulting cell density (OD_{600}) was 8 to 10, corresponding to 2.4 to 3.0 mg of dry weight ml^{-1}. The cells were incubated at 30°C and stirred rapidly with a magnetic stirrer. Samples for silicone oil centrifugation (20) were taken every 15 min over a period of 2 h. The separation of cellular and extracellular fractions as well as the quantification of the amino acids as their *o*-phthaldialdehyde derivatives via high-pressure liquid chromatography were carried out as described previously (49). The intracellular volume used for calculations was 2 μl mg of dry weight^{-1}.

In the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the determination of export rates was modified. The cells were precultivated, loaded with the appropriate peptide, and harvested as described above. After the cells were washed in ice-cold export buffer (100 mM morpholineethanesulfonic acid [MES], Tris [pH 7.5], 100 mM NaCl, 100 mM KCl, 500 mM mannitol, 20 mM glucose), amino acid excretion was initiated by resuspension of the cells in the same but prewarmed (30°C) buffer to a cell density (OD_{600}) of 8 to 10. The CCCP concentration used was 20 μM. For extracellular amino acid determinations, samples of 200 μl were taken every 3 min over a period of 20 min and centrifuged (10,000 × *g*, 1 min), and amino acids in the supernatants were quantified. The diffusion constant, K_d, was calculated according to the equation $v = K_d[\text{Thr}]_{in}$, where v is the rate of L-threonine efflux in nanomoles per minute per milligram of dry weight and $[\text{Thr}]_{in}$ is the intracellular L-threonine concentration in nanomoles per microliter.

Sequence analysis. DNA sequencing was performed by using standard automated cycle sequencing protocols.

Nucleotide sequence accession numbers. The sequence data have been submitted to the GenBank database under accession numbers AF326510 (*thrE*), AF326511 (ORF22), and AF326512 (ORF81).

RESULTS

Increase in intracellular L-threonine levels results in growth delay. The deletion of the basic amino acid exporter of *C. glutamicum* results in growth arrest in the presence of lysine-containing peptides (49). This is due to the accumulation of peptide-derived L-lysine up to an extremely high intracellular concentration, more than 1 M. To address whether such an effect of impaired growth could serve as a basis for the isolation of an L-threonine export-deficient mutant, we assayed the response of the wild-type derivative *C. glutamicum* R127 to the addition of threonine-containing peptides (Fig. 1A). The addition of 1 mM Thr-Ala or Ala-Thr dipeptide resulted in a significant growth delay. The strongest growth reduction was obtained in the presence of 1 mM Thr-Thr-Thr. In a separate experiment, the intracellular L-threonine concentrations were quantified by silicone oil centrifugation (Fig. 1B). Whereas without the addition of peptide the L-threonine concentration was below 1 mM, the presence of the dipeptides resulted in about 50 mM intracellular L-threonine, and with the tripeptide a concentration of up to 130 mM was obtained.

As is already known from the overexpression of threonine biosynthesis genes (39), we found an extracellular accumulation of L-isoleucine for the high intracellular L-threonine concentrations. To achieve an even more elevated level of L-threonine accumulation, *ilvA*, encoding threonine dehydratase (26), the key enzyme of isoleucine synthesis, was deleted. This led to an approximately twofold increase in the intracellular L-threonine level (data not shown). Interestingly, the increased intracellular L-threonine concentration was at best only transiently present for 4.5 h (Fig. 1B). Nevertheless, the growth delay lasted for more than 20 h. These experiments show that peptide use can dramatically alter the growth behavior of the cell. Furthermore, the correlation of high intracellular L-threonine concentration with retarded growth suggests the suitability of the observed effect for isolating mutants deficient in L-threonine export as clones characterized by a strong sensitivity to the tripeptide Thr-Thr-Thr.

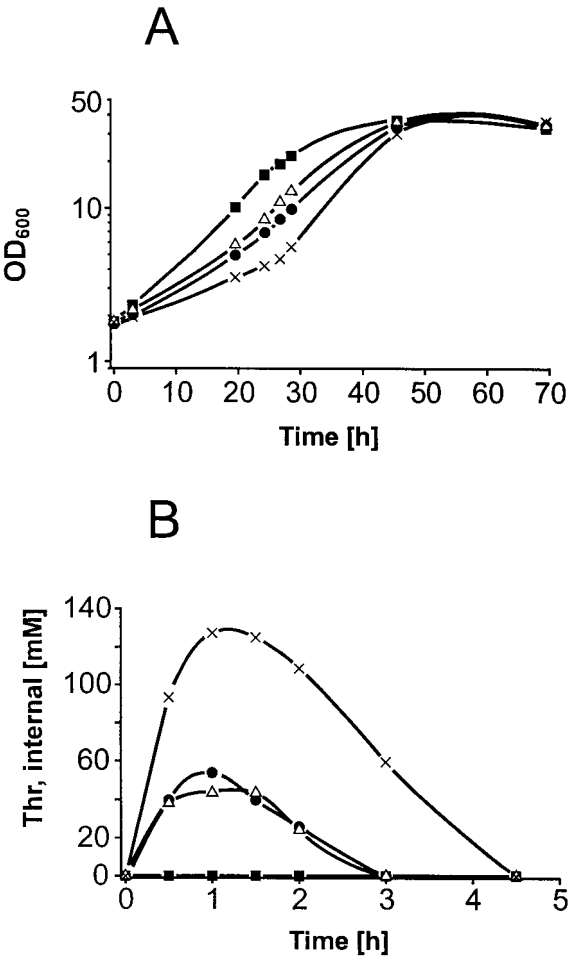

FIG. 1. Consequences of L-threonine peptide addition to *C. glutamicum* for growth and the intracellular L-threonine concentration. (A) Growth without peptide addition (■) and in response to the addition of 1 mM Ala-Thr (●), Thr-Ala (△), or Thr-Thr-Thr (×). (B) Time course for the intracellular L-threonine concentration within the first 5 h. Symbols are as described for panel A.

Isolation of threonine peptide-sensitive mutants and analysis of transposon insertion loci. Using transposon Tn*5531* (3) and strain *C. glutamicum* ATCC 14752 Δ*ilvA*, a transposon mutant bank was constructed. This strain had to be used because no transposon mutants were obtained with *C. glutamicum* R127 Δ*ilvA*. The strain used was confirmed to exhibit the Thr-Thr-Thr-dependent growth delay (data not shown). A to-

tal of 2,000 Km^r clones were tested individually for increased peptide sensitivity on agar plates. After retesting of 150 potential candidates, 21 clones remained which grew more slowly than the parent strain in the presence of peptide but normally in its absence. These clones were finally cultivated in liquid medium (CGXII with or without 2 mM Thr-Thr-Thr). Nine mutants repeatedly displayed retarded growth in the presence of Thr-Thr-Thr.

The upstream and downstream sequences of the transposon insertion loci of these mutants were cloned, sequenced, and finally analyzed using the BLASTX program (2). In one mutant, the fourth ORF within the *dapAB* operon of *C. glutamicum* was interrupted. This ORF is presumed to be involved in gene expression (34) and has now been identified in a large variety of bacteria, but its explicit function is still unknown. In a second mutant, the product of the interrupted ORF exhibited a high similarity (46%) to OtsB (trehalose-6-phosphate phosphatase) of *M. tuberculosis*. In all other mutants, the transposon was located within or close to an ORF whose derived gene product did not show any similarities to proteins of known function. Since we were interested in the actively driven component of L-threonine efflux, we focused on mutants 22, 53, and 81, since in these mutants the transposon was inserted into ORFs which could encode membrane proteins.

Analysis of ORF22 and ORF81. The deduced amino acid sequence of ORF22 revealed, among others, similarity to a putative amino acid transporter of *Bacillus halodurans*. We therefore first studied ORF22 in detail. For this purpose, a defined mutant of wild-type *C. glutamicum* ATCC 13032 was constructed using intergeneric gene transfer (44). Growth of the strain with ORF22 disrupted in the presence of 2 mM Thr-Thr-Thr in a liquid culture confirmed the growth delay in the type strain (Fig. 2A). However, in assays where pregrown cells were loaded with L-threonine and export rates were determined (see Materials and Methods), the inactivation mutant exhibited no decrease in L-threonine export compared to the control. This result suggests a function of the ORF different from catalysis of L-threonine export. The deduced gene product shares a similarity of 48% over a stretch of 61 aminoacyl residues with aquaporin of *Rattus rattus*. There is evidence that these water channel proteins and related proteins of the MIP family of channel proteins are involved in adaptation to osmotic stress conditions (8). We investigated the growth of the strain with ORF22 disrupted and of a strain with ORF22 overexpressed in the presence of up to 0.75 M NaCl and in media containing different osmolytes (glycine betaine and proline). As no growth alteration could be detected, an involvement of ORF22 in osmoregulation is unlikely.

Similar studies carried out with a set of *C. glutamicum* ATCC 13032-derived strains with ORF81 interrupted or overexpressed again confirmed growth retardation in the presence of Thr-Thr-Thr (Fig. 2A), but the L-threonine export rate was not altered.

Characterization of ORF53 (*thrE*) and its gene product. We therefore focused on ORF53 which, according to the subsequent functional analyses, was termed *thrE* (threonine exporter). The growth of strain 13032::*thrE* was indistinguishable from that of the wild type in the absence of Thr-Thr-Thr but was reduced in its presence (Fig. 2B). Overexpression of *thrE* in strain 13032(pZ1*thrE*) counteracted the negative Thr-Thr-Thr effect. Growth was even better than that of the wild type in the presence of the peptide, indicating a dose-effect relationship for *thrE* expression.

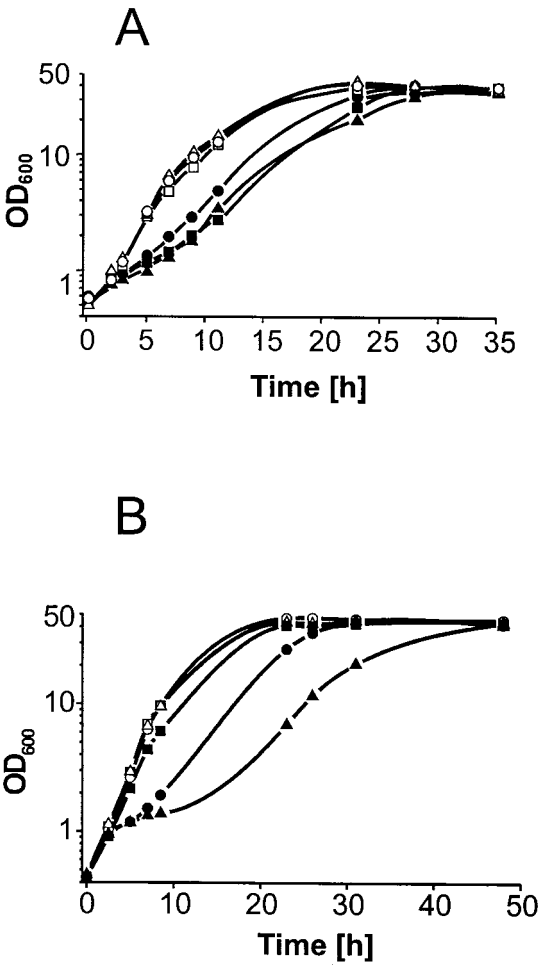

FIG. 2. (A) Growth of the wild type (circles), of strain 13032::ORF22 (triangles), and of strain 13032::ORF81 (squares) without (open symbols) and with (solid symbols) 2 mM Thr-Thr-Thr. (B) Growth of *C. glutamicum* 13032(pZ1*thrE*) (squares) and 13032::*thrE* (triangles) compared to that of the control strain 13032(pZ1) (circles) without (open symbols) and with (solid symbols) the addition of 2 mM threonine tripeptide.

An overview of the *thrE* locus with adjacent ORFs and selected fragments used for strain constructions is given in Fig. 3A. *thrE* is 1,467 nt long. The *thrE* gene product is predicted to be a hydrophobic protein of 489 amino acids with a molecular weight of 51,697. The only homologues in databases are Rv3737 of *M. tuberculosis* (9), with 29.6% identical amino acids, and a putative membrane protein of *Streptomyces coelicolor* (38), with 22.0% identical amino acids. A hydrophobicity analysis revealed pronounced local hydrophobicities within the stretch of the protein between aminoacyl residues 160 and 430 (Fig. 3B). Accordingly, application of the transmembrane prediction procedure PHD.htm (41) distinguished nine transmembrane-spanning helices within this stretch, placing the extremely long amino-terminal end in the periplasm and the carboxy-terminal end in the cytoplasm. Extensive alignments and database

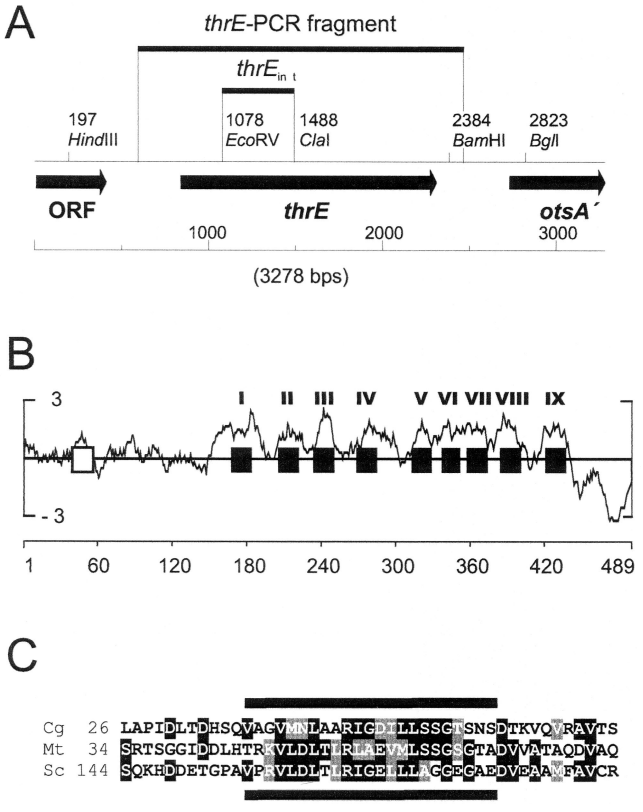

FIG. 3. Overview of the *thrE* locus of *C. glutamicum* and structural properties of ThrE. (A) DNA fragments used for *thrE* overexpression and inactivation as well as adjacent genes. (B) Average local hydrophobicity at each residue according to the algorithm of Kyte and Doolittle (22) using a window of 13 amino acids, as plotted on the vertical axis, versus the residue number on the horizontal axis. The transmembrane-spanning helices predicted by use of the neuronal network program PHD.htm (41) are highlighted as black squares and numbered I to IX. The amphipathic helix at the beginning of the protein is highlighted as an open box. (C) Part of a sequence alignment of ThrE of *C. glutamicum* (Cg) with putative proteins of *M. tuberculosis* (Mt) and *S. coelicolor* (Sc) in the region of the amphipathic helix, which is indicated by the thick lines. The numbers specify the amino acid positions at the start of the peptide stretches shown. Identical amino acid residues (black background) and conserved amino acid residues (gray shading) are indicated.

searches showed that ThrE does not belong to any characterized transporter family (43).

Upstream of *thrE* is a small ORF (399 nt) which might encode a regulator. It has a helix-turn-helix motif and exhibits an identity of 19.5% with the hypothetical transcriptional regulator MTH1328 of *Methanobacterium thermoautotrophicum* (45). The deduced amino acid sequence of the truncated ORF downstream of *thrE* exhibits a high identity, up to 63%, with trehalose-6-phosphate synthases (*otsA*) of several microorganisms.

Determination of the transcriptional start site of *thrE*. To define the *thrE* gene, its transcription initiation site was determined. For this purpose, a 267-bp *Bam*HI-*Kpn*I fragment was cloned into the promoter-probe vector pET2. The resulting plasmid made *C. glutamicum* resistant to chloramphenicol at an MIC of up to 40 μg/ml, indicating that the *thrE* promoter is of low strength (33). The result of the primer extension experiment with the sequencing reaction carried out in parallel with the same primer is shown in Fig. 4. The same initiation site was determined with a different primer. In front of *thrE*, an appropriate −10 hexamer is present, whereas a distinct −35 motif is not apparent. This is a typical feature of *C. glutamicum* promoters (34).

Expression of *thrE* correlates with export and is energy dependent. In order to functionally characterize *thrE* and to quantify its contribution to total cellular L-threonine efflux, export rates were determined with recombinant strains. A precondition for this test is the presence of a high internal L-threonine concentration, since the concentration is normally on the order of 1 mM (Fig. 1B). We therefore tested various conditions in order to achieve a greatly increased internal concentration remaining as constant as possible over an extended period of time. This is the case when cells are incubated for 2 h at 30°C with 1 mM Thr-Thr-Thr in CGXII minimal medium and, after being rinsed with cold medium, are transferred to identical fresh medium. In this way, a high internal L-threonine concentration is obtained at the start of the efflux experiment (Fig. 5A). Under these conditions, the efflux rate for the *thrE*-overexpressing strain is 3.8 nmol min^{-1} mg of dry $weight^{-1}$, and that of the wild type is 2.7 nmol min^{-1} mg of dry $weight^{-1}$ (Fig. 5B). This clear difference with almost identical internal concentrations is evidence that the *thrE* gene product catalyzes the export of L-threonine from the cell. The linear increase could indicate saturation of the exporter at 100 mM. The export rate is reduced to 1.1 nmol min^{-1} mg of dry $weight^{-1}$ for the *thrE* inactivation mutant, even though in this

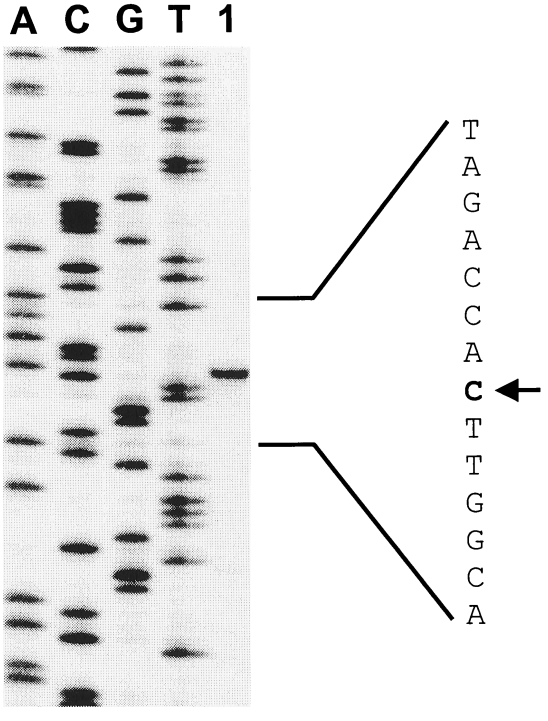

FIG. 4. Mapping of the transcriptional start site of *thrE* by primer extension analysis. The primer extension product was run in lane 1. The sequencing ladder (ACGT) of the coding strand was generated using the same primer as that used for primer extension. The transcriptional start site is indicated by the arrow.

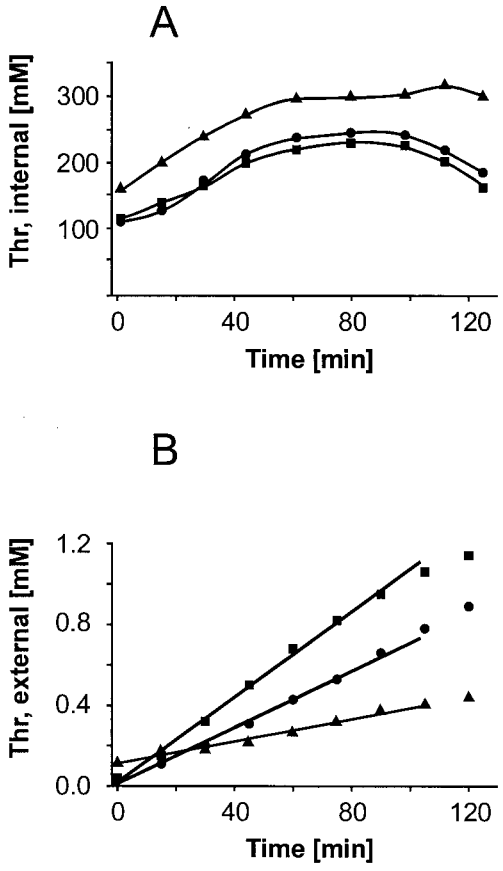

FIG. 5. Intracellular L-threonine concentration and export in recombinant *C. glutamicum* strains. The internal and external L-threonine concentrations are shown. The strains are *C. glutamicum* 13032 (pZ1*thrE*) (■), 13032::*thrE* (▲), and 13032(pZ1) (control) (●).

strain the internal threonine concentration may increase to about 300 mM, probably due to the absence of *thrE*.

Interestingly, the *thrE* inactivation mutant still excreted L-threonine at a substantial rate. Since the remaining efflux might be due in part to diffusion (32) or other, still-unknown active carriers, we used the proton ionophore CCCP to distinguish between these possibilities. Threonine export rates were determined as before, but in the presence of 20 μM CCCP. In this experiment, we used shorter measurement times to ensure a high internal L-threonine concentration, since deenergization might also influence peptide uptake. The measurements, made under slightly different conditions (see Materials and Methods), confirmed the export rates for the wild type and the inactivation mutant (Fig. 6). For both strains, however, the efflux rate was reduced to 0.6 nmol min^{-1} mg of dry $weight^{-1}$ with CCCP addition. Since active export is abolished under these conditions, the residual efflux is likely to represent passive diffusion. The calculated diffusion constant, K_d, is 0.004 μl min^{-1} mg of dry $weight^{-1}$. This experiment shows that the ThrE-mediated export of L-threonine is dependent on the proton motive force. It furthermore indicates that an additional active carrier catalyzing L-threonine export is present in *C. glutamicum* and verifies that passive diffusion, as a third component (32), contributes to total L-threonine efflux.

Substrate specificity of ThrE. It is well-known that the L-threonine uptake systems of *E. coli*, encoded by *tdcC* and *sstT*, also catalyze L-serine uptake (30, 46). We were therefore interested in analyzing whether this is also true of the new carrier which translocates L-threonine from the interior of the cell to the environment of the cell. We once again added peptides (Ser-Ala and Ser-Ser-Ser) to make these measurements possible. With the tripeptide it was possible to achieve an internal concentration, comparable to that of L-threonine, of about 180 mM L-serine (data not shown). The calculated export rates obtained from the linear increase in extracellular L-serine accumulation were 1.9, 1.4, and 0.6 nmol min^{-1} mg of dry $weight^{-1}$ for the overexpressing strain, the wild type, and the deletion mutant, respectively.

We also quantified glycine efflux by the three strains with different *thrE* expression levels. Efflux was determined in the same experiment as that shown in Fig. 5, since intracellular L-threonine is partly degraded to glycine in *C. glutamicum* (39). However, the level of glycine export in the isogenic strains was almost identical (0.9 nmol min^{-1} mg of dry $weight^{-1}$) to that of L-lysine export (about 1.1 nmol min^{-1} mg of dry $weight^{-1}$), which was quantified as a control.

DISCUSSION

This study offers an approach to identifying amino acid exporter genes. Its successful application resulted in the identification of *thrE*. The use of peptide-sensitive mutants is indirect compared to a recent approach in which the cysteine metabolite exporter of *E. coli* was identified by screening of a gene bank for increased extracellular cysteine accumulation (11). Therefore, some of the target genes inactivated might be more indirectly related to peptide sensitivity. This situation is already discernible from the fact that Thr peptide addition resulted in a short transient increase in intracellular L-threonine levels but a long-lasting growth lag (Fig. 1). These results indicate that in addition to the direct consequences of elevated L-threonine levels, an intracellular pulse of free amino acid initiates a chain of cellular events comparable, for instance, to the stringent response of enterobacteria. ORF22 and ORF70, exhibiting weak identities with aquaporin and trehalose-6-phosphate phosphatase, might be related to such secondary effects

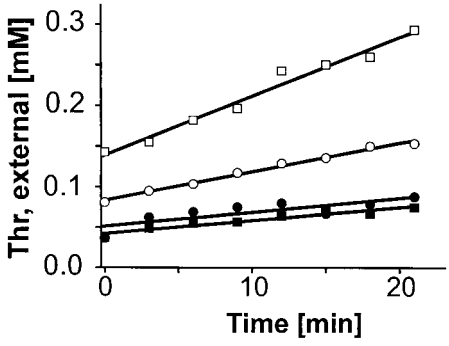

FIG. 6. Effect of the proton ionophore CCCP on L-threonine export in *C. glutamicum*. Extracellular L-threonine accumulation by 13032::*thrE* (circles) is compared to that of control strain 13032(pZ1) (squares) without (open symbols) and with (solid symbols) the addition of 20 μM CCCP.

as osmotic processes, although we have no experimental evidence to support this possibility.

The polypeptide sequence of the *thrE* gene product does not exhibit significant identities with known translocators. However, there are two putative proteins in *M. tuberculosis* and *S. coelicolor* which share more than 36% similar aminoacyl residues with ThrE. Obviously, ThrE is the prototype of a new translocator family of hitherto-unknown structure. In addition to the predicted nine transmembrane-spanning helices, the proteins are characterized by exceptional N- and C-terminal extensions. With 166 amino acids, the N terminus of ThrE is unusually long. It might be localized toward the periplasmic side, and it is rich in charged amino acids. With 13 positively and 15 negatively charged aminoacyl residues, it carries almost half of all the charged residues in ThrE. Interestingly, in all three homologues, a conserved amphipathic helix is present in this part (Fig. 3C), reminiscent of a similar structure in the long N terminus of ProW of *E. coli* (15). The C terminus of ThrE displays an even greater charge density. Of the 51 aminoacyl residues, 16 are positively charged and 4 are negatively charged. Such a strong preponderance of charged residues in the C-terminal region is known for the proline betaine transporter ProP of *E. coli* (10) and the glycine betaine uptake carrier BetP of *C. glutamicum* (36), where the extension is thought to play a role in regulation of the carrier activity.

ThrE actively exports L-threonine to the extracellular environment. However, there is also a diffusion component of efflux. In a mutant strain of *C. glutamicum* with deregulated biosynthesis (39), L-threonine excretion was attributed to active export and, to a minor extent, to diffusion (32). The identification of *thrE* enables the different efflux routes to be quantified in the wild type in detail. At an intracellular concentration in the range of 170 mM L-threonine, at least three separate components contribute to total L-threonine efflux. The major component, amounting to 59%, is the export driven by ThrE. This is evident from the analysis of the inactivation mutant. However, part of the remaining translocation is still dependent on the proton motive force. After CCCP addition (Fig. 6), the efflux due to passive diffusion contributes 22%. Therefore, a still-unknown carrier is expected to catalyze the remaining 19% of export. Since together with LysE we have now already found two novel export carriers (49; this work), it would not be surprising if there were other export carriers as well. Thus, for example, an assumed L-isoleucine transporter in *C. glutamicum* (52) could also export L-threonine, since it is known that the branched-chain amino acid import system LIV-I of *Pseudomonas aeruginosa* also accepts L-threonine with a low affinity (17).

A pertinent question is, of course, what the natural function of the discovered exporter might be. With respect to the basic amino acid exporter LysE of *C. glutamicum*, the absence of degrading activities for L-lysine and L-arginine and the control of *lysE* expression by these amino acids (4) are in agreement with the idea that LysE naturally serves to export these two amino acids. A special threonine-degrading activity, like that of the threonine dehydrogenase (*tdh*) in *E. coli* (7), is not present in *C. glutamicum* (unpublished results). The fact that the *thrE* deletion strain displays any phenotype at all at high internal L-threonine concentrations excludes a basic function of ThrE. However, the exporter could be required under special conditions. In this regard, it is interesting that *C. glutamicum* can be isolated only from soil samples contaminated with bird feces (51). In a special environment, such as the bird intestine, the export of selected low-molecular-weight compounds might be advantageous. This scenario, together with the fact that ThrE also accepts L-serine as a substrate, a feature typical of L-threonine uptake carriers like TdcC and SstT of *E. coli* (30, 46), leads us to assume that ThrE of *C. glutamicum* is structurally designed for the export of small solutes that have a structure similar to that of L-threonine.

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