JOURNAL OF BACTERIOLOGY, Nov. 2008, p. 7591–7594 Vol. 190, No. 22
0021-9193/08/$08.00+0 doi:10.1128/JB.01070-08

Identification of Genes Encoding the Folate- and Thiamine-Binding Membrane Proteins in Firmicutes▽‡

Aymerick Eudes,[1]† Guus B. Erkens,[2]† Dirk J. Slotboom,[2] Dmitry A. Rodionov,[3,4] Valeria Naponelli,[5] and Andrew D. Hanson[1]*

Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611[1]; Department of Biochemistry, University of Groningen, Groningen Biomolecular Science and Biotechnology Institute, Nijenborgh 4, 9747 AG Groningen, The Netherlands[2]; Burnham Institute for Medical Research, La Jolla, California 92037[3]; Institute for Information Transmission Problems RAS, Moscow 127994, Russia[4]; and Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611[5]

Received 30 July 2008/Accepted 27 August 2008

Genes encoding high-affinity folate- and thiamine-binding proteins (FolT, ThiT) were identified in the *Lactobacillus casei* genome, expressed in *Lactococcus lactis*, and functionally characterized. Similar genes occur in many Firmicutes, sometimes next to folate or thiamine salvage genes. Most *thiT* genes are preceded by a thiamine riboswitch.

The folate and thiamine transport systems of *Lactobacillus casei* were partially characterized 30 years ago by Henderson and colleagues (8, 9, 11, 12). These systems were shown to involve two small membrane proteins for specific substrate binding—one for folate and the other for thiamine—as well as an uncharacterized component shared by both systems.

To identify genes encoding the binding proteins (FolT and ThiT), we used the AACompIdent tool on the ExPASy server (27) to search the *L. casei* (strain ATCC 334) genome for open reading frames with amino acid compositions and molecular masses matching those published for FolT and ThiT (9, 12). The best match for FolT was LSEI_2252, a 19.0-kDa protein with five predicted transmembrane domains (Fig. 1A). LSEI_2252 has homologs in other Firmicutes, and in some cases, the corresponding genes are adjacent to *folC* (Fig. 1B). FolC is a salvage enzyme that mediates polyglutamylation of folates (2). The best match for ThiT was LSEI_1757, a 21.2-kDa protein with six predicted transmembrane domains, which belongs to the YuaJ family (InterPro accession number IPR012651) of predicted, uncharacterized thiamine transporters in the *Bacillus*/*Clostridium* group (20). LSEI_1757 is 32% identical to *Bacillus subtilis* YuaJ (Fig. 1C). In several Firmicutes, the *thiT* gene forms a putative operon with the thiamine pyrophosphokinase *thiN* gene (Fig. 1D). Like FolC, ThiN is a salvage enzyme that converts thiamine to its active pyrophosphate form (15).

To investigate whether *folT* and *thiT* indeed code for vitamin-binding proteins, the *folT* and *thiT* genes were PCR amplified from *L. casei* genomic DNA, cloned between the NcoI and SstI sites of pNZ8048, a vector carrying the nisin-inducible *nisA* promoter (14), and introduced into *Lactococcus lactis* strain NZ9000 (14). Transformants were grown at 30°C in M17 medium (Oxoid, Basingstoke, United Kingdom), supplemented with 1.0% (wt/vol) glucose, and 5 μg/ml chloramphenicol. Nisin was added when the optical density at 600 nm reached 0.7 (14), and cells were harvested 8 to 15 h later. Sodium dodecyl phosphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of membrane fractions prepared by differential centrifugation (23) showed that FolT and ThiT were abundantly expressed (Fig. 2A) and had apparent molecular masses (18 and 22 kDa, respectively) near those predicted. Cells expressing FolT or ThiT, and empty-vector controls, were assayed for binding of ^{3}H-labeled folates or thiamine after de-energization with 2-deoxyglucose to suppress interference by endogenous uptake systems (Fig. 2B to E). Cells expressing FolT bound large amounts of (6*S*)-[^{3}H]folinic acid or [^{3}H]folic acid (~17 pmol/mg protein), and those expressing ThiT bound a similar amount of [^{3}H]thiamine. Adding a polyglutamyl tail of 2 to 4 residues to [^{3}H]folic acid (16) markedly reduced binding, indicating that polyglutamyl folates are poor substrates for FolT, which is consistent with results from experiments using *L. casei* cells (22). In all cases, vitamin binding approached a plateau within 5 s and was rapidly reversed by adding an excess of unlabeled substrate. The observed vitamin acquisition, thus, has the characteristics of a binding process rather than those of an uptake process.

For further characterization, FolT and ThiT were tagged with N-terminal His$_8$ sequences. FolT-His and ThiT-His were produced in *L. lactis* as described above, except that cells were cultured in chemically defined medium (17, 19) without folic acid (for FolT-His) or thiamine (for ThiT-His) and harvested 3 h after induction. Membrane vesicles were prepared (24), and proteins were solubilized with dodecyl-β-D-maltoside (DDM) and purified to homogeneity by using nickel-Sepharose and gel filtration chromatography (3) (Fig. 3A and B). Vitamin binding was measured via quenching of intrinsic tryptophan fluorescence, using a Spex Fluorolog 322 spectrofluorometer (Jobin Yvon) and a 1-ml stirred cuvette at 25°C. The FolT-His and ThiT-His concentrations were 100 to 500 nM, and solutions of folinic acid, folic acid, or thiamine were added in 0.5- to 2-μl steps. Fluorescence was monitored at 340 nm for 20 to 30 s (excitation at 280 nm) after each substrate addition. Data were analyzed as described previously

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* Corresponding author. Mailing address: University of Florida, Horticultural Sciences Department, P.O. Box 110690, Gainesville, FL 32611. Phone: (352) 392-1928. Fax: (352) 392-5653. E-mail: adha@ufl.edu.

† A.E. and G.B.E. contributed equally to the paper.

‡ Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 5 September 2008.

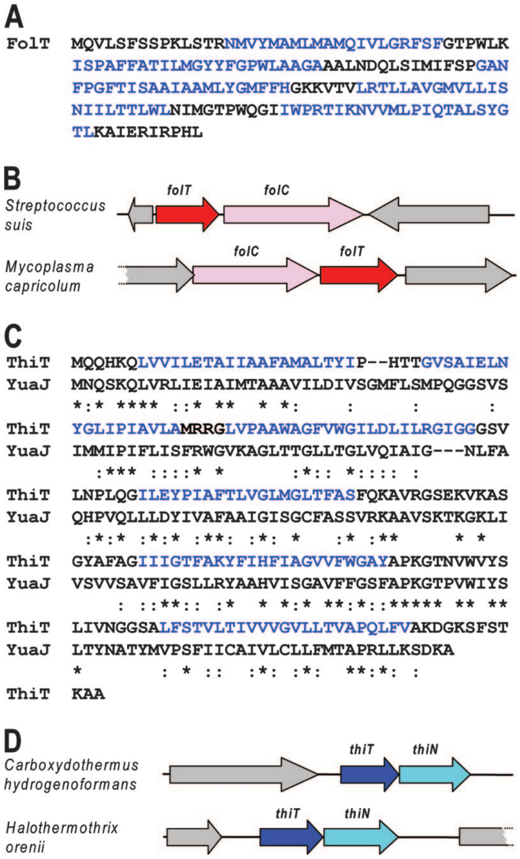

FIG. 1. FolT and ThiT proteins and the genomic context of *folT* and *thiT* genes. (A) Deduced protein sequence of *L. casei* FolT. Predicted transmembrane domains are colored blue. (B) Clustering of genes encoding FolT homologs with *folC* (folylpolyglutamate synthase-dihydrofolate synthase) in the genomes of two Firmicutes. Arrows indicate transcriptional direction. (C) Aligned sequences of *L. casei* ThiT and *Bacillus subtilis* YuaJ. Predicted transmembrane domains of ThiT are colored blue. Symbols beneath residues indicate identity (*) and similarity (:). (D) Clustering of genes encoding ThiT homologs with *thiN* (thiamine pyrophosphorylase) in genomes of the Firmicutes *Carboxydothermus hydrogenoformans* and *Halothermothrix orenii*.

(3, 25). Representative data for ThiT in the presence of increasing concentrations of thiamine are shown in Fig. 3C, and the corresponding fluorescence titration curve is shown in Fig. 3D. Comparable titration curves for FolT with (6*S*)-folinic acid and folic acid are given in Fig. 2E and F; (6*R*)-folinic acid (the unnatural isomer) produced no quenching. The proteins bind their substrates with high affinity. The dissociation constants of ThiT for thiamine (0.5 nM) and FolT for folic acid (9 nM) (Fig. 3) are within the range of values reported for *L. casei* cells (1 to 36 nM for folate binding and 0.03 to 10 nM for thiamine binding) (6, 7, 9, 12). The binding stoichiometries calculated from these data were far lower than 1:1 (0.17:1 for ThiT and 0.08:1 for FolT), compared to those calculated from the data for FolT and ThiT purified from *L. casei* (9, 12). A likely explanation is that the substrates copurified with the binding proteins, thereby obscuring binding sites, as occurred with the purified high-affinity riboflavin-binding protein RibU (3). Absorption spectra of purified FolT confirmed that substrate had indeed been copurified (not shown).

Analysis of prokaryotic genomes using the SEED comparative genomics resource (18) revealed that ThiT and FolT homologs occur commonly and almost exclusively in Firmicutes, many of which are pathogens. The multiple sequence alignments and maximum-likelihood phylogenetic trees for the FolT and ThiT protein families are shown in Fig. S1 to S3 in the supplemental material. The FolT family is substantially more diverse; while the majority of FolT proteins have five predicted transmembrane domains, two subgroups have insertions that add two more such domains, and a third subgroup has a C-terminal extension similar to aspartyl-tRNA amidotransferase subunit C (see Fig. S1A in the supplemental material). Folate-binding activity was verified experimentally for FolT proteins from three pathogens (*Mycoplasma capricolum*, *Clostridium novyi*, and *Streptococcus mutans*) by expression in *L. lactis* cells and by measuring [^{3}H]folinic acid binding as above (Fig. 4). Two of these bacteria, *C. novyi* and *S. mutans*, have complete folate biosynthesis pathways (2), as do various other pathogenic Firmicutes with *folT* genes, including *Bacillus anthracis* and *Clostridium botulinum*. It is likely that such organisms can both make and take up folates and that their folate transport capacity—which was hitherto unsuspected—confers intrinsic resistance to antibiotics targeting the folate pathway, as in malaria parasites (26).

Most of the genes encoding ThiT proteins, including that of *L. casei*, were found to be preceded by a thiamine pyrophosphate (TPP) riboswitch (see Fig. S1B in the supplemental material), and indeed, the ThiT/YuaJ family was previously predicted to participate in thiamine transport based on computational identification of these riboswitches (20). A marked feature of *L. casei* ThiT is its almost total repression by high levels of thiamine in the medium (8). TPP riboswitches located in 3′ noncoding gene regions attenuate expression of downstream genes upon binding TPP (20, 28), which readily suggests a mechanism for the observed repression.

The identification of the genes encoding the folate- and thiamine-binding proteins of *L. casei* and other Firmicutes opens the way for dissection of the corresponding transport systems at the molecular level. These systems are undoubtedly novel, as FolT and ThiT are integral membrane proteins without characterized homologs. In terms of size and hydrophobicity (but not sequence), they resemble an emerging group of integral membrane proteins implicated in vitamin and trace metal uptake. These include the following: RibU of *Lactococcus lactis*, involved in riboflavin uptake (3); BioY of *Rhodobacter capsulatus*, a component of a biotin uptake system (5); and CbiM and NikM, involved in uptake of cobalt and nickel (21). The latter three systems all include a characteristic transmembrane protein (e.g., BioN) and an ATPase similar to those of ABC-type transporters (e.g., BioM), both encoded by genes adjacent on the chromosome to genes encoding the FolT/ThiT-like component. Although there are no *bioN*- and *bioM*-related genes linked to *folT* or *thiT*, it is reasonable to infer that they lie elsewhere in the genome, given the evidence that *L. casei* FolT and ThiT require other, shared components to form an active transport system and that the energy source is ATP hydrolysis (10, 11). And indeed, the

L. casei genome contains a gene cluster encoding homologs of BioN (LSEI_2472) and BioM (LSEI_2473 and LSEI_2474), which are thus candidates for shared components of the folate and thiamine transporters.

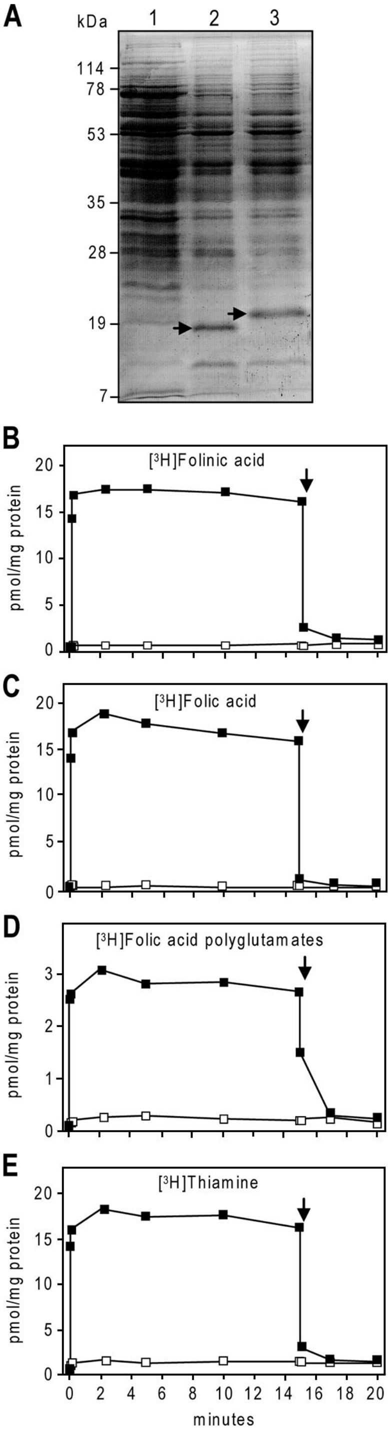

FIG. 2. Functional expression of *L. casei* FolT and ThiT in *L. lactis*. (A) SDS-PAGE (12% gel) of membrane fractions from *L. lactis* harboring pNZ8048 alone (lane 1; 50 μg protein), or containing FolT (lane 2; 25 μg protein) or ThiT (lane 3; 25 μg protein). Staining was with Coomassie brillant blue. The arrows indicate FolT and ThiT bands. Positions of molecular mass markers (kDa) are shown. (B to E) Binding of ^{3}H-labeled folates or thiamine to *L. lactis* cells harboring pNZ8048 alone (open squares) or expressing FolT or ThiT (filled squares). Assays (total volume, 1 ml) were performed in phosphate-buffered saline (PBS), pH 7.4, at 30°C with stirring. Cells were washed and resuspended (optical density at 600 nm, 20), and 0.5-ml aliquots were pretreated for 5 min with 2-deoxyglucose (25 mM final concentration). Assays were started by adding 0.5 ml of PBS containing ^{3}H-labeled vitamin (final concentration, 12.6 to 14.5 nM). At various times, cells (100 μl) were harvested by vacuum filtration on a cellulose nitrate membrane (0.45 μm). Filters were washed twice with 2 ml of ice-cold PBS, and their ^{3}H content was determined by scintillation counting. The arrows show when unlabeled vitamin was added to give a final concentration of 50 μM. Cells expressing FolT were incubated with (6*S*)-[3′,5′,7,9-^{3}H(N)]folinic acid diammonium salt (Moravek; 10 Ci/mmol) (B), [3′,5′,7,9,-^{3}H]folic acid diammonium salt (Moravek; 25.9 Ci/mmol) (C), or [^{3}H]folic acid polyglutamates (45 Ci/mmol) comprising 40% tri-, 56% tetra-, and 4% pentaglutamates (D). Cells expressing ThiT were incubated with [^{3}H(G)]thiamine hydrochloride (ARC; 10 Ci/mmol) (E). ^{3}H-Labeled substrates were chromatographically purified before use (4, 13).

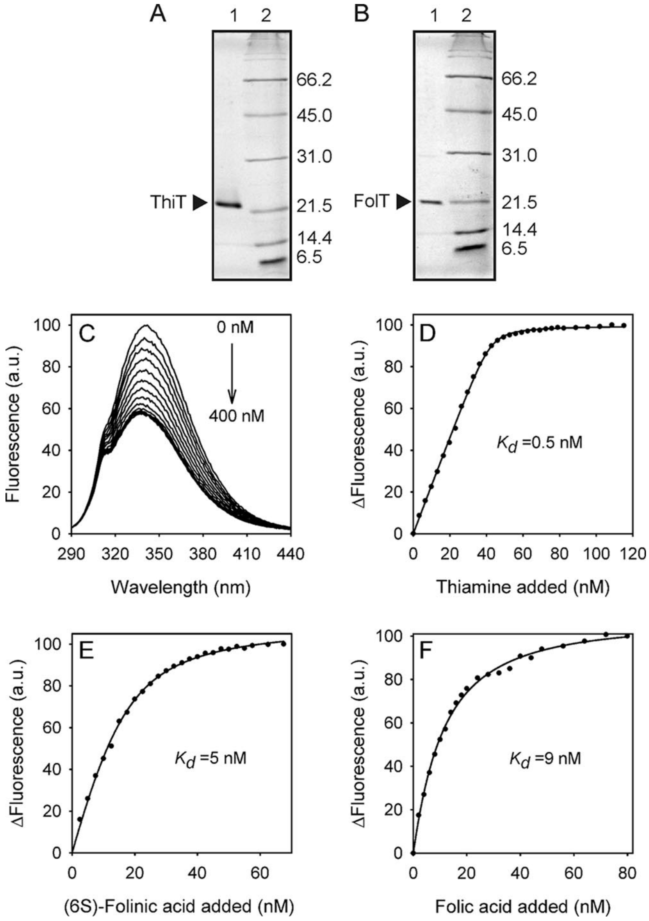

FIG. 3. Purification and characterization of His-tagged *L. casei* ThiT and FolT. (A and B) SDS-PAGE of purified ThiT-His and FolT-His, as in Fig. 2A. (C) Fluorescence spectrum of ThiT-His (320 nM in 50 mM K phosphate, 200 mM KCl, 0.05% [wt/vol] DDM, pH 7.0) in the absence of thiamine (uppermost trace) and in the presence of successively higher concentrations of thiamine (up to 400 nM). (D) Fluorescence titration of ThiT-His with thiamine. (E and F) Fluorescence titration of FolT-His (210 nM in 50 mM K phosphate, 200 mM KCl, 0.05% [wt/vol] DDM, pH 7.0) with (6*S*)-folinic acid (E) and folic acid (F).

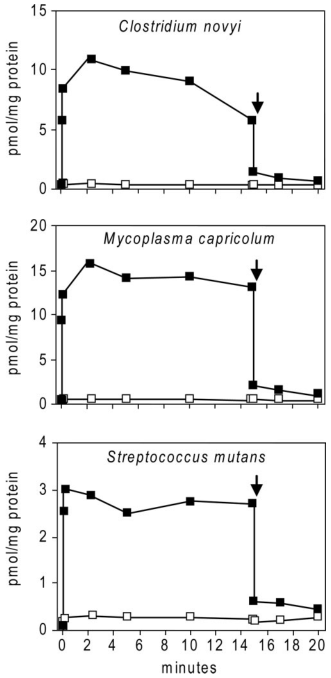

FIG. 4. Folate-binding by FolT homologs from pathogenic Firmicutes expressed in *L. lactis*. The *folT* genes from *Clostridium novyi* and *Streptococcus mutans* were obtained by PCR from genomic DNA; that of *Mycoplasma capricolum* was synthesized by GenScript (Piscataway, NJ). Cells harboring pNZ8048 alone (open squares) or containing FolT homologs (filled squares) were assayed for binding of (6*S*)-[^{3}H]folinic acid (final concentration, 13.5 nM) as in Fig. 2. The arrows show when unlabeled folinic acid was added to give a final concentration of 50 μM.

We thank Robert Burne (University of Florida) for *Streptococcus mutans* genomic DNA and Shibin Zhou (Johns Hopkins University School of Medicine) for *Clostridium novyi* genomic DNA.

This project was supported by National Institutes of Health grant R01 GM071382 (to A.D.H.), by The Netherlands Organization for Scientific Research (vidi grant to D.J.S.), and by an endowment from the C.V. Griffin, Sr. Foundation.

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