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

Aymerick Eudes,† Guus B. Erkens,‡ Dirk J. Slotboom, Dmitry A. Rodionov, and Andrew D. Hanson

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

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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 YuA family (InterPro accession number IPR012651) of predicted, uncharacterized thiamine transporters in the Bacillus/Clostridium group (20). LSEI_1757 is 32% identical to Bacillus subtilis YuA (Fig. 1C). In several Firmicutes, the thiT gene forms a putative operon with the thiamine pyrophosphokinase nisA 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 Ncol 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 3H-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 (6S)-[3H]folinic acid or [3H]folate (17 pmol/mg protein), and those expressing ThiT bound a similar amount of [3H]thiamine. Adding a polyglutamyl tail of 2 to 4 residues to [3H]folate (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 tags, 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 spectrophluorometer (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 folic 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.

* 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.
† E. and G.B.E. contributed equally to the paper.
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(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 FoIT with (6S)-folinic acid and folic acid are given in Fig. 2E and F; (6R)-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 FoIT 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 FoIT), compared to those calculated from the data for FoIT 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 FoIT confirmed that substrate had indeed been copurified (not shown).

Analysis of prokaryotic genomes using the SEED comparative genomics resource (18) revealed that ThiT and FoIT homologs occur commonly and almost exclusively in Firmicutes, many of which are pathogens. The multiple sequence alignments and maximum-likelihood phylogenetic trees for the FoIT and ThiT protein families are shown in Fig. S1 to S3 in the supplemental material. The FoIT family is substantially more diverse; while the majority of FoIT 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. SIA in the supplemental material). Folate-binding activity was verified experimentally for FoIT proteins from three pathogens (Mycoplasma capricolum, Clostridium novyi, and Streptococcus mutans) by expression in L. lactis cells and by measuring [3H]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 foIT 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. SIB 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 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 FoIT 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 FoIT/ThiT-like component. Although there are no bioN- and bioM-related genes linked to foIT or thiT, it is reasonable to infer that they lie elsewhere in the genome, given the evidence that L. casei FoIT 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
The L. casei genome contains a gene cluster encoding homologs of BioN (LSE1_2472) and BioM (LSE1_2473 and LSE1_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 brilliant blue. The arrows indicate FolT and ThiT bands. Positions of molecular mass markers (kDa) are shown. (B to E) Binding of [3H]-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 [3H]-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 3H 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 [6S]-[3H]-folinic acid diammonium salt (Moravek; 10 Ci/mmol) (B), [3H]-folic acid diammonium salt (Moravek; 25.9 Ci/mmol) (C), or [3H]-folic acid polyglutamates (45 Ci/mmol) comprising 40% tri-, 56% tetra-, and 4% pentaglutamates (D). Cells expressing ThiT were incubated with 1H(G)thiamine hydrochloride (ARC; 10 Ci/mmol) (E). [3H]-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 (6S)-folinic acid (E) and folic acid (F).

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