Characterization of Riboflavin (Vitamin B<sub>2</sub>) Transport Proteins from Bacillus subtilis and Corynebacterium glutamicum<sup>▼</sup>

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Received 17 April 2007/Accepted 3 August 2007

Riboflavin (vitamin B<sub>2</sub>) is the direct precursor of the flavin cofactors flavin mononucleotide and flavin adenine dinucleotide, essential components of cellular biochemistry. In this work we investigated the unrelated proteins YpaA from Bacillus subtilis and PnuX from Corynebacterium glutamicum for a role in riboflavin uptake. Based on the regulation of the corresponding genes by a riboswitch mechanism, both proteins have been predicted to be involved in flavin metabolism. Moreover, their primary structures suggested that these proteins integrate into the cytoplasmic membrane. We provide experimental evidence that YpaA is a plasma membrane protein with five transmembrane domains and a cytoplasmic C terminus. In B. subtilis, riboflavin uptake was increased when ypaA was overexpressed and abolished when ypaA was deleted. Riboflavin uptake activity and the abundance of the YpaA protein were also increased when riboflavin auxotrophic mutants were grown in limiting amounts of riboflavin. YpaA-mediated riboflavin uptake was sensitive to protonophors and reduced in the absence of glucose, demonstrating that the protein requires metabolic energy for substrate translocation. In addition, we demonstrate that PnuX from C. glutamicum also is a riboflavin transporter. Transport by PnuX was not energy dependent and had high apparent affinity for riboflavin (K<sub>m</sub> 11 μM). Roseoflavin, a toxic riboflavin analog, appears to be a substrate of PnuX and YpaA. We propose to designate the gene names ribU for ypaA and ribM for pnuX to reflect that the encoded proteins function in riboflavin uptake and that the genes have different phylogenetic origins.

Riboflavin consists of a ribityl side chain linked to an aromatic isoalloxazine ring structure. It is the precursor of the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which both are essential components of cellular metabolism. Riboflavin is phosphorylated to give FMN by flavokinase (EC 2.7.1.26), and FMN is subsequently converted to FAD by FAD synthetase (EC 2.7.7.2). Whereas free riboflavin does not have biological activity, the mostly noncovalently bound flavin cofactors FMN and FAD are the active groups of a large number of flavoproteins. These are involved in a wide range of redox reactions and catalyze the dehydrogenation of metabolites, one- and two-electron transfer reactions from and to redox centers, and hydroxylation reactions (9). Flavins are also known to act as chromophores in photoreceptors, such as the plant blue light sensors cryptochrome and phototropin (reviewed in reference 3). Moreover, flavins are the ligands of dodecin, a recently identified flavoprotein receptor, such as the plant blue light sensors cryptochrome (9). Flavins are also known to act as chromophores in photoreceptors, such as the plant blue light sensors cryptochrome and phototropin (reviewed in reference 3). Moreover, flavins are the ligands of dodecin, a recently identified flavoprotein receptor.
process requiring metabolic energy (5). Riboflavin uptake inversely correlates with the riboflavin concentration present during cell growth and increases in riboflavin-requiring mutants (5). Moreover, B. subtilis was also found to contain a membrane-associated riboflavin-binding activity with similar overall characteristics as the riboflavin transport activity, indicating that the binding component has a functional role for riboflavin uptake (5).

Identification of candidate genes for bacterial riboflavin transporters has been facilitated by comparative genomic analyses. The genome of B. subtilis contains two Rfn elements, one preceding the rib operon and the other preceding ypaA, a gene encoding a hypothetical membrane protein with five putative transmembrane domains (TMDs) (10, 23, 47). Transcription profiling experiments revealed that ypaA was more strongly expressed in ribC mutants (25) and that deletion of ypaA in a riboflavin-auxotrophic mutant drastically increased the demand for riboflavin, FMN, or FAD (23). Although these data and the presence of orthologs of YpaA in riboflavin-auxotrophic species (46) suggested a role of YpaA in riboflavin uptake, this possible function has not been experimentally studied before. Here, we report the characterization of YpaA as a riboflavin transporter and also study the Corynebacterium glutamicum gene pnuX. This gene is similar to ypaA in that it is preceded by an Rfn riboswitch and that it encodes a protein with multiple TMDs (46). YpaA from B. subtilis is related to the recently characterized RibU riboflavin transporter from Lactococcus lactis (4) and is a member of a protein family that includes riboflavin transporters, sodium symporters for bile acids, and exporters for arsenite from eukaryotic and bacterial organisms (29). In contrast, pnuX is not a member of the BART (bile/arсенic/riboflavin transporter) superfamily but is related to RibM from Streptomyces davawensis (12) and orthologous proteins in other sequenced gram-positive species.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The Escherichia coli strains used were BL21(DE3) (F− amp R strd gal dcm hsdS (r~ m~) XDE3) or the riboflavin-auxotrophic ribB11 mutant BSV11 (F− glnV44 lacZ− mecA rfbC endA1 ribB11:Tns spoT1 thi−1 merC hsdR29; E. coli Genetic Stock Center no. 6991) (2). DH5α (F− endA1 galV44 thi−1 recA1 relA1 gyrA96 deoR96 80lacZAM15 [lacZΔ8-argF] U169 hsdR17 (r m)−) was used for molecular cloning and for the experiment shown below in Fig. 5E. E. coli cells were grown in LB, 2TY, or M9 minimal medium supplemented with antibiotics as required (100 μg ml−1 ampicillin, 50 μg ml−1 kanamycin, or 34 μg ml−1 chloramphenicol). For growth of BSV11, 20 mg/liter riboflavin was added. LB medium or SP medium (8 g liter−1 nutrient broth, 1 mM MgSO4, 10 mM KCl, 0.5 mM CaCl2, 10 μM MnCl2, 4.4 mg liter−1 ferric ammonium citrate) (30) was used to propagate B. subtilis strains. All B. subtilis strains used were derived from wild-type strain 168. Strain QBB350 [pyc2 psfH-H15A amyE(p-lacZ [uphA3]) (44)] has a point mutation in the phoH gene and is devoid of PTS-dependent sugar transport activity. CSEG, which is C-medium (30) with added sodium succinate (6 g liter−1), potassium glutamate (8 g liter−1), and glucose (1 g liter−1), is a chemically defined medium devoid of vitamins and was used for growth and uptake assays. For energization of fructose uptake, glucose was replaced by the same amount of sorbitol. For marker selection, erythromycin/ lincomycin (1 mg ml−1/5 μg ml−1), kanamycin (5 μg ml−1), or phleomycin (0.5 μg ml−1) was added to the medium. Solid 2TY medium and SP plates were prepared using 2% agaragar (Roth), and CSEG and M9 minimal media were solidified with 2% Bacto agar (Difco). Cells for use in plate growth assays were diluted to an optical density at 600 nm (OD600) of 0.06, and 5 μl of the cell suspension (approximately 50,000 cells) was transferred to agar plates.

DNA manipulations. B. subtilis was transformed using a two-step protocol (24) and selected on SP plates supplemented with antibiotics as required. To create the ΔrpaB::Kan1 knockout strain, 1-kb flankng regions from either side of ypaA were amplified from B. subtilis genomic DNA and inserted upstream or downstream of the kanamycin resistance gene in the plasmid pDG780. The plasmid was linearized with Scal, which cuts in the AmpR gene, and transformed into B. subtilis. To delete rnb, we replaced rnb with 1-kb up- and downstream regions and cloned it into a pUC19 derivative with a single NotI site. Making use of naturally occurring sites (EcoRV, which truncates the preceding ribd gene by 27 bp, and CiaI, which cuts within rnb), we replaced rnb with the erythromycin resistance gene. Alternatively, a ΔribB::Tet reporter cassette was made by substituting the Erm1 marker with the Tet1 gene. The knockout cassettes were liberated with NotI, and B. subtilis wild-type cells or the ΔrpaA::Kan1 mutant was transformed with the DNA fragment. ypaA was C-termially His tagged using a specific primer encoding eight consecutive histidine residues. The PCR product was inserted into the integrative plasmid pMUTIN-HA (19), thereby removing the hemagglutinin (HA) tag. B. subtilis wild-type cells or the ΔribB::Tet mutant was directly transformed with the resulting plasmid followed by selection for erythromycin resistance. Correct loop-in of the plasmid into the genomic ypaA locus was verified by PCR and Western blot analysis. Overexpression of ypaA was achieved by amplification of the ypaA open reading frame with specific adaptor primers for cloning into pDG148-Stu that allows isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression (17). The promoter region of ypaA including the Rfn element was amplified by PCR and fused to the lacZ reporter gene in vector pAc64 (46). The plasmid was linearized with Scal and integrated into the amylE locus of B. subtilis wild-type cells or the ΔribB::Erm1 mutant. LacZ assays were performed as described elsewhere (32). Construction of GFP, phoA, and lacZ fusions to ypaA. Topological predictions of the C. glutamicum transporter pnuX in E. coli. For pDG148-pnuX-6His, pnuX was amplified without the stop codon from C. glutamicum strain ATCC 13032 genomic DNA. The PCR product was cloned into pQE60 (QiAGEN), isolated as a HindIII fragment including the His tag, and ligated into pDG148 (42). The pET21a (+)/pnuX-3HA plasmid was made by cloning a tagged version of pnuX into pET21a (+). A stop codon after the 3HA epitope tag prevented the translation into the vector-borne His tag. Construction of GFP, phoA, and lacZ fusions to ypaA. Topological predictions of the C. glutamicum transporters (containing the first four TMDs, amino acids [aa] 1 to 146) or ypaA-3 (containing the first three TMDs, aa 1 to 103) were amplified and cloned into pUC18. Next, the green fluorescent protein (GFP) gene, in which the start methionine was replaced with a VDGAGA linker, or alkaline phosphatase from E. coli (phoA, starting after aa 14 to remove the signal peptide) were PCR amplified and inserted into the pUC18 plasmids containing the different forms of ypaA. For lacZ tagging, the β-galactosidase gene from E. coli lacking the first two amino acids was amplified by PCR and cloned into pUC18. Next, the various fragments of ypaA were inserted in front of lacZ. Finally, all fusion constructs were amplified by PCR using specific adaptor primers, cloned downstream of the IPTG-inducible Pbus promoter of pDG148-Stu (17), and transformed into B. subtilis 168.

Analysis of cells containing GFP, phoA-, and lacZ-tagged ypaA. For all assays, the cells containing the proteins described above were grown in LB medium supplemented with 0.5 μg ml−1 phleomycin overnight, transferred to fresh medium containing 0.5 mM IPTG to an OD600 of 0.15, and aerobically cultured at 37°C until they reached an OD600 of 1.0. For PhoA assays, 1 mM iodoacetamide was added to the culture 10 minutes before harvesting to prevent spontaneous activation of cytosolic PhoA (36). Iodoacetamide was also added to all subsequently used buffers. PhoA activity was determined with 3 ml of the cells as described elsewhere (27). The activity of β-galactosidase was measured with a standard procedure (32). For plate overlay assays, cells expressing the lacZ fusions were spotted on SP plates and grown at 37°C overnight. The overlay mixture consisted of 5 g ml−1 low-melting-point agarose in 25 ml 50 mM K2HPO4/KH2PO4 pH 7.0, 0.05% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 7 mM β-mercaptoethanol, 0.1 mg ml−1 lysoyctene, and 2% Triton X-100 (vol/vol). The plates were incubated at 30°C until the color developed.

GFP was determined fluorometrically with cells washed once with 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 15 mM EDTA pH 8.0 and resuspended at an OD600 of 0.9. GFP was excited at 488 nm, and the emission at a 512-nm wavelength was recorded on a Fluoromax-2 instrument (ISA Instruments). Confluent macrophage cell monolayers were prepared in a Zeiss LSM 510 META system.

Preparation of B. subtilis membranes and Western blot assays. B. subtilis membranes were prepared from 40-ml cultures as described elsewhere (31). Briefly, after harvesting and washing with 50 mM Tris-HCl pH 8.0, the cells were resuspended in 1 ml freshly prepared and prewarmed TMS buffer (50 mM
Tris-HCl pH 8.0, 16 mM MgCl₂, 33% [wt/vol] sucrose. Lysozyme (final concentration, 300 μg ml⁻¹) and 1 mM phenylmethylsulfonfonylfluoride were added, and the suspension was incubated for 30 min at 37°C. The protoplasts were cooled to 4°C, harvested by centrifugation (1 min, 7,500 x g), resuspended in 1 ml ice buffer (50 mM Tris-HCl pH 8.0, 5 mM MgSO₄) containing phenylmethylsulfonfonyl fluoride, and sonified on ice. Membranes were collected by centrifugation (15 min, 110,000 x g), washed once with 50 mM Tris-HCl pH 8.0, and resuspended in 50 μl 50 mM Tris-HCl pH 8.0. Samples of 15 μl were analyzed on 10% (see Fig. 4D and E, below) or 15% (see Fig. 2B, below) polyacrylamide gels. After transfer to nitrocellulose membranes, His-tagged YpaA was detected with the Penta-His mouse monoclonal antibody (QIAGEN), and GFP-tagged YpaA was detected with mouse anti-GFP monoclonal antibody 11E5 (Molecular Probes). Anti-mouse peroxidase-coupled secondary antibodies were obtained from Sigma (A-9044). PhoA fusion proteins were detected with rabbit anti-alkaline phosphatase antiserum (Polyscienes, Inc.), followed by peroxidase-coupled anti-rabbit antibodies (A-6154; Sigma). Chemiluminescence reagents (GE Healthcare) were used to develop the blots.

**Uptake experiments.** B. subtilis cells for use in riboflavin uptake experiments were grown overnight in CSEG, and E. coli cells were grown in M9 minimal medium supplemented with antibiotics, IPTG, or riboflavin as required. Medium was inoculated to an OD₆₀₀ of 0.15, and the cells were grown at 37°C until they reached an OD₆₀₀ of 0.5 to 0.6. B. subtilis cells were used directly, and E. coli cells were induced with 0.5 mM IPTG and grown for 3 h (OD ₆₀₀, 0.8). The cells were harvested, washed with ice-cold water and with transport buffer (50 mM KH₂PO₄/KH₂PO₄, 50 mM MgCl₂ pH 7.0), resuspended in transport buffer (10 OD₆₀₀ ml⁻¹), and stored on ice. Uptake experiments were performed with 500 μl of cells that were vigorously stirred at 30°C. After warming for 2 min, glucose was added to a final concentration of 1 mM, and the assay was started 1 min later by adding [¹⁴C]riboflavin (specific activity, 5.54 MBq/mg; a gift of R. Krämer, Köln, Germany) for a final concentration of 1.6 or 2.2 μM. Several aliquots were removed over the next 3 or 4 minutes, filtered on 0.45-μm GN-6 membrane filters (Pall), washed with an excess of water, and analyzed by liquid scintillation counting. Roseoflavin was obtained from MPI Biochemicals, acriflavin and luciferin were from Sigma, and FMN and FAD were from Merck. Carbonyl cyanide m-chlorophenyldrazone (CCCP; 130 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 130 μM), or NaN₃ (1 mM) was added 3 min before addition of the labeled substrate. To test fructose uptake, B. subtilis wild-type cells or ptsH mutants were grown in CSE medium containing sorbitol instead of glucose as a carbon source. The substrate, a mixture of [U-¹⁴C]fructose (CFR847, GE Healthcare) and unlabeled fructose, was added to a final concentration of 58 μM.

**RESULTS**

YpaA is involved in riboflavin uptake. The gene *ypaA* from *B. subtilis* is regulated by an RFN element and is expressed when the cells need flavins (25). It encodes a hydrophobic protein with five putative TMDs that was predicted to catalyze riboflavin uptake across the plasma membrane (47). However, this function was not experimentally verified. To functionally characterize YpaA, we generated a *B. subtilis* mutant in which *ypaA* was deleted (ΔypaA::Kan'). In addition, we overexpressed *ypaA* in *B. subtilis* using the plasmid pDG148-Stu (17). Riboflavin uptake was monitored using [¹⁴C]riboflavin, and it was found that deletion of *ypaA* abolished riboflavin uptake, whereas overexpression of *ypaA* significantly increased riboflavin uptake relative to wild-type controls (Fig. 1). Furthermore, overexpression of *ypaA* resulted in a rapid increase in the cellular radioactivity after adding the labeled substrate. This may indicate that YpaA, like its *L. lactis* ortholog RibU, has a high binding activity for its substrate (8). These results strongly suggest that YpaA corresponds to the *B. subtilis* riboflavin transporter first described in 1979 (5).

We noted that YpaA also has weak similarity to membrane-localized subunits of phosphoenol-pyruvate:carbohydrate phosphotransferase systems (PTS). In addition, riboflavin contains a ribityl side chain and thus is structurally related to the carbohydrate substrates of PTS. Moreover, riboflavin taken up by *B. subtilis* is quickly converted to the phosphorylated derivatives FMN and FAD (5). These findings suggested a role for YpaA as a membrane component of a putative riboflavin-specific PTS. We performed uptake experiments in ptsH mutants that lack HPr, the universal phosphoryl donor for all membrane-bound enzymes II and thus are unable to take up PTS substrates (43). ptsH mutants displayed normal riboflavin uptake but, as expected, the uptake of the PTS sugar fructose was strongly reduced (data not shown). Thus, it is unlikely that YpaA functions as a membrane component of a PTS that mediates riboflavin uptake.

**Regulation of riboflavin transport activity.** As a next step in the characterization of *ypaA*, we checked if the activity of riboflavin uptake was influenced by the ability of the cells to synthesize riboflavin or by the amount of riboflavin present in the growth medium. To generate a riboflavin-auxotrophic *B. subtilis* strain, the ribB gene encoding the α-subunit of riboflavin synthase was inactivated. The ribB deletion strain (ΔribB::Erm') and a wild-type control were grown in a synthetic medium containing various concentrations of riboflavin, and the cells were used in uptake experiments. The experiments revealed that deletion of *ribB* caused an increased riboflavin uptake activity. This was most obvious in media with low riboflavin concentrations, where ΔribB mutants possessed at least eightfold more riboflavin transport activity than wild-type cells (Fig. 2A).

It was also analyzed whether the observed changes in the riboflavin uptake activity were reflected by the amount of YpaA present in the cells. To visualize YpaA in Western blot assays, a plasmid carrying a C-terminally His-tagged version of the Riboflavin Transport Proteins 7369

**FIG. 1.** Riboflavin uptake in *B. subtilis* depends on YpaA. Uptake assays were performed with *B. subtilis* strains grown in riboflavin-free CSEG medium. The strains were either wild type (wt), contained a plasmid for overexpression of *ypaA* (*ypaA-OE*), or had a deletion of the *ypaA* gene (ΔypaA::Kan'). The cells were resuspended in transport buffer (50 mM KH₂PO₄/KH₂PO₄, 50 mM MgCl₂, pH 7.0), energized with 1 mM glucose, and the experiment was started by adding [¹⁴C]riboflavin for a final concentration of 1.6 μM. Aliquots were withdrawn, filtered, and extensively washed with water, and the radioactivity was determined by liquid scintillation counting. The experiment was performed twice with similar results.
ypaA was integrated into the ypaA locus of a wild-type strain or the ΔribB::Tet' deletion strain (YpaA-His). This ensured that riboflavin deficiency leads to an increased production of riboflavin uptake. In summary, these experiments show that riboflavin uptake activity of the ΔribB strain in riboflavin-depleted medium was caused by an increased amount of YpaA. In contrast, wild-type cells had similar levels of YpaA at all riboflavin concentrations tested (Fig. 2B).

To confirm that ypaA is regulated by riboflavin, we generated a transcriptional fusion of the ypaA promoter including the RFN element to lacZ and integrated this construct into the amyE locus of wild-type cells or ΔribB::Erm' mutants. When both strains were grown in liquid medium containing 0.05 μM riboflavin, the ΔribB::Erm' mutant possessed sevenfold more β-galactosidase activity than the wild-type strain (data not shown). These findings are consistent with transcription profiling experiments that showed that ypaA is not regulated by riboflavin in a wild-type strain but that its expression is highly increased in a ribC mutant (25).

Finally, a ΔribB::Erm' ΔypaA::Kan' double mutant was generated and compared to the strains used in the experiments described above. Whereas ΔribB single mutants showed normal growth on medium containing riboflavin at concentrations of 20 μg/liter or more, ΔribB ΔypaA double mutants required at least 20 mg/liter riboflavin to grow. A ΔribB strain producing YpaA-His showed similar growth as the ΔribB strain producing untagged YpaA, demonstrating that the His tag did not interfere with the function of YpaA. As expected, ΔypaA::Kan' cells behaved like wild-type cells and were proficient for riboflavin biosynthesis (Fig. 2C). Characterization of the activity of YpaA. Riboflavin uptake experiments in the presence of putative competitors demonstrated that the activity was strongly reduced when an excess of riboflavin or FMN was present (Fig. 3A). Riboflavin, FMN, and FAD improved the growth of ΔribB mutants when added on a filter disk and placed on riboflavin-free plates (Fig. 3B). In these assays, riboflavin was more potent than FMN, which was more potent than FAD, reflecting the relative activities of these compounds in the uptake experiment (Fig. 3A). Rosoeoflavin (8-dimethyl-amino-8-demethyl-D-riboflavin), a toxic riboflavin analog produced by Streptomyces davawensis (34), strongly competed with riboflavin uptake (Fig. 3A). On plates containing high concentrations of rosoeoflavin, B. subtilis wild-type cells grew more poorly than ΔypaA::Kan' mutants, demonstrating that YpaA mediates rosoeoflavin uptake and sensitivity (Fig. 3C). Flavins lacking the ribityl side chain, such as lumichrome and acriflavine, did not significantly reduce riboflavin uptake when present in a 10-fold excess (Fig. 3A).

Earlier studies in B. subtilis revealed that riboflavin uptake depended on metabolic energy and that uptake was drastically reduced in the presence of protonophors or metabolic inhibitors (5). In contrast, the recently characterized riboflavin transporter RibU could not be demonstrated to require metabolic energy for transport and likely uses a facilitated diffusion mechanism (4). These contradictory findings for two related proteins prompted us to reinvestigate riboflavin uptake in B. subtilis. To test if riboflavin uptake depends on metabolic energy, wild-type cells were stored in transport buffer lacking glucose for 2 hours, and uptake assays were performed in the presence or absence of glucose. In these experiments, the lack of glucose reduced the transport activity by more than 90% (Fig. 3D). Moreover, addition of the protonophors FCCP and

FIG. 2. Regulation of riboflavin uptake activity by external riboflavin. (A) B. subtilis wild-type cells or a B. subtilis ΔribB::Erm' mutant cells were grown in synthetic CSEG medium to which riboflavin was added to give the desired concentrations. Standard uptake experiments with [14C]riboflavin were performed, and the uptake velocities were calculated for the first 4 minutes of the experiments. Bars represent means, and error bars indicate standard deviation values of three independent experiments. (B) YpaA was genomically tagged with a C-terminal his tag either in a wild-type strain or a strain carrying the ΔribB::Tet' disruption. After growth in CSEG medium containing the indicated concentrations of riboflavin, the cells were collected and membrane extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane and detection with the Penta-His mouse monoclonal antibody. This analysis revealed that the increased riboflavin uptake activity of the ΔribB strain in riboflavin-depleted medium was caused by an increased amount of YpaA. In contrast, wild-type cells had similar levels of YpaA at all riboflavin concentrations tested (Fig. 2B).

To confirm that ypaA is regulated by riboflavin, we generated a transcriptional fusion of the ypaA promoter including the RFN element to lacZ and integrated this construct into the amyE locus of wild-type cells or ΔribB::Erm' mutants. When both strains were grown in liquid medium containing 0.05 μM riboflavin, the ΔribB::Erm' mutant possessed sevenfold more β-galactosidase activity than the wild-type strain (data not shown). These findings are consistent with transcription profiling experiments that showed that ypaA is not regulated by riboflavin in a wild-type strain but that its expression is highly increased in a ribC mutant (25).

Finally, a ΔribB::Erm' ΔypaA::Kan' double mutant was generated and compared to the strains used in the experiments described above. Whereas ΔribB single mutants showed normal growth on medium containing riboflavin at concentrations of 20 μg/liter or more, ΔribB ΔypaA double mutants required at least 20 mg/liter riboflavin to grow. A ΔribB strain producing YpaA-His showed similar growth as the ΔribB strain producing untagged YpaA, demonstrating that the His tag did not interfere with the function of YpaA. As expected, ΔypaA::Kan' cells behaved like wild-type cells and were proficient for riboflavin biosynthesis (Fig. 2C). In summary, these experiments show that riboflavin deficiency leads to an increased production of YpaA and that this enhances riboflavin uptake.
FIG. 3. Characterization of the activity and energy requirements of YpaA. (A) Uptake of \( [14C] \) riboflavin (initial outside concentration, 1.6 \( \mu \)M) was studied in \( B. \ subtilis \) \( \Delta ribB:Erm^r \) mutants grown in CSEG minimal medium containing 20 \( \mu \)g/liter riboflavin. Uptake was determined in the absence of competitors (mean uptake velocity, 8.29 pmol riboflavin/OD cells \(^{-1}\) min \(^{-1}\); corresponds to 100%) or in the presence of a 10-fold excess (16 \( \mu \)M) of the given compounds. Bars represent means, and error bars indicate standard deviation (SD) values of three independent determinations. (B) \( B. \ subtilis \) \( \Delta ribB:Erm^r \) mutants were plated on riboflavin-free CSEG minimal medium to produce a lawn. Next, a filter disc was placed in the middle of the plates and impregnated with 20 \( \mu \)l of a solution containing 540 \( \mu \)M riboflavin, FMN, or FAD. The area of growth around the disc was scored after incubation for 1 day at 37°C and had a diameter of 76 mm for riboflavin, 50 mm for FMN, and 42 mm for FAD. (C) \( B. \ subtilis \) wild-type cells (wt) or the \( \Delta ypaA::Kan^r \) mutant were spotted on CSEG plates containing the indicated concentration of roseoflavin. Growth was recorded after incubation for 1 day at 42°C. (D) Uptake of \( [14C] \) riboflavin was determined using a \( \Delta ribB:Erm^r \) mutant grown in CSEG medium containing 20 \( \mu \)g/liter riboflavin. The transport activity was determined without further additions (uptake velocity, 7.13 pmol riboflavin \( \times \) OD cells \(^{-1}\) min \(^{-1}\); corresponds to 100%) or after adding CCCP (130 \( \mu \)M) or FCCP (130 \( \mu \)M) 3 min before starting the test by addition of the labeled substrate. To investigate if glucose stimulated riboflavin uptake, we stored the cells on ice for 2 hours in transport buffer. After this time, uptake activity was measured with \( [14C] \) riboflavin in the absence or presence of 1 mM glucose. In this experiment, 100% corresponds to an uptake activity of 5.1 pmol riboflavin \( \times \) OD cells \(^{-1}\) min \(^{-1}\). Bars represent means, and error bars indicate SD values of three independent experiments.

CCCP caused a strong reduction of riboflavin uptake (Fig. 3D). In summary, these findings support a proton-symport mechanism for riboflavin uptake in \( B. \ subtilis \).

Membrane topology of YpaA. Computational analysis of the YpaA primary structure led to the prediction that YpaA spans the membrane with five hydrophobic \( \alpha \)-helices. This finding is consistent with a recent phylogenetic analysis suggesting that YpaA belongs to a family of transport proteins that includes members with 5, 6, and 10 TMDs (29). Some of the six-TMD proteins contain catalytic C-terminal domains likely facing the cytoplasm, and TMDs 2 to 6 of these proteins have the highest similarity to the five TMDs of YpaA. Thus, it was concluded that the N terminus of YpaA lies on the extracellular side of the plasma membrane and that the C terminus faces the cytoplasm (29).

To investigate the topology of YpaA, we generated fusion proteins in which alkaline phosphatase (PhoA), GFP, or \( \beta \)-galactosidase (LacZ) was fused to the C terminus of YpaA. Alkaline phosphatase is only active when localized in the extracellular space, because it requires oxidative conditions for proper folding (27, 28). GFP is only active in the cytoplasm and does not form an active protein when exposed to the extracellular side (7). Similar to GFP, LacZ is only active in the cytoplasm (27). We found that shorter segments containing only the first or the first two TMDs of YpaA gave ambiguous results with the various tags, probably because the topological information contained in these fragments was insufficient to give the fusion proteins a distinct orientation (data not shown). However, experiments with longer fragments of YpaA, including the first three (YpaA-3) or four (YpaA-4) TMDs or the full-length protein (YpaA-5), produced conclusive results. Cells expressing YpaA-3 and YpaA-5 fused to GFP had high levels of fluorescence, whereas the YpaA-4 fusion had only background activity (Fig. 4A). The complementary pattern was obtained with the PhoA fusions, where YpaA-4 produced much more activity than YpaA-3 and YpaA-5, indicating an extracellular position of the loop following TMD4 (Fig. 4B). The pattern of GFP fluorescence was matched by \( \beta \)-galactosidase assays that demonstrated higher activity when LacZ was fused to YpaA-5 than to YpaA-4 (Fig. 4C). Control experiments demonstrated that the PhoA fusions described above were well expressed in the cells analyzed for PhoA activity (Fig. 4D). For the LacZ fusions, \( \beta \)-galactosidase activity was observed in a plate overlay assay only for YpaA-5 but not for
YpaA-4 (Fig. 4F). As expected, confocal microscopy localized the YpaA-5 fusion to GFP to the periphery of *B. subtilis* cells, a position likely corresponding to the plasma membrane (Fig. 4G). To check the activity of the fusion proteins derived from YpaA-5, they were expressed in the *H9004* *ribB*::Ermr *ypaA*::Kanr strain. Overproduction of any of the three fusion proteins allowed growth at low riboflavin concentrations, demonstrating that the fusion proteins were functional in transporting riboflavin (Fig. 4H). In summary, these results are consistent with a protein model that contains five TMDs with the N terminus localized to the extracellular space and the C terminus facing the cytoplasm (Fig. 4I).

**Characterization of pnuX from Corynebacterium glutamicum.**
Phylogenetic analyses of genes regulated by upstream RFN elements identified three different types of putative bacterial riboflavin transporters. These are (i) orthologs of YpaA and RibU, which are bona fide riboflavin transporters, (ii) ImpX of *Fusobacterium nucleatum* and *Desulfotobacterium hafniense*, and (iii) orthologs of PnuX which are present in the riboflavin operons of *Thermonospora fusca*, *Streptomyces coelicor*, and *S. davawensis* and as a solitary gene in *C. glutamicum* and *Corynebacterium diphtheriae* (12, 46). YpaA, ImpX, and PnuX have unrelated primary structures, a fact that is also reflected in the predicted protein topologies that suggest 5 TMDs for YpaA and orthologs, 6 to 7 TMDs for PnuX and orthologs, and 8 to 10 TMDs for most members of the ImpX family.

Of these proteins, PnuX of *C. glutamicum* (NCgl0063) was investigated with respect to riboflavin uptake. *E. coli* cells producing a tagged version of PnuX had increased rates of riboflavin uptake relative to control *E. coli* cells which, in agreement with previous observations (1), showed no endogenous riboflavin uptake activity (Fig. 5A). Expression of *pnuX* in *E. coli* allowed riboflavin uptake, which was satu-
flavin reduced the uptake of [14C]riboflavin by 40%. This is an effect. We observed that a 10-fold excess of unlabeled riboflavin decreased the uptake activity by 5.76 pmol riboflavin per cell and minute. The bars represent means and error bars indicate standard deviation values of three independent determinations. The uptake activity in the absence of competitors was 5.76 pmol riboflavin per cell and minute.

FIG. 5. Characterization of PnuX from Corynebacterium glutamicum as a riboflavin transporter. All uptake experiments were performed in the presence of 1 mM glucose with E. coli BL21(DE3) expressing a 3HA-tagged version of pnuX from plasmid pET21a(+) or harboring an empty pET21a(+) plasmid (control). (A) E. coli BL21 cells were either measured directly (standard assay; 100%) or incubated with [14C]riboflavin for 5 min. In the presence of the protonophores CCCP (100 μM) or FCCP (100 μM), glucose is not transported, resulting in a 10-fold reduction in the uptake of [14C]riboflavin. The graph represents the Lineweaver-Burk plot of an experiment from which a Km value of 11 μM was calculated. Two repetitions of this experiment yielded Km values of 5 μM and 17 μM, respectively. (C) Uptake experiments were performed with 2.2 μM [14C]riboflavin in the presence of a 10-fold excess of unlabeled riboflavin or riboflavin analogs. (A and C), bars represent means and error bars indicate standard deviation values of three independent determinations. The uptake activity in the absence of competitors was 5.76 pmol riboflavin per cell and minute (100%). (D) E. coli ribBII mutants were plated on riboflavin-free M9 minimal medium to produce a lawn and growth was assayed as in Fig. 3B. The area of growth had a diameter of 53 mm for riboflavin and 18 mm for FAD. FAD produced no growth, even after incubation for one day. (E) E. coli DH5α cells expressing pnuX from plasmid pDG148-pnuX-6His (pnuX) or containing an empty pDG148 plasmid (control) were spotted onto M9 minimal medium plates containing various concentrations of [14C]riboflavin in the presence of various concentrations of sodium azide. Two repetitions of this experiment yielded Km values of 5 μM and 17 μM, respectively.

DISCUSSION

B. subtilis is a model organism to study riboflavin uptake and biosynthesis and is used in industrial processes for riboflavin production (1, 5, 26, 35). Expression of the B. subtilis riboflavin operon is modulated by the flavin cofactor FMN, which, if available in a sufficient amount, binds to the RFN element and prevents the expression of the downstream structural genes (47). A similar RFN element is also present upstream of ypaA. This gene codes for a membrane protein that leads to an increased requirement for riboflavin when deleted in riboflavin auxotrophic mutants (23), making it a candidate riboflavin transporter.

The biochemistry of riboflavin transport in B. subtilis was thoroughly investigated in earlier studies (5). It was found that riboflavin is taken up by a high-affinity transport mechanism requiring metabolic energy. This activity can now be fully ascribed to YpaA. We characterize YpaA as a protein of the B. subtilis plasma membrane that requires metabolic energy for substrate translocation and whose expression is regulated by the availability of riboflavin. In many respects, YpaA is similar to the related riboflavin transporter RibU from L. lactis (4, 8). Although RibU is predicted to contain six TMDs, the fifth potential TMD of RibU is postulated to be part of a loop rather than a TMD (4). For YpaA, we found evidence for the presence of five TMDs and a C terminus located in the cytoplasm (Fig. 4). This topology is consistent with the finding that longer members of the superfamily of BART transporters, to which YpaA and RibU belong, have catalytic domains that face the cytoplasm only when the position corresponding to the C terminus of YpaA is cytosolic (29). Duurkens et al. (8) demonstrated a high level of riboflavin binding to RibU, and our data (Fig. 1), as well as data from earlier experiments (5), are compatible with riboflavin binding to YpaA. A distinct difference between YpaA and RibU appears to lie in their
energy requirements. Whereas RibU could not be demonstrated to require metabolic energy for transport (4), our data and the data of Cecchini et al. (5) clearly show that riboflavin uptake in B. subtilis requires metabolic energy. Although some of the cellular radioactivity in our transport assays might reflect riboflavin binding, we also found that the amount of radioactivity was strongly reduced in the presence of metabolic inhibitors such as the protonophors CCCP and FCCP or when no glucose was present during the uptake experiment. In agreement with earlier work, we thus postulate that YpaA is a proton-riboflavin symporter. YpaA is strikingly different from the C. glutamicum riboflavin transporter PnuX, which appears to be a facilitator for riboflavin. Moreover, PnuX has a lower apparent affinity for riboflavin ($K_m$, 11 ± 6 µM) (Fig. 5B) than YpaA ($K_m$, 2 to 20 nM) (5). As a facilitator, PnuX allows the flux of riboflavin down a concentration gradient. Since incoming riboflavin might become metabolically trapped by conversion to FMN, which does not appear to be a substrate of PnuX (Fig. 5A), PnuX is likely acting as an importer in vivo. Based on the biochemical characterization of the two proteins described above, we propose to designate the gene names ribU for ypaA and ribM for pnuX. These names reflect that both proteins function in riboflavin uptake, although they have different phylogenetic origins and no detectable sequence similarity.

It is interesting that PnuX from C. glutamicum has some homology (21% identity and 43% similarity) to transporters for nicotinamide riboside, such as PnuC from Haemophilus influenzae (39). Like riboflavin, nicotinamide riboside consists of a heterocyclic ring structure, fused to a sugar side chain, and is the building block of a redox-active cofactor, NAD$^+$. Another bacterial transporter with a putative function in riboflavin uptake is encoded by impX. ImpX is unrelated to YpaA and PnuX and not functionally characterized (46). The identification of these bacterial proteins by comparative genomics (46) impressively demonstrates the power of computational analyses to predict protein function. Eukaryotic plasma membrane transporters for riboflavin are not related to any of these bacterial transporters. We have identified the Saccharomyces cerevisiae transporter Mch5p, which is a member of the major facilitator superfamily having 12 TMDs and working as a facilitator (38). Moreover, a mammalian riboflavin exporter has recently been identified that secretes riboflavin into milk and belongs to the ATP-binding cassette family of transport proteins (45).

With respect to the substrates transported by YpaA, we observed that riboflavin uptake is reduced in the presence of FMN or FAD (Fig. 3A). Moreover, the growth of the riboflavin-auxotrophic B. subtilis ΔribB strain was also supported by both flavin cofactors (Fig. 3B). At first sight, this indicates that FAD is transported in a YpaA-dependent fashion. However, another possibility is that FAD is degraded by extracellular enzymes and riboflavin is taken up. There are indications that this is indeed the case. First, whereas riboflavin, roseoflavin, and FMN bind with nanomolar affinities to the YpaA-related L. lactis riboflavin transporter RibU, this protein shows no binding of FAD (8). A second hint comes from the analysis of B. subtilis ribC mutants. RibC is the bifunctional B. subtilis flavokinase/FAD synthetase (26). B. subtilis ribC mutants can be complemented by FMN only when a heterologous mono-functional FAD synthetase accounts for the missing enzymatic activity (26). In contrast, FAD does not complement ribC mutants, indicating that FAD is not taken up (26). Thus, although riboflavin uptake is reduced in the presence of FAD, FAD does not appear to be a substrate of YpaA. Extracellular degradation of FAD and uptake of riboflavin is not without precedent. For example, the blood-borne bacterium Haemophilus influenzae cannot synthesize NAD$^+$ or salvage nicotinate or nicotinamide. Instead, this organism depends on the uptake of nicotinamide riboside via the plasma membrane transporter PnuC (39). Nicotinamide riboside derives from NAD$^+$ by the extracellular activity of NadN, an NAD$^+$ pyrophosphatase that produces nicotinamide mononucleotide and AMP and also has nucleotide phosphatase activity to dephosphorylate nicotinamide mononucleotide and produce nicotinamide riboside (11, 20, 37). The dephosphorylation step can also be performed by e(P4), a periplasmic phosphatase with higher activity than NadN (11, 20, 37). In H. influenzae, neither NAD$^+$ nor nicotinamide mononucleotide is transported by PnuC, which has specificity for nicotinamide riboside only (39). The NAD$^+$ pyrophosphatase activity of H. influenzae has been studied in vitro and also has activity towards FAD, producing FMN and AMP (18). Homologs of NadN and other proteins that could act on FAD are encoded in the B. subtilis genome. These proteins have not been studied for a role in FAD degradation, but it is possible that their activity is similar to NadN. In the light of these findings, it is also questionable if FMN is transported by YpaA. Although FMN binds to RibU (8), represses the riboflavin operon in a RibU-dependent manner (4), reduces riboflavin uptake via YpaA (Fig. 3), and supports growth of B. subtilis ribB mutants (Fig. 3), these findings do not unequivocally prove that FMN is transported by YpaA. To resolve if FMN or FAD or both are substrates of bacterial riboflavin transporters, it will be necessary to study the degradation of these compounds by extracellular enzymes and to generate mutants that are defective in these pathways.

ACKNOWLEDGMENTS

This work was supported by the DFG ( GK640 and DFG STO434/2-1). We thank Reinhard Krämer (Köln) for providing the C. glutamicum genomic DNA and [14C]riboflavin and Mary Berlin from the E. coli Genetic Stock Center for the ribB11 mutant BSV11. Plasmids were donated by Mohamed A. Mahariel (Marburg), Wolfgang Schumann (Bayreuth), and the Bacillus Genetic Stock Center. We also thank Birgit Flauger, Ina Weig-Meckl, Bert Poolman, Rainer Deutzmann, Widmar Tanner, Alons Penzkofer, and Joachim Reidl for discussions and contributions and Peter Hegemann for initiating this project within GK640.

REFERENCES


