The Riboflavin Transporter RibU in Lactococcus lactis: Molecular Characterization of Gene Expression and the Transport Mechanism

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This study describes the characterization of the riboflavin transport protein RibU in the lactic acid bacterium Lactococcus lactis subsp. cremoris NZ9000. RibU is predicted to contain five membrane-spanning segments and is a member of a novel transport protein family, not described in the Transport Classification Database. Transcriptional analysis revealed that ribU transcription is downregulated in response to riboflavin and flavin mononucleotide (FMN), presumably by means of the structurally conserved RFN (riboflavin) element located between the transcription start site and the start codon. An L. lactis strain carrying a mutated ribU gene exhibits altered transcriptional control of the riboflavin biosynthesis operon ribGBAH in response to riboflavin and FMN and does not consume riboflavin from its growth medium. Furthermore, it was shown that radio-labeled riboflavin is not taken up by the ribU mutant strain, in contrast to the wild-type strain, directly demonstrating the involvement of RibU in riboflavin uptake. FMN and the toxic riboflavin analogue roseoflavin were shown to inhibit riboflavin uptake and are likely to be RibU substrates. FMN transport by RibU is consistent with the observed transcriptional regulation of the ribGBAH operon by external FMN. The presented transport data are consistent with a uniport mechanism for riboflavin translocation and provide the first detailed molecular and functional analysis of a bacterial protein involved in riboflavin transport.

The water-soluble vitamin riboflavin (vitamin B2) is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide. These flavins are essential cofactors in enzymes catalyzing redox reactions and are obtained by phosphorylation of riboflavin in all living organisms. Plants, fungi, and many microorganisms have the biosynthetic ability to synthesize riboflavin, whereas higher animals must obtain it from their diet. The abilities of various gram-positive and gram-negative bacteria to synthesize riboflavin de novo have been described by various authors (3, 10, 27).

Certain bacteria such as Escherichia coli are dependent on endogenous biosynthesis because they apparently lack an uptake system for the vitamin (2). It is for this reason that E. coli riboflavin auxotrophs require extremely high levels of riboflavin (720 μM) in their growth medium, and it is likely that at this concentration the vitamin crosses the membrane by diffusion rather than by means of a dedicated transport system. Bacillus subtilis not only possesses a functional riboflavin biosynthetic pathway but can also import the vitamin from the growth environment (6). The ypaA gene of Bacillus subtilis has been suggested to be a riboflavin transporter (17), since a B. subtilis riboflavin auxotroph carrying a mutated ypaA gene was shown to require unusually high riboflavin concentrations in its growth medium. Although the role of the ypaA gene in riboflavin transport has not been unequivocally demonstrated, its putative function is consistent with the presence, immediately upstream of the ypaA coding sequence, of a so-called RFN element (11). RFN elements are found upstream of prokaryotic riboflavin biosynthesis operons, where they play a regulatory role in the expression of the riboflavin biosynthesis genes by a mechanism involving FMN-mediated folding of the RFN-comprising mRNA that causes premature transcription termination and/or ribosome seclusion (25, 41).

In Lactococcus lactis, the riboflavin biosynthesis operon has been recently characterized and the RFN element was shown to be instrumental in transcriptional regulation of the riboflavin biosynthesis genes (4). An L. lactis strain containing a mutation in the ribA gene, which is essential for riboflavin biosynthesis and codes for a GTP cyclohydrolase II/3,4-dihydroxy-2-butanoate-4-phosphate synthase, is dependent on the presence of riboflavin in the growth medium (4). However, such a mutant needed much lower levels of riboflavin (5 μM) than the levels required by E. coli riboflavin auxotrophs (720 μM), suggesting the presence of a dedicated transport system in L. lactis. The current study describes the characterization of a homologue of ypaA, designated ribU, in Lactococcus lactis subsp. cremoris NZ9000 and demonstrates its direct role in riboflavin uptake.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. L. lactis strains were grown in M17 medium supplemented with 0.5% glucose (GM17) (38) or in chemically defined medium (CDM) (adapted by removal of folic acid, riboflavin, and nucleotides) (26, 28). Where appropriate, growth medium contained tetracycline (5 μg ml−1) or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml−1).

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TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ9000</td>
<td>MG1363 pepN::ntrRK, wild-type strain</td>
<td>19</td>
</tr>
<tr>
<td>CB101</td>
<td>Riboflavin-overproducing spontaneous mutant of NZ9000</td>
<td>4</td>
</tr>
<tr>
<td>NZ9000ΔribA</td>
<td>NZ9000 derivative with a 783-bp deletion in ribA</td>
<td>4</td>
</tr>
<tr>
<td>NZ9000ΔribU</td>
<td>NZ9000 derivative with a 414-bp deletion encompassing P_ribU and 69 bp of ribU</td>
<td>This study</td>
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**Plasmids**

- pOR1280
  - Em’ LacZ- ori+ of pWV01, replicates only in strains where repC is provided in trans
- pOR1280ΔribU
  - pOR1280 derivative containing a truncated version of NZ9000 ribU and the surrounding regions
- pPTPL
  - Tet’ LacZ-; promoter probe vector
- pPTPLΔribU
  - pPTPL derivative containing the promoter region of NZ9000 ribU
- pPTPLΔopI
  - pPTPL derivative containing the promoter of NZ9000 rib operon

**Bioinformatics.** All sequence data were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Sequence database interrogations were performed using BLASTP (1). Potential membrane-spanning regions were identified using DAS (dense alignment surface) (7), TMHMM (transmembrane hidden Markov model) (18), and TMpred (transmembrane prediction) (13). The proteins were also analyzed for similarity to families of transport proteins using the BLAST program on the Transport Classification Database (www.tcdb.org) (5). Potential RFP elements were identified using RFAM (RNA family) (http://www.sanger.ac.uk/Software/Rfam/) (12). Folding of the 5' mRNA leader region was predicted using mFold (http://www.bioinf.ku.edu/applications/mfold/old/ma/) (43).

**DNA manipulations and transformations.** Plasmid DNA was isolated from E. coli using the JETquick plasmid miniprep kit (Genomed, Löhne, Germany), following the instructions of the manufacturer. Plasmid DNA was isolated from L. lactis using the same kit except that cells were preincubated in cell resuspension solution containing 20 mg ml⁻¹ lysozyme at 55°C for 30 minutes to effect cell lysis. Transformation of E. coli was carried out as described by Sambrook and Russell (36). Transformation of L. lactis was achieved according to the protocol of de Vos et al. (9). Chromosomal DNA was isolated from L. lactis as described previously (22, 23). Southern blot assays were carried out using standard protocol (36), and detection was accomplished using ECL labeling (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer’s instructions.

**Plasmid construction.** Primers containing a BglII site and an XbaI recognition sequence within the forward and reverse primer, respectively, were used to amplify the predicted promoter and regulatory region of ribU. The amplified product was digested with BglII and XbaI and cloned into the promoter probe vector, pPTPL (4), digested with the same two enzymes. The resulting plasmid, listed in Table 1, was constructed using E. coli EC1000 as a cloning host and was subsequently transferred into the lactococcal strain NZ9000. X-Gal was used in plates as a qualitative indicator of promoter activity.

**Transcriptional analysis.** β-Galactosidase assays (16) were performed on crude cell extracts of L. lactis strains which had been grown in CDM with or without riboflavin or CDM supplemented with 5 μM or 50 μM riboflavin or 5 μM FMN as appropriate. Total RNA was isolated at mid-logarithmic phase by the Macaloid solution containing 20 mg ml⁻¹ SDS and 0.5 M NaCl for 30 minutes to effect cell lysis. Northern hybridization was performed by denaturing 5 μg RNA at 65°C and followed by electrophoretic separation on a 0.8% formaldehyde agarose gel. The ribosomal RNA bands were then detected by exposure to a Kodak Biomax MR film at −70°C for 4 h.

**Determination of transcription start site.** A reverse primer was designed approximately 120 bp downstream of the assumed transcription start site upstream of ribU. Primer extension analysis was performed by annealing 10 pmol of 5’-32P-labeled primer to 50 μg NZ9000 RNA isolated from mid-logarithmic-phase cells (32). A GATC sequence ladder which was run alongside the primer extension product was produced using the same labeled primer with the T7 DNA polymerase sequencing kit (USB Corp, Ohio). Detection was carried out by exposure to Kodak Biomax MR film at −70°C for 4 h.

**Construction of a chromosomal deletion in ribU.** Primers were designed to amplify the sections overlapping and flanking either end of ribU and its promoter region. Splicing by overlap extension PCR (15) was used to create a PCR product which contained a 414-bp deletion encompassing the entire promoter and regulatory region and the first 23 codons of ribU. This PCR product was inserted into pOR1280 (Table 1) using the Ncol and BamHI restriction sites present on the outermost primers. The resulting plasmid, designated pOR1280ΔribU, was used to introduce the deletion into the NZ9000 chromosome by replacement recombination (21), creating strain NZ9000ΔribU. PCR, sequencing, and Southern blot analyses were used to confirm the integrity of the deletion.

**Quantitative analysis of riboflavin in culture medium.** Extracellular riboflavin concentrations were measured using reverse-phase high-pressure liquid chromatography (HPLC) as described previously (4). Samples taken from GM17 were diluted 1 in 2 with HPLC-grade water before analysis. Commercially obtained riboflavin was used as a reference and to obtain a standard curve.

**Riboflavin uptake using whole cells.** Cultures were grown to early stationary phase in GM17 (optical density at 600 nm [OD₆₀₀] of approximately 1.8), and cells were then harvested by centrifugation at 7,700 × g for 4°C at 10 min. Harvested cells were washed with 50 mM KPF, pH 7.0; resuspended in the same buffer to obtain a cell suspension with an OD₆₀₀ of 10; and kept on ice until use. Before each uptake assay, the cells were preenergized for 5 min with 10 mM glucose at 30°C or decerengized with 10 mM 2-deoxyglucose (30, 39).

Uptake was started by the addition of [3H]riboflavin (Campro Scientific, Veenendaal, The Netherlands) to a final concentration of 1 μM, unless otherwise specified. The sample volume was 200 μl, and at the indicated time points uptake was quenched with ice-cold 50 mM KPF, pH 7.0, and rapidly filtered through 0.45-μm cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany). The filters were washed once with 2 ml ice-cold 50 mM KPF, pH 7.0, and then dried using an infrared lamp, and the radioactivity was determined by liquid scintillation counting. In some cases rothoflavin or FMN was added at various concentrations at the same time as the radiolabeled riboflavin.

For imposition of an artificial proton motive force, NZ9000 cells were washed in 50 mM KPF, pH 7.0, plus 100 mM potassium acetate; treated for 10 min at 30°C with 10 mM 2-deoxyglucose (Fluka, Buchs, SG, Switzerland) to deenergize the cells; and then resuspended in 50 mM KPF, pH 7.0, plus 100 mM potassium acetate and 10 mM 2-deoxyglucose to a final OD₆₀₀ of 100. The cells were then diluted 1 to 10 in either 50 mM KPF, pH 7.0, plus 100 mM potassium acetate (no gradient) or 50 mM NaPF, pH 7.0, plus 100 mM NaCl in order to generate a proton motive force. In each case, the outside buffer contained valinomycin at a final concentration of 2 μM. Dilution of the energy-depleted cells in the sodium ion-containing buffer will create a membrane potential (by valinomycin-mediated K⁺ efflux down the concentration gradient), a pH gradient (by efflux of acetic acid directly through the lipid bilayer), and a Na⁺ ion gradient (imposed by the buffer composition). For an overview of the procedures see the work of Poolman et al. (29). Either [3H]riboflavin at a final concentration of 1 μM or [3H]galaline at a final concentration of 1.7 μM was used. At various time points, the reaction mixtures were quenched with ice-cold outside buffer and the cells were rapidly filtered through cellulose nitrate filters. For the calculations, it was assumed that an L. lactis cell suspension with an OD₆₀₀ of 5 contains 1 mg of protein per ml (31).

**Nucleotide sequence accession numbers.** The nucleotide sequence data for L. lactis subsp. cremoris NZ9000 ribU and the regulatory region reported in this
RESULTS

Identification of a putative riboflavin transporter, RibU, in L. lactis NZ9000. The amino acid sequence of a putative riboflavin transporter, encoded by *ypaA* in *B. subtilis* (17), was employed to identify a single 618-bp homologue, designated here as *ribU*, on the *L. lactis* MG1363 genome. In a situation analogous to that in *B. subtilis* (41), a conserved RNA regulatory region is present upstream of *ribU*. Homologues of RibU and YpaA are present in gram-positive bacteria belonging to the *Bacillales*, the *Lactobacillales*, and the clostridia as well as in *Thermotoga maritima* and in archaea belonging to the *Thermococcales* (*Pyrococcus* and *Thermococcus* species). These proteins have a length of approximately 200 amino acids. A multiple sequence alignment of selected RibU homologues is shown in Fig. 1.

RibU and its homologues were analyzed for the presence of transmembrane segments (TMSs) using a number of computational tools. Although RibU from *L. lactis* is predicted to contain six TMSs by TMHMM and DAS (7, 18) (Fig. 1), the region corresponding to the fifth TMS is lacking in the other identified homologous proteins including YpaA (represented by gaps in the multiple sequence alignment). Since the overall structure and topology of these highly similar proteins are most likely conserved, the predicted fifth TMS in RibU may actually be part of a loop structure. Figure 1 shows an alignment of the

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**FIG. 1.** Alignment of RibU homologues from various bacterial strains. The positions of the predicted TMSs are shaded and numbered I to V. The region between TMSs IV and V is predicted to be membrane spanning in both *L. lactis* RibU proteins, while in the other aligned sequences a large part of this region is absent and not predicted to be traversing the membrane.
proteins with the TMSs predicted by TMHMM highlighted. Neither RibU nor any of the homologues displayed significant sequence similarity to any known family of the Transport Classification Database (5) (data not shown).

The DNA regions upstream of the ribU homologues were analyzed for the presence of a potential RFN element using RFAM (12). All identified ribU genes are predicted to possess an RFN element in the region upstream of ribU. These upstream regions were analyzed using the program mFOLD, which predicts RNA and DNA folding structures (43). For ypaA of B. subtilis, it has been suggested that regulation occurs at the level of translation by means of a Shine-Dalgarno sequence sequestering mechanism (41). For a number of the putative regulatory elements analyzed in this study a similar Shine-Dalgarno sequence sequestering mechanism may be predicted, as such folding is energetically favorable. However, in NZ9000 a putative rho-independent terminator structure can be identified in this conserved regulatory region, which suggests that regulation takes place at the level of transcription. Such a terminator structure is also present in the leader region of Lactobacillus plantarum WCFS1 (data not shown).

Transcriptional analysis of ribU in NZ9000 and the riboflavin-overproducing derivative CB010. In order to identify the promoter, \( P_{ribU} \) of the L. lactis NZ9000 ribU gene, primer extension analysis was performed (Fig. 2). The transcription start site was identified as a thymine, upstream of which \(-10\) and \(-35\) sequences were identified with a clear resemblance to the consensus vegetative RNA polymerase recognition sequences for L. lactis (8). To analyze transcription of ribU in further detail, Northern hybridization was carried out using a PCR product encompassing ribU as a probe with RNA isolated from L. lactis strains NZ9000 and CB010, grown to mid-logarithmic phase in the presence or absence of 5 \( \mu \)M riboflavin. L. lactis CB010 is a roseoflavin-resistant mutant that constitutively produces riboflavin due to deregulation of transcription of the rib operon (4). This analysis showed that ribU is transcribed as a monocistronic transcript with an estimated length of 0.95 kb (Fig. 3A). This transcript would encompass the predicted RFN element and is predicted to end at a putative rho-independent terminator located downstream of ribU. The highest level of ribU transcription occurred when NZ9000 was grown in the absence of riboflavin, although a low level of transcription was still observed in the presence of the vitamin (lanes 1 and 2, Fig. 3A). In contrast, for CB010 ribU transcription was undetectable regardless of the exogenous riboflavin status, indicating that RibU is not essential for riboflavin pro-

![FIG. 2. Primer extension (PE) analysis of \( P_{ribU} \) run alongside a sequencing ladder. The deduced \(-35\) and \(-10\) boxes are indicated by boldface in the sequence displayed on the right side of this figure. The bent arrow indicates the identified transcription start site. The identified RFN element is underlined. The assumed ribosomal binding site is boxed, and the ribU start codon is in boldface.](image)

![FIG. 3. Transcriptional analysis of ribU. (A) Northern hybridization analysis of ribU in NZ9000 and CB010. Lane 1, NZ9000 RNA from CDM; lane 2, NZ9000 RNA from CDM plus 5 \( \mu \)M riboflavin; lane 3, CB010 RNA from CDM; lane 4, CB010 RNA from CDM plus 5 \( \mu \)M riboflavin. An RNA size ladder (in kilobases) is indicated on the left. The size of the transcripts is indicated to the right. (B) \( \beta \)-Galactosidase activities of NZ9000 containing pPTPL\( ribU \) grown in CDM or CDM plus 5 \( \mu \)M riboflavin are represented by circles and inverted triangles, respectively. \( \beta \)-Galactosidase activities produced by CB010 containing pPTPL\( ribU \) grown in CDM or CDM plus 5 \( \mu \)M riboflavin are represented by squares and diamonds, respectively. The dashed line indicates growth of the strains plotted on a semilog scale.](image)
This apparent transcriptional regulation is consistent with the results from the analysis of NZ9000 and CB010 containing a P\textsubscript{ribU}-\textit{lacZ} transcriptional fusion on plasmid pPTPL\textsubscript{ribU} (Fig. 3B), which showed that in the absence of riboflavin 
P\textsubscript{ribU} activity is high in NZ9000, while in the presence of the vitamin in the growth medium the activity of this promoter is low. In contrast, when the \textit{ribU}-reporter fusion plasmid was present in CB010, essentially no \textbeta-galactosidase activity was observed, regardless of the presence of extracellular riboflavin.

**Chromosomal deletion of ribU in NZ9000.** It has previously been shown that NZ9000 consumes riboflavin from its growth medium, if the vitamin is present (37). To examine whether \textit{ribU} in \textit{L. lactis} plays a role in this observed vitamin consumption phenotype, a deletion strain, designated NZ9000Δ\textit{ribU}, was created in which the \textit{ribU} promoter, the presumed regulatory region, translation initiation signals, and the first 23 codons of \textit{ribU} were removed from the genome. To determine whether this partial \textit{ribU} deletion affects riboflavin consumption, strains NZ9000 and NZ9000Δ\textit{ribU} were grown in GM17. Samples were taken from the cultures periodically, and the riboflavin content of the cell-free supernatant was determined. Figure 4 illustrates that, in contrast to the wild-type strain, NZ9000Δ\textit{ribU} does not consume riboflavin from the medium, indicating that the riboflavin uptake mechanism has been rendered nonfunctional. The riboflavin-consuming phenotype could be restored in NZ9000Δ\textit{ribU} by complementation in trans, that is, following introduction of intact \textit{ribU} on a plasmid (data not shown).

\[ \text{FIG. 4. Analysis of growth and riboflavin levels of NZ9000 and NZ9000Δ\textit{ribU} in GM17. The solid lines represent log OD}_{600} \text{ and the dashed lines represent riboflavin levels as measured by HPLC in the cell-free supernatant following growth. Data obtained using NZ9000 are shown with black solid circles, and those obtained using NZ9000Δ\textit{ribU} are depicted with empty inverted triangles.} \]

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{P\textsubscript{ribGBAH} activity in NZ9000 and NZ9000Δ\textit{ribU} in various media. The solid circles represent NZ9000, and the empty inverted triangles represent NZ9000Δ\textit{ribU}. The solid lines represent growth (on a semilog scale), and the dashed lines represent \textbeta-galactosidase activity. (A) CDM; (B) CDM plus 5 μM riboflavin; (C) CDM plus 5 μM FMN; (D) CDM plus 50 μM riboflavin.}
\end{figure}
Effect of chromosomal deletion of ribU on transcription of the riboflavin biosynthesis operon. To study the effect of the ribU deletion on expression of the riboflavin biosynthesis genes, the activity of P\textsubscript{ribGBAH} was examined in the wild-type strain NZ9000 and in NZ9000ΔribU, grown in CDM or CDM supplemented with 5 or 50 μM riboflavin or 5 μM FMN. In CDM high promoter activity of P\textsubscript{ribGBAH} was observed in both NZ9000 and NZ9000ΔribU, although for unknown reasons it is lower in the latter strain (Fig. 5A). Regardless of the presence of 5 μM riboflavin or 5 μM FMN the activity of the P\textsubscript{ribGBAH}-lacZ fusion in NZ9000ΔribU was high (Fig. 5B and C). This is in contrast to the wild-type situation where P\textsubscript{ribGBAH} activity was dramatically reduced in the presence of this concentration of riboflavin or FMN. However, when the riboflavin concentration in the medium was increased from 5 to 50 μM, the P\textsubscript{ribGBAH} activity was reduced in both NZ9000 and NZ9000ΔribU (Fig. 5D). This suggests that, in the presence of high levels of riboflavin, the vitamin is able to enter the cell independently of RibU and consequently exert its effects on transcription of the rib operon.

Riboflavin uptake and substrate specificity of RibU. Riboflavin transport was analyzed in glucose-metabolizing whole cells of NZ9000, NZ9000ΔribU, and NZ9000ΔribA, a strain rendered incapable of riboflavin biosynthesis (4). Both the wild-type strain NZ9000 and NZ9000ΔribA showed high rates of riboflavin transport (Fig. 6) and reached similar end levels of uptake. The level of accumulation of riboflavin was approximately 30-fold, assuming a specific internal volume of 3 μl per mg of protein. In agreement with the lack of riboflavin consumption from the external medium, essentially no uptake of riboflavin could be measured in NZ9000ΔribU. In deenergized wild-type cells (depleted of ATP and the proton motive force [Δp] poised to zero by incubation with 2-deoxyglucose) the uptake of riboflavin was significantly lower than in glucose-metabolizing cells but still an apparent eightfold accumulation was found. To determine whether FMN and roseoflavin are substrates of RibU, these compounds were used in competition assays (Fig. 7). Clearly, both roseoflavin and FMN inhibited uptake of radiolabeled riboflavin, but higher concentrations of FMN than roseoflavin were needed to compete with riboflavin uptake. Although the inhibition of riboflavin uptake by FMN and roseoflavin could be due to binding of these compounds to RibU without transport taking place, transport of FMN by RibU would be consistent with the reduction of the activity of the P\textsubscript{ribGBAH} promoter by FMN in the growth medium that is observed in the wild-type strain but not in the RibU deletion (Fig. 5C).

To determine the driving force for riboflavin accumulation in the wild-type strain an artificial Δp was generated in cells that previously had been deenergized (depleted of ATP and Δp poised to zero). The proton motive force was not capable of driving accumulation of riboflavin (Fig. 8A). The amount of riboflavin taken up in cells with an artificially imposed Δp was similar to the uptake observed in deenergized cells. As a control for the generation of the proton motive force, uptake of radiolabeled l-alanine was measured and was found to be significantly higher in cells containing an artificially imposed Δp than in deenergized cells (Fig. 8A, inset).

The apparent accumulation of radiolabeled riboflavin, which is not dependent on the proton motive force, is likely to be driven by equilibration of internal and external riboflavin pools via an exchange (counterflow) mechanism. This possibility is supported by the fact that accumulated radiolabeled riboflavin in energized cells could be chased out of the cell with excess nonlabeled external riboflavin (Fig. 8B). Exchange would also explain the low but significant apparent riboflavin uptake in deenergized cells, because deenergized cells may not be completely depleted of riboflavin. Because the RibU primary sequence does not reveal any indications for a role of ATP (no Walker A and B motifs, no similarity to ABC or P-type motifs), this role could be fulfilled by an interaction of RibU with some other, unknown factor.
ATPases, etc.), it is unlikely that ATP directly provides the driving force for transport. Moreover, the reversibility of the transport reaction and the exchange transport both point towards a facilitated diffusion mechanism.

DISCUSSION

The current work is to the best of our knowledge the first molecular and functional analysis of a protein responsible for the transport of riboflavin into a bacterial cell. Vitamin transporters in bacteria appear to fall into a number of different classes of membrane transport proteins. The thiamine (14), vitamin B12 (24), and possibly biotin (34) transporters belong to the ABC superfamily, whereas vitamin C transport is mediated by a phosphotransferase system (42), while the Na+/H+ pantothenate symporter in E. coli (40) is an example of secondary transport of a vitamin. Bacteria thus employ different transport mechanisms to internalize various vitamins from their environment. In humans riboflavin is absorbed in the small intestine via a specialized, Na+/H+-independent carrier-mediated system (35), and in the yeast Saccharomyces cerevisiae a riboflavin transporter belonging to a family of monocarboxylate transporters was found that mediates facilitated diffusion (33). We have found that the lactococcal protein RibU most likely mediates riboflavin translocation via a facilitated diffusion mechanism.

Transport assays using radiolabeled riboflavin showed that a deletion in ribU renders L. lactis incapable of transporting the vitamin. This result was confirmed in the riboflavin-overproducing strain CB010, in which ribU expression was essentially undetectable and which consequently exhibited very low riboflavin uptake (not shown). Homologues of RibU are present in gram-positive bacteria and archaea, but homologues were not found in the analyzed genomes of gram-negative bacteria (data not shown). Notably, E. coli does not contain a RibU homologue, consistent with its inability to transport riboflavin. Various lactic acid bacteria have also been shown to contain a riboflavin biosynthesis operon (4). The presence of a tightly regulated, dedicated transport system as well as a functional biosynthetic process indicates that such organisms can be flexible and economical in the acquisition of this vitamin depending on environmental supply. However, some species such as Streptococcus thermophilus and Oenococcus oeni do not appear to possess a riboflavin biosynthetic pathway (4) and therefore must completely depend on a riboflavin uptake system. Members of the RibU family are well conserved, have a length of approximately 200 residues, and are predicted to have five hydrophobic membrane-spanning regions. No homologues of RibU were found in the Transport Classification Database (5), indicating that the family of RibU proteins constitutes a novel addition to the large number of recognized transporter families in the database.

An RFN element is conserved upstream of the ribU genes in the analyzed genomes, but the length of this regulatory region varies between species and has different predicted folding conformations. In B. subtilis the predicted FMN-responsive element upstream of the ribU homologue ypaA encompasses 349 nucleotides (41), whereas in L. lactis NZ9000 this region is just 246 nucleotides in length as was shown by our transcriptional analysis. Northern hybridization and lacZ fusions indicated that transcriptional downregulation occurs in the presence of riboflavin. This would suggest that when riboflavin is present the gene is expressed at a low level but that under riboflavin starvation conditions it increases expression in order to import the vitamin. This is most likely mediated by alternative folding conformations of the 5′ leader region as has been described for

FIG. 8. (A) Effect of the proton motive force on riboflavin uptake in whole cells of L. lactis NZ9000. Shown is the uptake of radiolabeled riboflavin and alanine (inset) in cells deenergized with 2-deoxyglucose (inverted triangles) and in deenergized cells in which an artificial proton motive force was applied (circles). The expected level of radiolabeled riboflavin inside the cells when no accumulation would take place (the concentration inside equals the concentration in the uptake buffer) is indicated by the dashed line. (B) Chase of internalized radiolabeled riboflavin from NZ9000 cells with unlabeled riboflavin. Uptake of [3H]riboflavin was performed as for Fig. 6 (black circles). After 30 min, unlabeled riboflavin at a final concentration of 70 μM was added, and at the given time points the samples were filtered and radioactivity was counted (white circles).
the rib operon in *B. subtilis* (41) and *L. lactis* (4), that is, in the presence of FMN or riboflavin the formation of a terminator structure upstream of the rib operon is energetically favorable, thus resulting in premature transcription termination. However, in *B. subtilis* it has been suggested that regulation of YpaA expression would occur at the level of translation by means of a Shine-Dalgarno sequence sequestration mechanism (41). In *L. lactis* it is apparent that transcriptional regulation is the dominant means of controlling the expression of the riboflavin transporter, although this does not rule out the possibility that translational regulation may add a second level of control to RibU expression.

The observation that in the ribU deletion strain the effect of riboflavin or FMN on regulation of the rib operon promoter $P_{ribGABH}$ is absent suggests that the transporter facilitates uptake of both FMN and riboflavin. Indeed excess FMN competes with uptake of radiolabeled riboflavin. This is in contrast to the situation in *B. subtilis*, where it was reported that FMN is not a substrate of the riboflavin transport system. This implies that at these high levels it is possible for the vitamin to permeate the membrane by some other manner, possibly by diffusion directly through the lipid bilayer or by another, non-miscuous transport protein. This is analogous to the situation in *E. coli*, which does not appear to have a dedicated riboflavin transport system. It should be noted, however, that *E. coli* riboflavin auxotrophs require even higher concentrations of riboflavin (720 $\mu$M) in their growth medium (2).

It has been shown that disruption of the ribU homologue ypaA in a *B. subtilis* strain leads to roseoflavin resistance at up to concentrations of 3 mM in contrast to the wild-type strain, which is inhibited at 250 $\mu$M to 375 $\mu$M (17). *E. coli*, which naturally lacks a riboflavin transport system, has also been found to be resistant to 250 $\mu$M roseoflavin, a level which is toxic to *L. lactis* NZ9000. Even though roseoflavin competes with riboflavin uptake by RibU and is likely transported by RibU, NZ9000$\Delta$ribU is not resistant to roseoflavin and is even sensitive to concentrations as low as 12 $\mu$M (no growth observed; unpublished data). These results suggest that *L. lactis* is more permeable to roseoflavin than either *E. coli* or *B. subtilis*, and that in *NZ9000$\Delta$ribU* the compound enters the cell by some other, as yet unknown means. We found no indication that the proton or sodium motive force plays a role in riboflavin transport. Most likely, RibU mediates facilitated diffusion of riboflavin and equilibration of internal and external riboflavin pools drives the apparent accumulation observed in our experiments. In growing cells, the ATP-dependent conversion of riboflavin into FMN and subsequently into flavin adenine dinucleotide will keep the internal substrate concentration low and ensure net influx of riboflavin. In this way ATP may be indirectly involved in transport.

In conclusion, RibU belongs to a novel protein family involved in riboflavin transport. It is likely a secondary transporter mediating facilitated diffusion of riboflavin and the related compounds roseoflavin and to a lesser extent FMN. Further work is required to establish whether RibU catalyzes uptake alone or in conjunction with other proteins.

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**REFERENCES**


