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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 immediately upstream of the riboflavin biosynthesis operon. RibU is predicted to act as a riboflavin transporter, since a mutant strain carrying a mutated ribU gene exhibits altered transcriptional control of the riboflavin biosynthesis operon, and FMN and FMN do not consume riboflavin from its growth medium. Furthermore, it was shown that radiolabeled 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 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).

In *Lactococcus lactis*, the riboflavin biosynthesis operon has recently been 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 tetra-cycline (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::nisRK, wild-type strain</td>
<td>19</td>
</tr>
<tr>
<td>CB10</td>
<td>Riboflavin-overproducing spontaneous mutant of NZ9000</td>
<td>4</td>
</tr>
<tr>
<td>NZ9000ΔribU</td>
<td>NZ9000 derivative with a 783-bp deletion in ribU</td>
<td>4</td>
</tr>
<tr>
<td>NZ9000ΔribU</td>
<td>NZ9000 derivative with a 414-bp deletion encompassing P_rnb, and 69 bp of ribU</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmids

| pOR1280           | Em' LacZ' _ori_ of pMV1, replicates only in strains where repA is provided in trans | 21 |
| pOR1280ΔribU       | pOR1280 derivative containing a truncated version of NZ9000 ribU and the surrounding regions | This study |
| pPTPL             | Tet' LacZ' _ori_; promoter probe vector | 4 |
| pPTPL•ribU         | pPTPL derivative containing the promoter region of NZ9000 ribU | This study |
| pPTPL•lop          | pPTPL derivative containing the promoter region of NZ9000 rib operon | 4 |

**Bioinformatics.** All sequence data were obtained from the National Center for Biotechnology Information (NCBI) ([http://www.ncbi.nlm.nih.gov](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 TMFFRED (transmembrane prediction) (13). The proteins were also analyzed for similarity to families of transport proteins using the BLAST program on the Transport Classification Database (http://www.tcdb.org) (5). Potential RFN elements were identified using RFAM (RNA family) ([http://www.sanger.ac.uk/software/RFam](http://www.sanger.ac.uk/software/RFam)) (12). Folding of the 5' mRNA leader region was predicted using MFOLD (http://www.bioinfo.rpi.edu/applications/mfold/old/mb/) (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 a 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 lacticoccal 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 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 Macilaid method (28) from _L. lactis_ strain grown in CDM in the presence or absence of 5 μM riboflavin. Northern hybridization analysis was performed by denaturing 5 μg RNA at 65°C and followed by electrophoretic separation on a 0.8% formaldehyde agarose gel. RNA was then transferred to a Hybond N membrane (Amersham, Buckinghamshire, United Kingdom) by capillary transfer using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as buffer transfer. Purified ribU-encompassing PCR product was used as a probe and was labeled with [α-32P]dATP with the Prime-a-Gene kit (Promega, Madison, Wis.) according to the manufacturer’s instructions. The prehybridization and hybridization steps were carried out at 48°C in 10 ml UltraHyb (Ambion, Austin, Tex.), and washes were executed at 48°C according to the manufacturer’s instructions. Detection was performed 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'-[γ-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 each end of ribU and its promoter region. Sparing 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 NcoI 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 [OD_600] of approximately 1.8), and cells were then harvested by centrifugation at 7,700 × _g_ at 4°C for 10 min. Harvested cells were washed with 50 mM KPi, pH 7.0; resuspended in the same buffer to obtain a cell suspension with an OD_600 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 deenergized with 10 mM 2-deoxyglucose (30, 39).

Uptake was started by the addition of [3H]riboflavin (Campro Scientific, Veennendaal, 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 KPi, 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 KPi, pH 7.0, and then dried using an infrared lamp, and the radioactivity was determined by liquid scintillation counting. In some cases rosoflavin 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 KPi, 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 KPi, pH 7.0, plus 100 mM potassium acetate and 10 mM 2-deoxyglucose to a final OD_600 of 100. The cells were then diluted 1 to 10 in either 50 mM KPi, pH 7.0, or 50 mM NaPi, 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⁺/Cl⁻ exchange across the membrane 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]glycine 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 OD_600 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
paper have been submitted to the GenBank database under accession number AY994156.

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

![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.](image)
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 μM 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-
duction. This apparent transcriptional regulation is consistent with the results from the analysis of NZ9000 and CB010 containing a ribU-lacZ transcriptional fusion on plasmid pPTPL (Fig. 3B), which showed that in the absence of riboflavin P_{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 ribU-reporter fusion plasmid was present in CB010, essentially no β-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 ribU in L. lactis plays a role in this observed vitamin consumption phenotype, a deletion strain, designated NZ9000ΔribU, was created in which the ribU promoter, the presumed regulatory region, translation initiation signals, and the first 23 codons of ribU were removed from the genome. To determine whether this partial ribU deletion affects riboflavin consumption, strains NZ9000 and NZ9000ΔribU 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, NZ9000ΔribU 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 NZ9000ΔribU by complementation in trans, that is, following introduction of intact ribU on a plasmid (data not shown).

**FIG. 4.** Analysis of growth and riboflavin levels of NZ9000 and NZ9000ΔribU in GM17. The solid lines represent log OD_{600}, 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 NZ9000ΔribU are depicted with empty inverted triangles.

**FIG. 5.** P_{ribGBAH} activity in NZ9000 and NZ9000ΔribU in various media. The solid circles represent NZ9000, and the empty inverted triangles represent NZ9000ΔribU. The solid lines represent growth (on a semilog scale), and the dashed lines represent β-galactosidase activity. (A) CDM; (B) CDM plus 5 μM riboflavin; (C) CDM plus 5 μM FMN; (D) CDM plus 50 μM riboflavin.
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 $P_{ribGAH}$ was examined in the wild-type strain NZ9000 and in NZ9000delribU, grown in CDM or CDM supplemented with 5 or 50 μM riboflavin or 5 μM FMN. In CDM high promoter activity of $P_{ribGAH}$ was observed in both NZ9000 and NZ9000delribU, 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 $P_{ribGAH}$-lacZ fusion in NZ9000delribU was high (Fig. 5B and C). This is in contrast to the wild-type situation where $P_{ribGAH}$ 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 $P_{ribGAH}$ activity was reduced in both NZ9000 and NZ9000delribU (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 $\Delta\mu$ 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 radio labelled 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 $P_{ribGAH}$ promoter by FMN in the growth medium that is observed in the wild-type strain but not in the RibU deficient (Fig. 5C).

To determine the driving force for riboflavin accumulation in the wild-type strain an artificial $\Delta\mu$ was generated in cells that previously had been deenergized (depleted of ATP and $\Delta\mu$ 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 $\Delta\mu$ 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 $\Delta\mu$ 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
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 \( \text{Na}^+/\text{H}^+ \)-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, \( \text{Na}^+/\text{H}^+ \)-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 *yphaA* 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

![Graph](image-url)
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<sub>ribGBAH</sub> 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 (6). The presence of a high concentration of riboflavin (50 μM) resulted in downregulation of P<sub>ribGBAH</sub> in *NZ9000ΔΔribU*, and the effects were similar to those of 5 μM in the wild-type strain. 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 μM) 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 μM to 375 μM (17). *E. coli*, which naturally lacks a riboflavin transport system, has also been found to be resistant to 250 μM 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, NZ9000ΔΔribU is not resistant to roseoflavin and is even sensitive to concentrations as low as 12 μM (no growth observed; unpublished data). These results suggest that *L. lactis* is more permeable to roseoflavin than either *E. coli* or *B. subtilis*, and it appears that in *NZ9000ΔΔribU* 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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