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Riboflavin Production in *Lactococcus lactis*: Potential for In Situ Production of Vitamin-Enriched Foods

Catherine Burgess,[1] Mary O'Connell-Motherway,[1,2] Wilbert Sybesma,[3] Jeroen Hugenholtz,[3] and Douwe van Sinderen[1,2]*

Department of Microbiology[1] and Alimentary Pharmabiotic Centre,[2] Biosciences Institute, National University of Ireland Cork, Cork, Ireland, and Wageningen Centre for Food Sciences, Wageningen, The Netherlands[3]

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This study describes the genetic analysis of the riboflavin (vitamin B_2) biosynthetic (*rib*) operon in the lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* strain NZ9000. Functional analysis of the genes of the *L. lactis rib* operon was performed by using complementation studies, as well as by deletion analysis. In addition, gene-specific genetic engineering was used to examine which genes of the *rib* operon need to be overexpressed in order to effect riboflavin overproduction. Transcriptional regulation of the *L. lactis* riboflavin biosynthetic process was investigated by using Northern hybridization and primer extension, as well as the analysis of roseoflavin-induced riboflavin-overproducing *L. lactis* isolates. The latter analysis revealed the presence of both nucleotide replacements and deletions in the regulatory region of the *rib* operon. The results presented here are an important step toward the development of fermented foods containing increased levels of riboflavin, produced in situ, thus negating the need for vitamin fortification.

Riboflavin (vitamin B_2) is an essential component of basic cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The latter two biomolecules play a central role in metabolism acting as hydrogen carriers in biological redox reactions involving enzymes such as NADH dehydrogenase (for a review of this topic, see reference 32). Many microorganisms, plants, and fungi possess the biosynthetic ability to produce riboflavin. However, vertebrates, including humans, lack this ability and must therefore obtain this vitamin from their diet.

Dietary riboflavin is present in liver, egg yolk, milk, and meat, whereas the vitamin is commercially synthesized for nutritional use in the fortification of various food products such as bread and breakfast cereals. Because of its intense yellow color it is also used in small amounts as a coloring agent in foods such as ice cream and sauces, and as a medical identification aid. The recommended daily requirement of riboflavin is set at 1.3 mg (14) and sufficient amounts of riboflavin need to be ingested regularly since the body is unable to store the vitamin. Symptoms of riboflavin deficiency (ariboflavinosis) in humans, which still occurs in both developing and developed countries (6, 34), include sore throat, hyperemia, edema of oral and mucous membranes, cheilosis, and glossitis (48). Furthermore, riboflavin is used as a treatment for nucleoside analogue-induced type B lactic acidosis, which can occur as a result of AIDS treatment (9), for migraine (23), and for malaria (1). Commercially available riboflavin has traditionally been produced by chemical processes, but in recent times this has been replaced by biotechnological and more economical processes with *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* (43).

Riboflavin biosynthesis has been studied in both gram-positive and gram-negative bacteria, in most detail in *B. subtilis* (36) and *Escherichia coli* (4). The precursors of riboflavin are GTP and ribulose-5′-phosphate and the biosynthesis of riboflavin occurs through seven enzymatic steps (36) (Fig. 1) with a slight difference between bacteria and fungi (43). (For a recent review of this subject, see reference 3.) In order to perform its metabolic function, riboflavin must be biochemically transformed to the coenzymes FMN and FAD. In all bacteria that have been analyzed, these conversions are catalyzed by an essential bifunctional flavokinase/FAD synthetase encoded by a gene that is not genetically linked to the biosynthetic genes, if the latter are present (4, 31).

In *B. subtilis*, strict transcriptional regulation of the *rib* operon takes place by means of a mRNA regulatory region transcribed from the 5′ end of the *rib* operon. This regulatory mRNA region is conserved in several bacteria and is predicted to fold into a specific secondary structure (RFN element) comprising five stem-loops and a single root stem (15, 46).

Roseoflavin is a riboflavin analogue, and from previous work in *B. subtilis* it is known that exposure to this compound leads to spontaneous mutants that are constitutive riboflavin overproducers (37). Mutations in the regulatory region of the *rib* operon have been shown to have this effect (20), as have certain mutations in *ribC* (8, 22).

Lactic acid bacteria (LAB) are industrially important microbes that are used all over the world in a wide variety of industrial food fermentations. Of the group of microorganisms, *Lactococcus lactis* is by far the most extensively studied LAB, and many efficient genetic tools have been developed for the organism. We describe here the characterization of riboflavin biosynthesis in *L. lactis* subsp. *cremoris* NZ9000, a bacterium which can be used as a model for the development of strains that have the potential to produce an essential vitamin

* Corresponding author. Mailing address: Alimentary Pharmabiotic Centre, Department of Microbiology and Biosciences Institute, National University of Ireland Cork, Western Rd., Cork, Ireland. Phone: 353214901365. Fax: 353214903101. E-mail: d.vansinderen@ucc.ie.

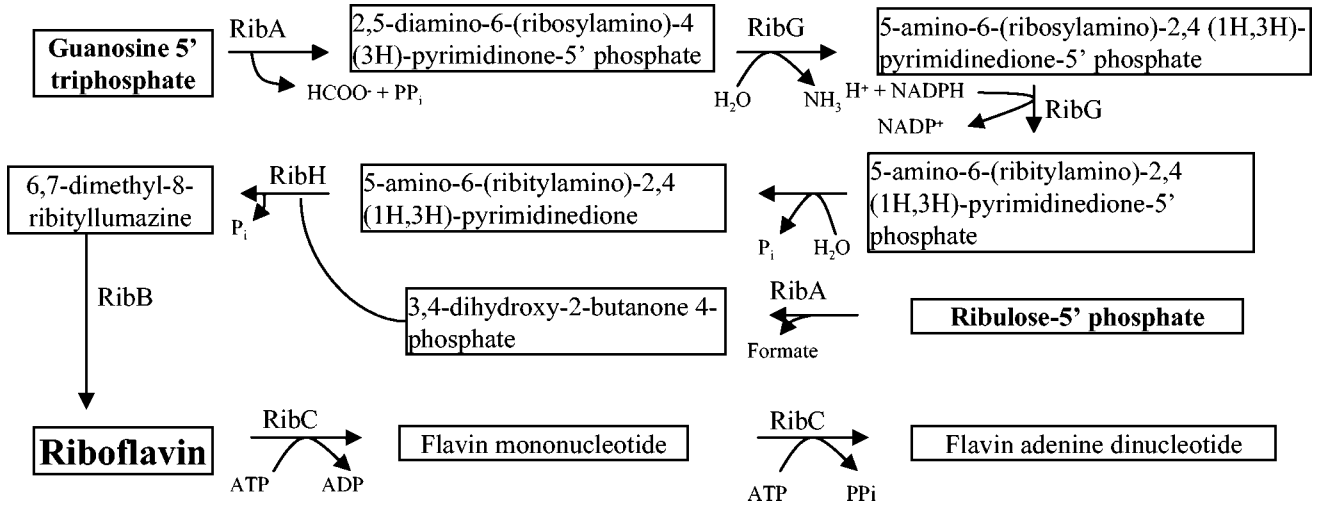

FIG. 1. Riboflavin biosynthetic pathway in bacteria. The enzymes encoded by *L. lactis* responsible for each step are indicated. RibG, riboflavin-specific deaminase/reductase; RibB, riboflavin synthase (alpha subunit); RibA, GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase; RibH, lumazine synthase (beta subunit).

in situ which would contribute significantly to the functional value of certain fermented foods.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium at 37°C (40). *L. lactis* strains were grown in M17 medium supplemented with 0.5% glucose (45) or in chemically defined medium (CDM) (adapted by removal of folic acid, riboflavin, and nucleotides) (35, 38). Where appropriate, growth medium contained chloramphenicol (5 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (5 μg ml^{-1}), ampicillin (50 μg ml^{-1}), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml^{-1}).

Bioinformatics. Putative riboflavin biosynthesis genes and *ribC* were identified from the *L. lactis* subsp. *cremoris* MG1363 sequencing project (21) by homology with the *B. subtilis rib* genes (26). These sequences were used to design PCR primers for NZ9000, a direct derivative of MG1363 (24), and the sequences of the amplified *rib* operon and *ribC* were determined by using the respective PCR products as templates. The obtained *L. lactis* sequences were used for comparative analysis by using tBLAST-N (2) with other LAB genomes obtained from the URL sites of The Institute for Genomic Research (http://www.tigr.org), the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and the DOE Joint Genome Institute (http://www.jgi.doe.gov).

DNA manipulations and transformations. Plasmid DNA was isolated from *E. coli* by using the JETquick plasmid miniprep kit (Genomed, Löhne, Germany) according to the instructions of the manufacturer. Plasmid DNA was isolated from *L. lactis* with the same kit, except that the cells were preincubated in cell resuspension solution containing 20 mg of lysozyme ml^{-1} at 55°C for 30 min to cause cell lysis. Transformation of *E. coli* was carried out as described by Sambrook et al. (40). Transformation of *L. lactis* was achieved according to the protocol of De Vos et al. (12). Isolation of chromosomal DNA from *L. lactis* was performed as described by Leenhouts et al. (29, 30). Southern blotting was done by a standard protocol (40), and detection was accomplished by using digoxigenin labeling (Roche, Lewes, United Kingdom) according to the manufacturer's instructions.

Construction of a chromosomal deletion in *ribA*. The primers were designed to amplify the sections overlapping and flanking either end of *ribA*. Splicing-by-overlap-extension PCR (17) was used to create a PCR product which contained *ribA* with a 783-bp in frame deletion (nucleotides 2631 to 3414 of AY453633) of the *ribA* coding sequence. This PCR product was inserted into pORI280 (Table 1) by using the PstI and NcoI restriction sites present on the outermost primers and using EC1000 as a cloning host. The resulting plasmid, designated pORI280Δ*ribA* was used to introduce the deletion into the NZ9000 chromosome by replacement recombination (28), creating strain NZ9000Δ*ribA*. The deletion was confirmed by PCR and sequence analysis and by Southern hybridization analysis.

Plasmid constructions. Primers were used to amplify the entire *ribA* gene, the 5′ portion of *ribA* which was predicted to encode 3,4-dihydoxy-2-butanone-4-phosphate synthase (nucleotides 2311 to 2944 of AY453633), the 3′ portion of *ribA* that was assumed to specify GTP cyclohydrolase II (nucleotides 2919 to 3507 of AY453633), as well as the complete *ribB* gene. The mutated *ribA* from NZ9000Δ*ribA* was also amplified. The individual PCR products were cloned into pCR-II-TOPO (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions by using *E. coli* TOP-10 as the host. The resulting plasmids are listed in Table 1.

The lactococcal plasmid pNZ8048 is a vector used in nisin-controlled expression (10). Various gene combinations of the *rib* operon were amplified from NZ9000 chromosomal DNA by using primers that contained an NcoI site and a PstI recognition sequence within the forward and reverse primers, respectively. The amplified product was digested with NcoI and PstI and cloned into pNZ8048 digested with the same two enzymes. The resulting plasmids, listed in Table 1, were constructed by using *E. coli* EC1000 as a cloning host and were subsequently transferred to the lactococcal strain NZ9000.

pPTPL is a promoter probe vector containing the *E. coli* promoterless *lacZ* gene and multiple cloning site from pORI13 (41). It replicates as a low-copy-number plasmid in both *E. coli* and *L. lactis* by virtue of the *E. coli* pSC101 (47) and *L. lactis* pIL252 (42) replication regions. It contains the *Staphylococcus aureus*-derived *tetK* gene (16) as a selective antibiotic resistance marker. The region upstream of the *rib* operon (nucleotides 2 to 702 of AY453633) was amplified by PCR with primers containing BglII and XbaI sites in the forward and reverse primers, respectively, by using chromosomal DNA from NZ9000 (wild-type strain) and CB010 (roseoflavin-induced riboflavin overproducer) as templates. Plasmid pPTPL and amplified PCR products were digested with the restriction enzymes mentioned above and ligated to generate plasmids pPTPLop and pPTPLcbop in which the *rib* promoter regions of NZ9000 and CB010, respectively, were placed upstream of the *lacZ* gene of pPTPL. The plasmids were constructed by using *E. coli* EC1000 as a host and subsequently transferred to NZ9000 or CB010. X-Gal was used in plates as a qualitative indicator of promoter activity. In the same manner, a region spanning the distal 3′ end of *ribB* and the proximal 5′ end of *ribA* (nucleotides 1904 to 2421 of AY453633) was probed for promoter activity by cloning this region into pPTPL creating plasmid, pPTPL-P2.

Isolation of roseoflavin-resistant mutants and sequence analysis of roseoflavin-resistant mutants. Spontaneous roseoflavin-resistant NZ9000 mutants were isolated by plating mid- to late-log-phase cells on CDM containing 100 mg of roseoflavin $liter^{-1}$. In an effort to identify the mutations that cause roseoflavin resistance and riboflavin overproduction, *ribC* and the regulatory region upstream of *ribG* were amplified by PCR, purified by using the JETquick PCR

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
L. lactis		
NZ9000	MG1363 *pepN*::*nisRK*; wild-type strain	24
NZ9000Δ*ribA*	NZ9000 derivative with a 783-bp deletion in *ribA*	This study
CB010 to CB021	NZ9000 derivatives that have increased resistance to roseoflavin	This study
E. coli		
BSV11	Mutation in 3,4-dihydroxy-2-butanone 4-phosphate synthase	5
BSV13	Mutation in riboflavin synthase α chain	5
BSV18	Mutation in GTP cyclohydrolase II	5
EC1000	Km^r; MC1000 derivative, carrying a single copy of pWV01 *repA* in *glgB*	28
TOP10	Commercial cloning host used for pCR2.1-TOPO	Invitrogen, Groningen, The Netherlands
Plasmids		
pNZ8048	Cm^r; inducible expression vector carrying the *nisA* promoter	10
pORI280	Em^r; LacZ+, *ori*+ of pWV01; replicates only in strains providing *repA in trans*	28
pCR2.1-TOPO	Km^r Amp^r; commercial cloning vector	Invitrogen, Groningen, The Netherlands
pORI280Δ*ribA*	pORI280 derivative containing a truncated version of *ribA* and the surrounding regions	This study
pPTPL	Promoter probe vector	This study
pCB001	pCR2.1-TOPO with NZ9000 *ribA* inserted	This study
pCB002	pCR2.1-TOPO with NZ9000 *ribA* 5′ section inserted	This study
pCB003	pCR2.1-TOPO with NZ9000 *ribA* 3′ section inserted	This study
pCB004	pCR2.1-TOPO with a truncated NZ9000 *ribA* inserted	This study
pCB005	pCR2.1-TOPO with NZ9000 *ribB* inserted	This study
pNZdhbp	pNZ8048 containing NZ9000 *ribA* 5′ section under the control of the *nisA* promoter	This study
pNZgchII	pNZ8048 containing NZ9000 *ribA* 3′ section under the control of the *nisA* promoter	This study
pNZA	pNZ8048 containing NZ9000 *ribA* under the control of the *nisA* promoter	This study
pNZB	pNZ8048 containing NZ9000 *ribB* under the control of the *nisA* promoter	This study
pNZBA	pNZ8048 containing NZ9000 *ribA* and *ribB* under the control of the *nisA* promoter	This study
pNZBAH	pNZ8048 containing NZ9000 *ribA*, *ribB*, and *ribH* under the control of the *nisA* promoter	This study
pNZGBA	pNZ8048 containing NZ9000 *ribG*, *ribB*, and *ribA* under the control of the *nisA* promoter	This study
pNZGBAH	pNZ8048 containing NZ9000 *ribG*, *ribB, ribA*, and *ribH* under the control of the *nisA* promoter	This study
pPTPLop	pPTPL containing the promoter region upstream of the NZ9000 *rib* operon	This study
pPTPLcbop	pPTPL containing the promoter region upstream of the CB010 *rib* operon	This study
pPTPL-P2	pPTPL containing the promoter region within *ribB* of NZ9000	This study

purification kit (Genomed, Löhne, Germany), and subjected to sequence analysis (MWG Biotech AG, Ebersberg, Germany).

Nisin-induced riboflavin production. Overnight cultures of NZ9000, which contained the various pNZ8048 constructs, were diluted 1:100 in CDM supplemented with chloramphenicol and grown to an optical density at 600 nm of ca. 0.5. The cells were then induced with 0, 1, or 5 ng of nisin A ml^{-1} and allowed to grow for a further 3 h; the riboflavin concentration of the cell-free supernatant was then determined, and cellular proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27).

Transcriptional analysis. β-Galactosidase assays (19) were performed on NZ9000(pPTPLop), CB010(pPTPLcbop), and NZ9000(pPTPL-P2) during growth in CDM either in the presence or absence of 5 μM riboflavin or FMN.

Total RNA was isolated at mid-logarithmic phase by the Macaloid method (25) from the strains NZ9000 and its riboflavin-overproducing derivative CB010 grown in CDM in the presence or absence of 5 μM riboflavin. Northern hybridization analysis was performed by denaturing 5 μg of RNA at 65°C and separating it on a 0.8% formaldehyde agarose gel. The RNA was then transferred to a Hybond N+ charged nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary transfer by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer. Purified *ribH* and *ribB* PCR products were used as probes and were labeled with [α-^{32}P]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 of UltraHyb (Ambion, Austin, Tex.), and washes were performed at 48°C according to the manufacturer's instructions. Detection was carried out by exposure to a Kodak Biomax MR film at −70°C for 4 h.

Determination of transcription start site. A reverse primer was designed ca. 120 bp downstream of the assumed transcription start site, upstream of the first gene of the *rib* operon (nucleotides 368 to 390 of AY453633). Primer extension analysis was performed by annealing 10 pmol of 5′γ-^{32}P-labeled primer to 50 μg of NZ9000 RNA (39). A GATC sequence ladder which was run alongside the primer extension product was produced by using the same labeled primer with the T7 DNA polymerase sequencing kit (USB Corp., Cleveland, Ohio). Detection was carried out by exposure to Kodak Biomax MR film at −70°C for 48 h.

Quantitative analysis of riboflavin in culture medium. Extracellular riboflavin concentrations were measured by reversed-phase high-pressure liquid chromatography. An Ultrasphere RP 4.6-mm-by-25-cm column (Beckman Coulter, Fullerton, Calif.) was used, and riboflavin was eluted with a linear gradient of acetonitrile from 3.6 to 30% at pH 3.2. Fluorescence detection was used, and the excitation and emission wavelengths were 440 and 520 nm, respectively. Commercially obtained riboflavin and FMN were used as references and to obtain a standard curve (Sigma, Steinheim, Germany).

Nucleotide sequence accession numbers. The nucleotide sequence data of *L. lactis* subsp. *cremoris* NZ9000 *rib* operon and regulatory region reported in the present study were submitted to the GenBank database under accession number AY453633, and NZ9000 *ribC* was submitted under accession number AY456331.

RESULTS

Comparative analysis of the lactococcal genes presumed to be involved in riboflavin biosynthesis. The presumed riboflavin biosynthesis genes of *L. lactis* subsp. *cremoris* MG1363 were identified by homology with the characterized *B. subtilis rib* genes and the annotated *L. lactis* IL-1403 *rib* genes (7, 26).

TABLE 2. Comparative analysis of riboflavin biosynthesis genes among various LAB strains and *B. subtilis*

Strain[c]	RFN element	% Homology to *L. lactis* NZ9000[a]					Source[b]
		ribG	*ribB*	*ribA*	*ribH*	*ribC*	
Lactococcus lactis subsp. *cremoris* SK11[1]	+	95	98	96	98	99	JGI
Lactococcus lactis subsp. *lactis* IL1403[2]	+	72	74	81	82	94	NCBI
Streptococcus pneumoniae TIGR4[2]	+	52	58	57	69	47	TIGR
*Streptococcus pneumoniae*R6[2]	+	52	58	57	69	47	TIGR
Streptococcus agalactiae 2603V/R[2]	+	47	50	59	60	48	NGBI
Leuconostoc mesenteroides[1]	+	39	37	42	47	36	JGI
Pediococcus pentosaceus ATCC 25745[1]	+	36	46	50	49	39	JGI
Lactobacillus brevis ATCC 367[1]	+	35	40	48	42	39	JGI
Lactobacillus plantarum WCFS1[2]			–[d]	47	51	40	NCBI
Lactobacillus gasseri ATCC 33323[1]					47	37	JGI
Lactobacillus casei ATCC 334[1]						37	JGI
Lactobacillus bulgaricus ATCC BAA365[1]						35	JGI
Streptococcus thermophilus LMD-9[1]						46	JGI
Streptococcus pyogenes MGAS8232[2]						45	NCBI
Streptococcus mitis[1]						47	TIGR
Oenococcus oeni PSU1[1]						35	JGI
Enterococcus faecalis V583[2]						44	TIGR
Bacillus subtilis	+	39	50	57	58	36	

[a] The figures indicate the degree of homology to *L. lactis* NZ9000 genes.
[b] Sequences were obtained from The Institute for Genomic Research (TIGR), the Joint Genome Institute (JGI), or The National Center for Biotechnology Information (NCBI) website as indicated.
[c] Superscript numbers: 1, uncompleted genomes; 2, completed genomes.
[d] –, 3′ present.

These homologies were used to design primers for NZ9000 in order to obtain and determine the sequence of the entire *rib* operon, as well as *ribC* (accession numbers AY453633 and AY456331, respectively). The translated genes of the *rib* operon, as well as *ribC* in NZ9000, were in turn used to search for homologues in available chromosomal sequences of various LAB strains. The observed levels of homology between the genes are given in Table 2. As expected, all screened bacterial chromosomes contain a homologue of *ribC*, the gene necessary to convert riboflavin to its cofactor derivatives. The two *L. lactis* subspecies, as well as *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* ATCC 25745, harbor a complete set of riboflavin biosynthetic genes in the same order as in *B. subtilis* (46). Also, both *Streptococcus pneumoniae* strains TIGR4 and R6 and *S. agalactiae* 2603V/R contain the full complement of similarly organized riboflavin biosynthesis genes, in contrast to the streptococcal species *S. thermophilus* LMD-9 and *S. mitis*, which do not appear to contain such homologues. For sequenced members of the genus *Lactobacillus*, it was found that *Lactobacillus brevis* ATCC 367 contains all four genes required for riboflavin biosynthesis, whereas *Lactobacillus plantarum* WCFS1 only contains an intact copy of *ribA* and *ribH* and a truncated copy of *ribB*, and *Lactobacillus gasseri* ATCC 33323 only contains a *ribH* homologue. *Lactobacillus casei* ATCC 334 and *Lactobacillus delbrueckii* ATCC BAA365 do not appear to contain any of the riboflavin biosynthetic genes. Also, other genera of LAB such as *Oenococcus oeni* PSU1 and *Enterococcus faecalis* V583, appear to lack the riboflavin biosynthesis genes. In *S. pneumoniae* and *L. lactis* IL-1403 structurally conserved RFN elements have been previously identified (46). This region has also been identified in other LAB containing the complete *rib* operon. It appears that some species have lost the entire *rib* operon (or never possessed it), whereas in some cases, such as *Lactobacillus plantarum*, only part of the operon appears to have been lost. However, it should be noted that at this time some of these genomes are not complete (http://www.tigr.org; http://www.jgi.doe.gov).

Chromosomal deletion in *ribA*. In order to investigate the functionality of the presumed *L. lactis rib* operon, a derivative of NZ9000, named NZ9000Δ*ribA*, was constructed containing a 783-bp in-frame deletion in the *ribA* gene (see Materials and Methods). The deletion was confirmed by Southern hybridization analysis and sequence analysis of a PCR product encompassing the relevant region in NZ9000Δ*ribA* (data not shown). In contrast to the wild type, the deletion mutant is unable to grow in CDM in the absence of riboflavin but will grow when it is supplemented in the medium. The strain is also capable of growth in CDM in the presence of FMN and FAD (data not shown). No growth difference was observed between the two strains in complex medium. The introduction of the intact *L. lactis ribA* gene on a plasmid into the deletion strain restored the ability to grow in the absence of riboflavin in the medium (data not shown).

Complementation of *E. coli* auxotrophic mutants. The *E. coli* strain BSV11 carries a mutation in the gene encoding the enzyme 3,4-dihydroxy-2-butanone-4-phosphate synthase, whereas strain BSV18 carries a mutation in the gene specifying GTP cyclohydrolase II (5). These mutations result in an inability of such strains to synthesize riboflavin and therefore to grow in the absence of added riboflavin. Based on homology with the *B. subtilis ribA* gene, it was assumed that the *L. lactis ribA* gene encodes both of these enzymatic functions within distinct sections of the encoded RibA product. In order to verify this assumption the *L. lactis ribA* gene was introduced into BSV11 and BSV18 as a complete gene (in pCB001), as the 5′-proximal portion of *ribA* predicted to encode 3,4-dihydroxy-2-butanone-4-phosphate synthase (in pCB002), or as the 3′ distal portion of the gene assumed to specify GTP cyclohydrolase (pCB003).

TABLE 3. Complementation study of *L. lactis ribA* and *ribB*

Plasmid	Growth (+) or no growth (−) of strain[a]:					
	BSV11		BSV13		BSV18	
	LB with Ribo	LB without Ribo	LB with Ribo	LB without Ribo	LB with Ribo	LB without Ribo
pCB001	+	+	NA	NA	+	+
pCB002	+	+	NA	NA	+	−
pCB003	+	−	NA	NA	+	+
pCB004	+	−	NA	NA	+	−
pCB005	NA	NA	+	+	NA	NA
No plasmid	+	−	+	−	+	−

[a] Ribo, 200 mg of riboflavin per liter; LB, Luria-Bertani medium; NA, not applicable.

A deleted version of *L. lactis ribA* in which 783 bp were removed from the center of the gene (in pCB004), corresponding to the chromosomal deletion introduced in *L. lactis* NZ9000Δ*ribA* (see above), was also transformed into these *E. coli* strains. The results are summarized in Table 3. The presence of the complete *L. lactis ribA* in pCB001 was shown to complement the riboflavin auxotrophy of both BSV11 and BSV18, indicating that it encodes the two enzymatic functions lacking in those two strains. Plasmid pCB002, which contains the DNA region specifying 3,4-dihydroxy-2-butanone-4-phosphate synthase, was shown to complement only BSV11, whereas the DNA region specifying GTP cyclohydrolase II present in pCB003 only complemented BSV18. A deletion in *ribA* spanning both 3,4-dihydroxy-2-butanone-4-phosphate synthase and GTP cyclohydrolase II (in plasmid pCB004) prevents complementation, proving that it is the intact *ribA* or sections thereof that were complementing the strain. Complementation was also used to prove the functionality of the lactococcal *ribB*, which is shown to encode riboflavin synthase α chain since a plasmid encompassing this gene, pCB005, was able to complement the *E. coli* mutant strain BSV13. These complementation experiments therefore showed that the *L. lactis* NZ9000 *ribA* and *ribB* genes are functional in *E. coli* and thus involved in riboflavin biosynthesis. *E. coli* or *B. subtilis ribG* and *ribH* mutants are not available, and the assumed *L. lactis ribG* and *ribH* could therefore not be used for complementation studies.

Controlled overexpression of *rib* genes. A targeted approach was taken to prove the involvement of *ribG* and *ribH* in riboflavin biosynthesis, as well as to identify which genes of the *rib* operon need to be overexpressed in order to result in significant extracellular riboflavin production. The genes of the *rib* operon were cloned in different combinations into the nisin-inducible expression vector pNZ8048 (Fig. 2) and introduced into *L. lactis* NZ9000. The cell-free supernatant of each of the resulting lactococcal strains was analyzed for riboflavin content 3 h after nisin induction. The cellular protein profile of each strain was also examined after nisin induction to verify that overexpression of the targeted *rib* genes had occurred, which was indeed the case (data in Fig. 2 illustrate the protein profile for *L. lactis* NZ9000 containing pNZGBAH). Minute amounts of riboflavin (<0.01 mg liter^{-1}) can be detected by high-pressure liquid chromatography analysis upon overexpression of intact *ribA* on its own and in other combinations, whereas 0.18 mg of riboflavin liter^{-1} is detected upon concurrent overexpression of *ribG*, *ribB*, and *ribA*. In contrast, substantial riboflavin overproduction (24 mg liter^{-1}) is seen when all four biosynthetic genes are overexpressed simultaneously (in *L. lactis* NZ9000 containing pNZGBAH).

Isolation and sequence analysis of roseoflavin-resistant mutants. Exposure of NZ9000 to the toxic riboflavin-analogue roseoflavin was used in an attempt to isolate *L. lactis* mutants with increased levels of riboflavin production. In this way, 12 roseoflavin-resistant mutants were isolated which upon analysis were also shown to exhibit riboflavin overproduction (Fig. 3). Since it is known in *B. subtilis* that roseoflavin resistance may be caused by mutations in *ribC* or in the regulatory region upstream of the *rib* operon (8, 20, 22), the DNA regions en-

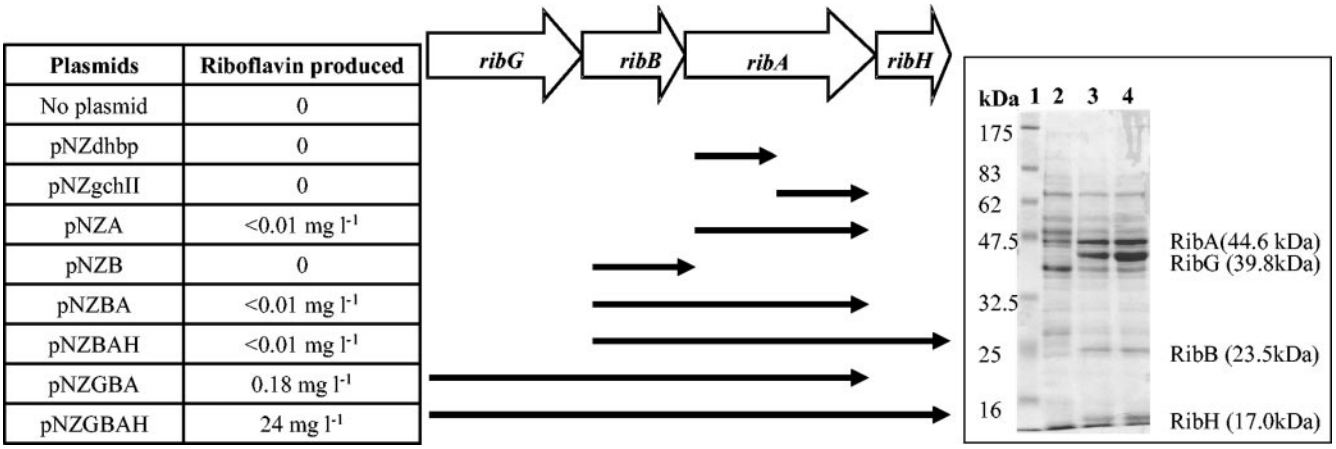

FIG. 2. Constructs made in pNZ8048 and effect on extracellular riboflavin production upon nisin induction. The black arrows indicate the various regions of the *rib* operon cloned into pNZ8048. The effect of nisin induction on riboflavin production is indicated in the table. Sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis shows a protein profile of NZ9000(pNZGBAH) after induction with various amounts of nisin. Lane 1 contains a size marker with sizes indicated in the left-hand margin, lane 2 contains uninduced NZ9000(pNZGBAH), lane 3 contains NZ9000(pNZGBAH) induced with 1 ng of nisin ml^{-1}, and lane 4 contains NZ9000(pNZGBAH) induced with 5 ng of nisin ml^{-1}. The overexpressed Rib proteins with their calculated molecular masses are indicated in the right-hand margin.

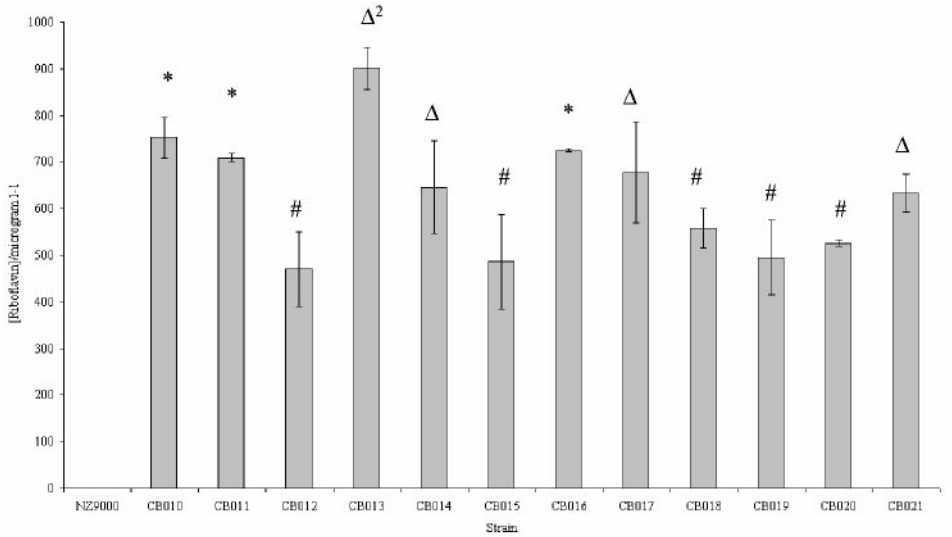

FIG. 3. Riboflavin levels in the cell-free supernatant of roseoflavin-resistant strains grown in CDM for 8 h. ✻, values for isolates that were shown to carry a G→T mutation in the first stem of the RFN regulatory element; #, values for isolates that were shown to carry a G→A mutation in the third loop of the RFN element; Δ, values for isolates that were shown to carry a 90-bp deletion; Δ^2, values for the isolate that was shown to carry a 138-bp deletion.

compassing the *rib* mRNA leader and the *ribC* gene of each of the 12 lactococcal mutants was subjected to sequence analysis to identify mutations that might explain the observed riboflavin overproduction. In the present study, no mutations were identified in *ribC* of the mutant strains. Five of the isolated mutants contain a G-to-A mutation (mutation indicated in Fig. 4) in a conserved base in the predicted third loop of the RFN (15). Three isolates contained a G-to-T mutation in the right side of the first stem of the RFN element (in the anti-antiterminator sequence). Three isolates were found to contain a 90-bp deletion in the region encompassing part of the RFN-encoding sequence including the complete anti-antiterminator, while one isolate contained a 138-bp deletion between the RFN-encoding DNA and the ribosomal binding site of the *rib* operon, removing the terminator structure (deletions indicated in Fig. 4). These mutations are the most likely cause of the observed increased riboflavin production phenotype through increased transcription of the *rib* operon, as was indeed demonstrated for isolate CB010 (see below).

Transcriptional analysis of the *rib* operon in NZ9000 and its riboflavin-overproducing derivative CB010. Primer extension analysis was executed to identify the main promoter, *rib*P1, immediately upstream of the *L. lactis rib* operon (Fig. 4). The transcription start site was identified as an adenine, upstream

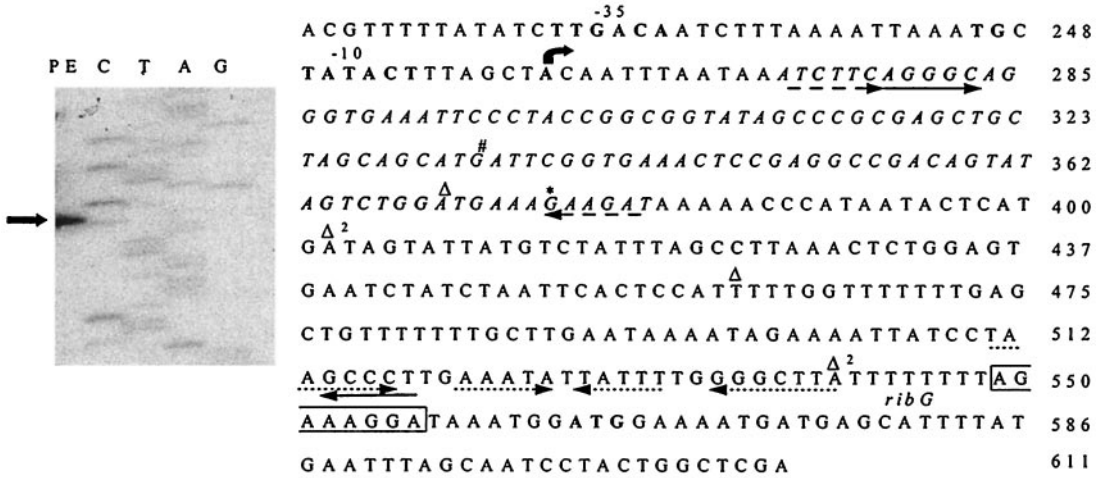

FIG. 4. Primer extension (PE) analysis of the *rib*P1 promoter run alongside a sequencing ladder. The deduced −35 and 10 boxes are indicated in boldface type in the sequence displayed on the right-hand side of the figure. The bent arrow indicates the identified transcription start site. The identified RFN element is marked in italics. The assumed ribosomal binding site is boxed, and the *ribG* start codon is in boldface. The dotted arrows beneath the sequence indicate the terminator. The solid arrows indicate the antiterminator. The dashed arrows indicate the anti-antiterminator. ✻ and #, positions of mutations found in roseoflavin-resistant mutants; Δ, start and end of a 90-bp deletion found in three roseoflavin-resistant mutants; Δ^2, start and end of a 138-bp deletion found in one strain.

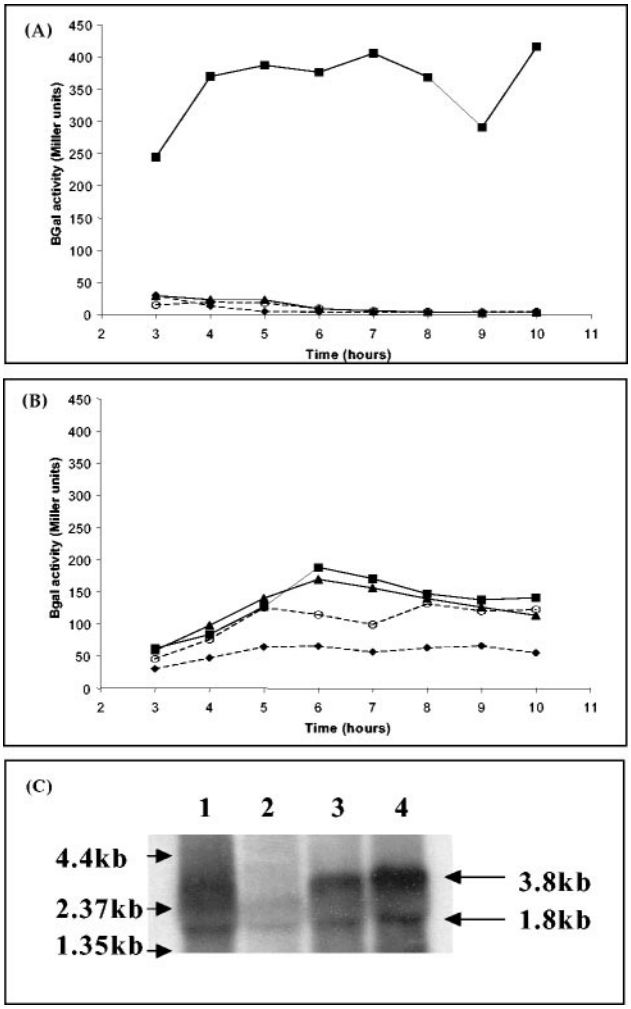

FIG. 5. (A) β-Galactosidase activity of NZ9000(pPTPLop). (B) β-Galactosidase activity of CB010 (pPTPLcbop). The dashed line with solid diamonds represents growth in GM17, the solid line with solid squares represents growth in CDM, the solid line with solid triangles represents growth in CDM plus riboflavin, and the dashed lines with open circles represents growth in CDM plus FMN. (C) Northern blot with *ribH* as a probe. Lane 1, NZ9000 RNA from CDM; lane 2, NZ9000 RNA from CDM plus riboflavin; lane 3, CB010 RNA from CDM; lane 4, CB010 RNA from CDM plus riboflavin. An RNA size ladder is indicated on the left. The sizes of the transcripts are indicated on the right.

of which −10 and −35 sequences were identified with a clear resemblance to the consensus vegetative RNA polymerase recognition sequences for *L. lactis* (11). Northern hybridization analysis was used to detect transcription of the *rib* operon in both NZ9000 and its roseoflavin-resistant derivative CB010 in the presence or absence of riboflavin. Using *ribH* as a probe, a 3.8-kb transcript is present in total RNA isolated from NZ9000 grown in CDM without riboflavin. However, this transcript is absent from RNA isolated from the same strain in the presence of riboflavin (Fig. 5C). In contrast to the wild-type situation, the 3.8-kb transcript is present in total RNA preparations from the riboflavin overproducer CB010 regardless of whether the strain was grown in the presence or absence of riboflavin. The *rib*P1 promoter region, including the regulatory region, was cloned into the promoter probe vector pPTPL from both the NZ9000 and the CB010 strains, and the β-galactosidase activity was measured for these two strains in complex media and CDM in the presence or absence of riboflavin or FMN. The NZ9000-derived *rib*P1 promoter activity was detected in CDM, but it can be seen that there is almost no promoter activity in the presence of riboflavin or FMN or in complex media (Fig. 5A). In the case of the CB010-derived *rib*P1 promoter, the flavin-dependent regulation observed for the NZ9000-derived *rib*P1 does not appear to be functional, and this promoter drives more or less constitutive β-galactosidase activity from the pPTPL reporter plasmid (Fig. 5B), although at a level that is lower than that observed for the NZ9000-derived *rib*P1 in CDM in the absence of riboflavin or FMN. Furthermore, expression of the CB010-derived *rib*P1 promoter in cells grown in complex medium is also constitutive but lower compared to the expression levels observed in CDM.

Interestingly, Northern hybridization analysis revealed the presence of a second transcript of 1.8 kb in RNA preparations from both NZ9000 and CB010, indicating the presence of a second promoter (designated *rib*P2) within the operon. Using *ribB* as a probe only the 3.8-kb transcript can be seen (data not shown), indicating that this transcript is initiated downstream of *ribB*. Using pPTPL to determine the location of this promoter activity within the *rib* operon of NZ9000, *rib*P2 was shown to be located on a 517-bp fragment encompassing the 3′ distal region of *ribB* and the 5′-proximal region of *ribA* (nucleotides 1904 to 2421 of AY453633).

DISCUSSION

Industry constantly strives to develop novel starter cultures for fermented foods with enhanced characteristics that result in a value-added product. The design of rational approaches to metabolic engineering and/or natural selection with such an aim requires an in-depth understanding of the pathway, the genes involved, and their regulation. The results presented here give a detailed analysis of riboflavin biosynthesis in the industrially relevant *L. lactis*.

The presence of the biosynthetic genes and thus biosynthetic ability is not conserved within all examined LAB genomes, as demonstrated by homology searches. The presence or absence of the *rib* biosynthetic genes does not appear to be linked to whether the LAB in question is a hetero- or homofermentative species, whether they are pathogenic species or phylogenetically closely related. It is worth noting that a homologue of *ribT*, a gene of unknown function found downstream of the *rib* operon in *B. subtilis*, is not present in *L. lactis*.

A deletion in *ribA* renders *L. lactis* NZ9000 incapable of growth in the absence of exogenous riboflavin, but growth is restored when the medium is supplemented with riboflavin or FMN. It should also be noted that growth could be restored by much lower levels of riboflavin than the levels required for *E. coli* riboflavin auxotrophs (4). This suggests the presence of a dedicated transport mechanism for this vitamin in *L. lactis*. The possibility of a flavin-specific transporter, encoded by *ypaA*, has been suggested in *B. subtilis* (36). A homologue of this gene is present in *L. lactis*, and an RFN element-encoding sequence is located immediately upstream of its coding region (unpublished data).

After it was established that the riboflavin biosynthesis pathway is functional in *L. lactis* and involves the predicted genes, a study of the operon's regulation was undertaken. The position of *rib*P1 was identified by primer extension analysis, and in vivo promoter activity studies showed that both riboflavin (converted to FMN within the cell by the action of RibC) and FMN decrease the *rib*P1 promoter activity in the same manner as has been demonstrated in *B. subtilis* (33). The effect of riboflavin on transcription of the operon was confirmed by Northern hybridization. A second promoter, *rib*P2, was detected within the *rib* operon at the 3′ end of *ribB*, a situation reminiscent of that found in *B. subtilis* (36). The means by which this promoter is regulated has not yet been elucidated.

Two approaches were taken to develop riboflavin-overproducing strains of *L. lactis*: targeted metabolic engineering and isolation of spontaneous mutants to a toxic riboflavin analogue. Resistance to roseoflavin was used as a selection method for riboflavin overproduction in *L. lactis*, resulting in the isolation of 12 mutants containing four types of mutation different from those previously observed in *B. subtilis* (20). Based on the predicted terminator, antiterminator, and anti-antiterminator structures (Fig. 4), it can be hypothesized that mutations at these positions affect the folding of the terminator and anti-antiterminator structures, allowing for transcription to occur even in the presence of FMN, resulting in elevated riboflavin production. It has been reported that *rib* expression is induced >20-fold in an engineered *B. subtilis* strain in which the *rib* leader terminator was deleted (33), although spontaneous deletions in this region have not been previously described.

In *B. subtilis ribA* has been reported to be the rate-limiting enzyme in the riboflavin biosynthetic process in this bacterium and that increased expression of *ribA* leads to up to 25% increase in riboflavin yield (18). Figure 2 illustrates the combinations in which the *rib* genes were overexpressed by using a nisin-inducible controlled expression system. In *L. lactis*, overexpression of *ribA* alone is not sufficient to elicit such a significant increase in riboflavin production as seen in *B. subtilis*. In fact, all four lactococcal riboflavin biosynthetic genes need to be overexpressed together to give rise to substantial riboflavin overproduction, although overexpression of *ribGBA* also results in clearly increased extracellular riboflavin production.

Previous work has shown *L. lactis* NZ9000 to be, where possible, a riboflavin consumer (44). Furthermore, it has also been suggested that yogurt bacteria may utilize B vitamins from their surroundings, thus decreasing their bioavailability upon ingestion (13). The present study successfully applied two different approaches by which *L. lactis* NZ9000 as a model strain can be converted from a vitamin consumer into a vitamin "factory." All riboflavin measurements in the present study were carried out extracellularly, indicating that riboflavin would be freely available upon consumption of a product fermented by such a riboflavin-producing strain. Preliminary results have shown that the overexpression strategy can be successfully used in a milk-based medium (data not shown), indicating the potential usefulness in fermented dairy foods. It is important to note that the genetic modifications of the riboflavin-producing strains (being either chemically induced or genetically engineered) did not appear to affect their acid production during growth, an important attribute in fermentation of foods (data not shown). Although the chemically induced mutants produce riboflavin at a much lower level than the engineered strain, they have a considerable advantage over the latter since such chemically induced strains are much easier to generate from existing industrial strains and are much more likely to be accepted by the general public. The presented results are thus an important step in the development of fermented foods, for which the traditional starter can be replaced by a riboflavin-producing equivalent, resulting in the vitamin being produced in situ, thereby contributing to the required intake of the vitamin.

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