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Identification and Functional Characterization of the *Lactococcus lactis rfb* Operon, Required for dTDP-Rhamnose Biosynthesis

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dTDP-rhamnose is an important precursor of cell wall polysaccharides and rhamnose-containing exopolysaccharides (EPS) in *Lactococcus lactis*. We cloned the *rfbACBD* operon from *L. lactis* MG1363, which comprises four genes involved in dTDP-rhamnose biosynthesis. When expressed in *Escherichia coli*, the lactococcal *rfbACBD* genes could sustain heterologous production of the *Shigella flexneri* O antigen, providing evidence of their functionality. Overproduction of the RfbAC proteins in *L. lactis* resulted in doubled dTDP-rhamnose levels, indicating that the endogenous RfbAC activities control the intracellular dTDP-rhamnose biosynthesis rate. However, RfbAC overproduction did not affect rhamnose-containing B40-EPS production levels. A nisin-controlled conditional RfbBD mutant was unable to grow in media lacking the inducer nisin, indicating that the *rfb* genes have an essential role in *L. lactis*. Limitation of RfbBD activities resulted in the production of altered EPS. The monomeric sugar of the altered EPS consisted of glucose, galactose, and rhamnose at a molar ratio of 1:0.3:0.2, which is clearly different from the ratio in the native sugar. Biophysical analysis revealed a fourfold-greater molecular mass and a twofold-smaller radius of gyration for the altered EPS, indicating that these EPS are more flexible polymers with changed viscosifying properties. This is the first indication that enzyme activity at the level of central carbohydrate metabolism affects EPS composition.

Bacterial polysaccharides can be present in the cell wall as components of the cell envelope. Information about the structure and biosynthesis pathway of these compounds is fragmented. Glucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylmannose, and rhamnose are often found to be constituents of cell wall polysaccharides (8, 41).

Rhamnose is a 6-deoxyhexose sugar which is widely distributed in O antigens of gram-negative bacteria as part of the lipopolysaccharide (LPS) (43). Furthermore, this compound is often found in capsular polysaccharides (CPS), which are covalently bound to the cell wall, and in exopolysaccharides (EPS), which are loosely associated with the cell wall. L-dTDP-rhamnose is the sugar-nucleotide precursor of these rhamnose moieties and is formed in a four-step reaction from glucose 1-phosphate. The reaction involves the enzyme activities of glucose-1-phosphate thymidylyl transferase, dTDP-glucose-4,6-dehydratase, dTDP-4-keto-L-rhamnose-3,5-epimerase, and dTDP-L-rhamnose synthase encoded by the genes that are commonly designated *rfbABCD*, respectively. These genes have been found in several gram-negative bacteria, including *Escherichia coli* (23), *Salmonella enterica* (30), *Xanthomonas campestris* (25), and *Shigella flexneri* (35). Various *rfb* mutant strains have been described, and the mutations have various effects on the rhamnose contents of the cell wall polysaccharides produced, including a loss of O antigen production (31), a reduced level of LPS production (23), or production of LPS with a reduced amount (25) or complete lack (29) of rhamnose.

In gram-positive bacteria *rfb* homologues, designated *rml* genes in *Streptococcus mutans* (49, 50) and *cps* genes in *Streptococcus pneumoniae* (15, 20), have been characterized, and these homologues appear to play an essential role in the production of serotype-specific, rhamnose-containing CPS antigens. In *S. mutans*, *rml* mutations resulted in a change in the composition of the cell wall polysaccharide, which lacked rhamnose, and in a complete lack of production of the serotype-specific O antigen (49, 50). *S. pneumoniae cps19fL* and *cps19fN* mutants exhibited a so-called rough phenotype and did not have the capacity to produce CPS, indicating that the *rfb* analogues play an essential role in CPS-19F production (38).

Various lactic acid bacteria, including lactobacilli (7, 11, 19, 59), streptococci (45), and lactococci (33, 46), characteristically contain rhamnose in their cell walls. Lactococcal cell wall polysaccharides decorate the peptidoglycan network (for a review see reference 8), and rhamnose is one of the major components of these sugar polymers (33, 46) and has been suggested to be the primary binding site for certain bacteriophages (for a recent review see reference 14). Moreover, it is also a component of the EPS produced by *L. lactis* SBT0495 (40), NIZO B40 (53, 56), and NIZO B39 (54). Since EPS-producing lactic acid bacteria are used in the food industry, in which the EPS produced in situ determines dairy product properties like texture, EPS could provide a potential new source for food-grade biothickeners (12).

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TABLE 1. Bacterial strains and plasmids used in this study and their relevant characteristics and sources

Strain or plasmid	Relevant characteristics[a]	Reference or source
L. lactis strains		
MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	16
NZ9000	MG1363 *pepN*::*nisRK*	26
NZ4114	Tet^r, NZ9000 derivative containing the *nisA* promotor upstream of *rfbB*, leading to conditional, nisin-controlled *rfbBD* expression	This study
E. coli strains		
MC1061		6
SΦ874	*E. coli* K-12 analogue	42
Plasmids		
pGEM-T	Ap^r	Promega
pGhost8	Ery^r	37
pGhost9	Tet^r	37
pK194	Km^r	21
pUC18	Ap^r	60
pUC19	Ap^r	60
pUC18Ery	Ap^r Ery^r	56
pPM2213	Ap^r, contains the *S. flexneri rfb* region and directs *S. flexneri* 4 O antigen production in *E. coli*	35
pPM2716	Ap^r, pPM2213 derivative lacking the *S. flexneri rfbBDAC* genes	36
pNZ4000	EPS$^+$, 43-kb plasmid encoding B40-EPS production	56
pNZ4030	Ery^r EPS$^+$, 27-kb derivative of pNZ4000	56
pNZ4104	Ap^r, pUC18 derivative containing the MG1363 3.8-kb *Sac*I/*Eco*RI chromosomal DNA fragment with the *rfb′ACBD* genes	This study
pNZ4105	Ap^r, Ery^r, pUC18Ery derivative containing the 1.2-kb *Sph*I/*Spe*I fragment with the *′rfbAC′* genes	This study
pNZ4106	Ap^r, pUC18 derivative containing the MG1363 2.4-kb *Hind*III chromosomal DNA fragment with the *rfbAC* genes	This study
pNZ4107	Ap^r, Ery^r, pUC18Ery derivative containing the *Acc*I/*Eco*RI fragment with the *rfbD* gene	This study
pNZ4108	Ap^r, Ery^r, pUC18Ery derivative containing the *Acc*I/*Eco*RI fragment with the *rfbD* gene and the *Hind*III fragment with the *rfb′AC* genes of pNZ4104	This study
pNZ4109	Ap^r Ery^r Tet^r, derivative of pNZ4108 containing the *tetR* gene from pGhost9	This study
pNZ4110	Km^r, derivative of pK194 carrying the lactococcal *rfbACBD* genes transcriptionally fused to the *nisA* promoter	This study
pNZ4111	Ap^r, derivative of pUC19 with the 1.4-kb *Stu*I/*Sac*I fragment from pNZ4118 containing the *cat* terminator sequence and the *nisA* promoter	This study
pNZ4112	Ap^r, pNZ4111 derivative containing the *Eco*RI/*Hind*III fragment of pNZ4104 with the *rfbAC* genes	This study
pNZ4113	Ap^r Ery^r, pNZ4112 derivative containing the *ery* gene from pUC18Ery	This study
pNZ4114	Ap^r Ery^r Tet^r, derivative of pNZ4113 containing the *tetR* gene from pGhost9	This study
pNZ4115	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbAC* genes under control of the putative *rfb* promoter	This study
pNZ4116	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbAC* genes transcriptionally fused to the *nisA* promoter	This study
pNZ4117	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbACBD* genes transcriptionally fused to the *nisA* promoter	This study
pNZ4118	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbB* gene translationally fused to the *nisA* promoter	This study
pNZ8048	Cm^r, lactococcal cloning and expression vector with the *nisA* promoter upstream of a multiple cloning site	26

[a] EPS$^+$, EPS-producing phenotype; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Ery^r, erythromycin resistant; Tet^r, tetracycline resistant; Km^r, kanamycin resistant.

Here we describe cloning and functional analysis of the *rfb* operon involved in dTDP-L-rhamnose biosynthesis in *L. lactis*, including complementation of an *E. coli rfb* mutant, the effects of homologous overexpression of the *rfb* genes in *L. lactis* on dTDP-rhamnose synthesis, and the impact of *rfb* expression modulation on the production of rhamnose-containing lactococcal EPS. The *rfb* operon is essential for growth of *L. lactis*, as shown by using an *rfbBD* conditional mutant. In addition, the *rfbBD* conditional mutant produced an altered EPS with novel physical characteristics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 1. *E. coli* MC1061 (6), which was used as a host in cloning experiments, was grown with aeration in tryptone yeast extract broth at 37°C (44). *L. lactis* was grown without aeration at 30°C in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose or in a chemically defined medium (32). When appropriate, the media contained chloramphenicol (10 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (2.5 μg ml^{-1}), kanamycin (25 μg ml^{-1}), or ampicillin (100 μg ml^{-1}). To analyze the effect of gene overexpression, the nisin-controlled expression system was used (9, 26). Briefly, for the overexpression studies *L. lactis* cells were grown until the optical density at 600 nm was approximately 0.5 and then split into two cultures. One nanogram of nisin per milliliter was added to one of the two cultures, and both cultures were grown for an additional 2 h. For the studies in which the nisin-controlled conditional mutant was used, *L. lactis* cells were grown overnight in medium containing different levels of nisin and were subsequently subcultured in medium lacking nisin for 25 h.

DNA manipulations and DNA sequence analysis. Small-scale isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook et al. (44). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Isolation and transformation of *L. lactis* DNA were performed as previously described (10).

Automated double-stranded DNA sequence analysis of both strands was performed with an ALF*red* DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands). Sequence reactions were performed with an Autoread kit (Amersham Biosciences, Roosendaal, The Netherlands), were initiated by using Cy5-labeled universal and reverse primers, and were continued with synthetic primers purchased from Pharmacia Biotech in combination with fluorescein

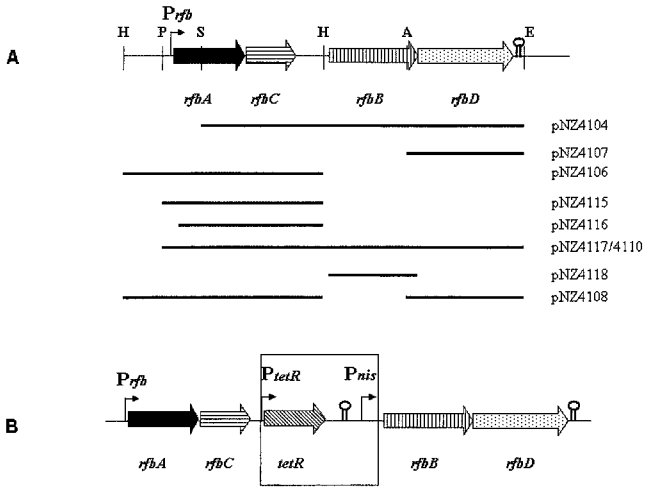

FIG. 1. (A) Schematic diagram of genetic organization of the MG1363 *rfbACBD* operon and DNA fragments of this operon cloned in vectors. (B) Diagram of the *rfb* locus in the nisin-controlled *rfbBD* conditional mutant strain NZ4114. Abbreviations for restriction sites: H, *Hin*dIII; P, *Pst*I; S, *Sac*I; A, *Acc*I; E, *Eco*RI.

15-dATP by following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed by using the PC/GENE program, version 6.70 (Intelli-Genetics, Inc., Mountain View, Calif.).

Construction of strains and plasmids. Cloning and characterization of the *rfb* operon from *L. lactis* MG1363 were performed prior to release of the *L. lactis* IL-1403 genome sequence (3). To do this, an internal fragment of the lactococcal *rfbA* gene was amplified by PCR by using chromosomal DNA of *L. lactis* MG1363 (16) as a template and the degenerate primers 5′-TAYGAYAARCC NATGATHTAYTAYCC-3′ and 5′-RTGNGTNCCNGTRTCNARCCA-3′ (where H is A, C, or T; N is A, C, G, or T; Y is C or T; and R is A or G), which were based on conserved regions in an alignment (PC/GENE package; Intelli-Genetics, Inc.) of amino acid sequences of the RfbA analogues RmlA and CPS19FL from *S. mutans* (accession no. D78182) and *S. pneumoniae* (U09239.1). The 0.6-kb PCR product generated was cloned in pGEM-T (Promega, Leiden, The Netherlands), and sequence analysis revealed a continuous open reading frame (ORF) that was predicted to encode a protein exhibiting high sequence homology with RfbA proteins. The resulting plasmid was used as a probe in Southern analysis. This fragment hybridized with a 3.8-kb *Sac*I/*Eco*RI fragment and a 2.4-kb *Hin*dIII fragment of the *L. lactis* MG1363 chromosomal DNA, which were cloned in similarly digested pUC18 (60), yielding pNZ4104 and pNZ4106, respectively (Fig. 1A). Sequence analysis of the inserts revealed the presence of four ORFs. These ORFs were predicted to encode proteins consisting of 289, 197, 350, and 300 amino acids that exhibited high sequence identity with RfbA, -C, -B, and -D homologues found in other gram-positive bacteria, including *L. lactis* IL-1403 (level of identity, >96%), *S. pneumoniae* (>71%), and *S. mutans* (>70%). Sequence comparisons were performed by using the BLAST module at the CMBI web site (www.cmbi.kun.nl).

The *rfbAC* overexpression plasmid was constructed by cloning a 2.0-kb *Pst*I-*Hin*dIII fragment of pNZ4106 containing *rfbAC* into similarly digested pNZ8048 (26), which yielded pNZ4115 (Fig. 1A). The *rfbAC* fragment cloned in pNZ4115 contained a putative termination sequence upstream of the *rfbA* coding sequence that hampered expression of the *rfbAC* genes (data not shown). Therefore, this sequence was deleted by using a PCR strategy. The 5′ coding region of the *rfbA* gene was amplified by using pNZ4115 as the template DNA and primers 5′-A ATGCAGGCTAATTAATTATGATTATGGAGGTCC-3′ and 5′-AGCATAT TGAAGATTGATACC-3′. The 223-bp PCR product generated was cloned into pNZ4115 digested with *Pst*I-*Sac*I by using the *Pst*I restriction site introduced in the forward primer (underlined) and the *Sac*I restriction site present in the PCR product generated, which yielded the functional *rfbAC* overexpression construct pNZ4116 (Fig. 1A; also see below). The *rfbACBD* overexpression plasmid was constructed by cloning the 3.8-kb *Sac*I-*Eco*RI fragment of pNZ4104, containing the *′rfbACBD* ORFs, into *Pst*I-*Hin*dIII-digested pNZ4116 after blunting of the *Eco*RI and *Hin*dIII sticky ends with the Klenow fragment, which yielded pNZ4117 (Fig. 1A). The *rfbB* gene was amplified by PCR by using pNZ4104 as the template DNA and primers 5′-CATGCCATGGCAACTGAATTTAAAAA TATCGTTGTGACAG-3′ and 5′-GCGCTCTAGAGCTAGGATTTCATCAG CAAATTTTGG-3′. The 1.1-kb PCR product generated was cloned into pNZ8048 (26) by using the *Nco*I and *Xba*I restriction sites that were introduced by the primers (underlined), which yielded the *rfbB* overexpression plasmid pNZ4118 (Fig. 1A). Plasmid pNZ4116, pNZ4117, or pNZ4118 was transformed into *L. lactis* strain NZ9000 (26). All PCR products that were cloned in vectors were sequenced to verify that no mutations were accidentally introduced during PCRs. The EPS-producing capacity was introduced into NZ9000 harboring pNZ4116, pNZ4118, or pNZ4117 by electroporation of plasmid pNZ4030 (56), which contained the B40 *eps* gene cluster.

To ascertain that the *L. lactis rfbACBD* operon encodes a functional dTDP-rhamnose biosynthesis pathway, a 3.9-kb *Sca*I-*Pst*I fragment of pNZ4117 (see below) was cloned into pK194 (21). The resulting plasmid, pNZ4110, was transformed into *E. coli* Sϕ874 containing pPM2716 (35).

Several strategies were employed to knock out one of the *rfb* genes. In the first strategy, which was used to knock out the *rfbA* gene by single-crossover plasmid integration, a 1.2-kb PCR fragment containing the 3′ end of RfbA and the 5′ end of RfbC was amplified by using primers 5′-TATCTATGATAAACCAATGAT TTATTATC-3′ and 5′ GCCCAGTAATCATTAACCAG-3′ and cloned into pGEMT (Promega). A 1.2-kb *Sph*I-*Spe*I fragment from the resulting plasmid was cloned into pUC18Ery (56). The resulting plasmid, pNZ4105, was transformed into *L. lactis* NZ9000, but despite several attempts, no erythromycin-resistant (Ery^r) colonies were obtained. This result provided the first suggestion that integration of this plasmid into the *rfb* locus could be lethal to *L. lactis*.

In the second strategy, which was used to knock out the *rfbB* gene by double-crossover gene replacement, an integration plasmid was constructed, which contained an erythromycin resistance gene cassette flanked by the up- and downstream regions of the *rfbB* gene. To do this, a 1.1-kb *Acc*I-*Eco*RI fragment of pNZ4104 containing the *rfbB* downstream region was cloned into similarly digested pUC18Ery (56), which yielded pNZ4107 (Fig. 1A). Subsequently, a 1.6-kb *Hin*dIII fragment of pNZ4104, containing the *rfbB* upstream region, was cloned into similarly digested pNZ4107, yielding pNZ4108. To facilitate direct double-crossover transformant selection, an additional selection marker, *tetR*, which was isolated as an *Sma*I-*Ecl*136 fragment from pGhost8 (37), was cloned into the *Sma*I restriction site of pNZ4108. The resulting plasmid, pNZ4109, was transformed into *L. lactis* NZ9000. No double-crossover transformants were obtained, which supported the postulated essential role of the *rfb* genes in *L. lactis*.

In the third strategy, which was used to knock out the *rfbB* gene by double-crossover gene replacement, plasmid pNZ4109 was transformed into *L. lactis* NZ9000 harboring pNZ4118. The latter plasmid contains a copy of a functional *rfbB* gene fused to the inducible *nisA* promoter. Ery^r colonies were screened by replica plating on GM17 plates containing tetracycline or erythromycin and 1 ng of nisin ml^{-1}. The addition of nisin was important to generate expression of the *rfbB* gene from pNZ4118. In contrast to the first two strategies, Ery^r Tet^s integrants were obtained only in *L. lactis* NZ9000 harboring pNZ4118. Southern analysis confirmed that all of the Ery^r Tet^s integrants contained a disrupted copy of the *rfbB* gene on the chromosome, and one colony, designated NZ4109, was selected for further analysis.

Since the *rfbB* gene could be disrupted only when another copy of the *rfbB* gene was present in *trans*, we used a fourth strategy to construct a nisin-controlled conditional *rfbBD* mutant. In this mutant expression of *rfbBD* was placed under control of the tightly controlled *nisA* promoter, while the *rfbAC* genes were constitutively expressed. This genetic organization allowed nisin-controlled modulation of *rfbBD* expression, including the shutting down of *rfbBD* expression by removal of nisin from the growth medium, which led to development of the *rfbBD* mutant phenotype in this strain. For construction of this nisin-controlled conditional *rfbBD* mutant, a 1.4-kb *Stu*I-*Sac*I fragment of pNZ4118, containing a chloramphenicol (*cat*) gene-derived termination sequence that originated from cloning vector pNZ8048 (26), the *nisA* promoter, and the *rfbB* gene, was cloned in pUC19 (60) digested with *Sma*I and *Sac*I, which yielded pNZ4111. In pNZ4111 the 1.5-kb *Hin*dIII-*Eco*RI fragment of pNZ4104, containing a *′rfbAC* fragment, was cloned, which yielded pNZ4112. To facilitate direct double-crossover selection, we cloned two resistance markers in pNZ4112. An erythromycin resistance gene cassette was isolated as a 1.2-kb *Hin*dIII-*Kpn*I fragment from pUC18Ery (56) and cloned in *Sma*I-*Kpn*I-digested pNZ4112, after the *Hin*dII site was filled with the Klenow fragment. In the resulting plasmid, pNZ4113, a second selection marker, *tetR*, was cloned. Therefore, a *Sma*I-*Ecl*136 fragment from pGhost8 (37) was cloned (after the cohesive ends were filled by using the Klenow fragment) in the *Hin*dIII restriction site of pNZ4113. The resulting plasmid, pNZ4114, was transformed into *L. lactis* NZ9000. Integrants were primarily selected on plates containing nisin and tetracycline. After this the integrants were screened for erythromycin resistance by replica plating. The

desired Tet^r Ery^s colonies that were obtained were further analyzed by Southern analysis, and a single colony, designated NZ4114, was selected. This integrant contained the *rfbAC* coding sequence, followed by the desired integration of the tetracycline resistance gene cassette, the *cat* gene-derived termination sequence, and the *nisA* promoter followed by the *rfbBD* coding sequence (Fig. 1B).

Preparation of CEs and protein analysis. Lactococcal cells (50 ml) were harvested by centrifugation (3,500 × *g*, 10 min, 4°C), and the cell pellets were suspended in 1 ml of 20 mM sodium phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The suspensions were mechanically disrupted by bead beating in the presence of zirconium beads (55), and cell debris was removed by centrifugation (3,500 × *g*, 10 min, 4°C). The protein content of the cell extract (CE) was determined by the method of Bradford (4) by using bovine serum albumin as the standard.

Each lactococcal CE was mixed with an equal amount of twofold-concentrated Laemmli buffer, and after boiling, 15 μg of each sample was analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (27).

Northern, Southern, and Western blot analyses. Southern blots were hybridized at 65°C with homologous DNA probes, which were labeled by nick translation by using established procedures (44), and the blots were subsequently washed with a solution containing 0.015 M NaCl and 0.0015 M sodium citrate at 65°C before exposure.

RNA was isolated from *L. lactis* cultures, and Northern blot analysis was performed as described by Luesink et al. (34). The blots were probed with internal fragments of the *rfbA* and *rfbD* genes. The internal fragment of the lactococcal *rfbA* gene was isolated as a 0.4-kb *Eco*RV-*Afl*II fragment from pNZ4105, and the internal fragment of the lactococcal *rfbD* gene was isolated as a 0.7-kb *Eco*RI-*Hin*dIII fragment from pNZ4104.

For Western blot analysis of *E. coli*, protein samples were prepared by harvesting 1 ml of a cell culture and then resuspending it in 100 μl of distilled water. Subsequently, the resuspended pellet was mixed with and equal volume of Laemmli buffer and boiled for 3 min, and 10 μl of the resulting suspension was applied to an SDS-PAGE gel (27). Proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) (48) by using electroblot equipment according to the instructions of the manufacturer (LKB 2051 Midget Multiblot). The filters were probed with rabbit antiserum raised against *S. flexneri* O antigen (Sifin, Berlin, Germany), used at a dilution 1:2,500. Primary, O antigen-bound antibodies were detected by using goat anti-rabbit peroxidase-conjugated antibodies at a dilution of 1:5,000 and a peroxidase-specific reaction performed according to the instructions of the manufacturer (Pierce, Rockford, Ill.).

Enzyme assays. Enzyme reactions were performed at 30°C in 1-ml (total volume) mixtures by using freshly prepared CEs at various concentrations. The formation of NAD(P)(H) was determined by measuring the change in absorbance at 340 nm. The values given below are the means of at least two independent measurements. Each blank contained the reaction buffer, cofactors, and the substrate but lacked the CE.

The glucose-1-phosphate thymidylyl transferase (RfbA; EC 2.7.7.24) reverse reaction assay was based on the assay described by Bernstein (1). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.8), 8 mM $MgCl_2$, 0.3 mM $NADP^+$, 2.1 U of α-phopsphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, 4 mM inorganic phosphate, and CE. The reaction was started by addition of 0.1 mM dTDP-glucose. One RfbA activity unit (U_{RfbA}) was defined as 1 nmol of $NADP^+$ converted per min per mg of total protein.

The overall activities of dTDP-glucose-4,6-dehydratase (RfbB; EC 4.2.1.46), dTDP-4-keto-6-deoxy-D-glucose-3,5-epimerase (RfbC; EC 5.1.3.13), and dTDP-4-keto-L-rhamnose reductase (RfbD; EC 1.1.1.133) were each determined in a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 0.5 mM NADH, and CE; 0.3 mM dTDP-glucose was added to start the reaction (adapted from the method described by Grobben et al. [18]). One unit for the overall reaction (U_{RfbBCD}) was defined as 1 nmol of NADH converted per min per mg of total protein.

The dTDP-D-glucose-4,6-dehydratase (RfbB) (EC 4.2.1.46) reaction mixture (final volume, 700 μl) contained 50 mM Tris-HCl buffer (pH 8.0) and CE. The reaction was started by addition of 43 mM dTDP-glucose. At different times 75-μl samples of the reaction mixture were taken and added to 600 μl of 0.5 M NaOH. After 10 min of incubation, the formation of dTDP-4-keto-6-deoxy-D-glucose was determined at 320 nm. The molar absorption coefficient of dTDP-4-keto-6-deoxy-D-glucose (6.5×10^3 liters mol^{-1} cm^{-1}) (61) was used to calculate the RfbB specific activity. One RfbB activity unit (U_{RfbB}) was defined as 1 nmol of dTDP-4-keto-6-deoxy-D-glucose converted per min per mg of total protein.

Sugar nucleotide and EPS analysis. Sugar nucleotides were separated from cell extracts, and individual sugar nucleotide contents were determined by high-performance liquid chromatography as previously described by Looijesteijn et al. (33). The values reported below are the averages of at least two independent determinations. EPS were isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (32). The molecular mass and the radius of gyration (R_g) were determined by using the program Insight II (Biosym MS I, Cambridge, United Kingdom). The intrinsic viscosity (η) was calculated as described by Tuinier et al. (51) by using the equation $\eta = [10\pi(R_g/1.27)^3 N_{AV}]/3M$, where N_{AV} is Avogadro's number and M is the molecular mass.

Isolation of cell wall sugars and characterizations of EPS and cell wall sugars. Isolation of cell wall sugars was performed as described by Looijesteijn et al. (33). Lactococcal cells (50 ml) grown in chemically defined medium were harvested in the stationary phase by centrifugation (3,500 × *g*, 10 min, 4°C) and washed twice with 0.85% (wt/vol) NaCl at 4°C. After disruption with a French press (twice at 18,000 lb/in^2), whole cells were removed by centrifugation (3,500 × *g*, 10 min, 4°C), and the supernatant was centrifuged (200,000 × *g*, 60 min, 4°C) to harvest cell envelopes. The crude cell envelope fraction obtained was resuspended in 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7) containing 140 μg of RNase per ml and 100 μg of DNase per ml and incubated for 90 min at 37°C. Cell envelopes were reisolated by centrifugation (200,000 × *g*, 60 min, 4°C) and then were resuspended in 0.5 mM MOPS buffer (pH 7) containing 2% SDS and incubated at 70°C for 1 h. After centrifugation (200,000 × *g*, 60 min, 4°C), the pellet was washed twice with distilled water to remove the SDS and subsequently freeze-dried, which resulted in a purified cell wall fraction. Isolated EPS or cell walls were hydrolyzed in 4 M HCl for 30 min at 100°C. Samples were dried under a vacuum and dissolved in distilled water. The monomeric sugar composition after hydrolysis was determined by high-performance liquid chromatography (58). The values presented below are averages based on at least two independent experiments.

Nucleotide sequence accession number. The nucleotide sequences of the *rfbACBD* genes have been deposited in the GenBank database under accession no. AF458777.

RESULTS

Cloning and functional analysis of the *rfb* gene cluster. We cloned and sequenced the *rfbACBD* genes from MG1363. Sequence alignments (see Materials and Methods) indicated that these genes most likely encode the enzymes involved in the conversion of glucose 1-phosphate to dTDP-rhamnose. The lactococcal *rfbACBD* genes are organized in an operon-like structure (Fig. 1A) and are all preceded by typical Shine-Dalgarno sequences. A putative promoter region, containing possible −10 (5′-TATAAT-3′) and −35 (5-TTGTGT-3′) sequences, was found to precede the *rfbA* coding sequence. An inverted repeat sequence (5′-TAATGACTTTGTCATTA-3′) followed by an AT-rich region downstream from the *rfbD* coding sequence could function as a rho-independent transcriptional terminator (Fig. 1A).

To assess the transcriptional organization of the *rfb* gene cluster, RNA was isolated from strain *L. lactis* MG1363 and used for Northern analysis. Internal fragments of the *rfbA* and *rfbD* genes were generated by PCR, labeled, and used as DNA probes. Both probes hybridized with a transcript that was approximately 3.8-kb long, and no other transcripts were detected. These results confirmed that the *rfb* genes are transcribed as a single 3.8-kb polycistronic mRNA, which probably starts at the postulated promoter upstream of *rfbA* and terminates at the putative terminator.

To ascertain whether the *L. lactis rfbACBD* operon encodes a functional dTDP-rhamnose biosynthesis pathway, these genes were cloned into pK194 (21). The resulting plasmid, pNZ4110, was transformed into *E. coli* Sϕ874 containing pPM2716, which is a derivative of pPM2213. pPM2213 contains the complete *S. flexneri* 4 *rfb* region and directs expression of *S. flexneri* O antigen production in *E. coli*. The difference

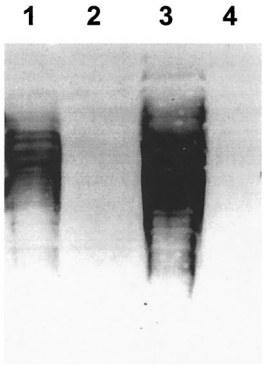

FIG. 2. Complementation of *S. flexneri rfbBDAC* by *L. lactis rfbACBD*. *E. coli* lysates were separated by SDS-PAGE and subjected to Western blot analysis by using rabbit antiserum raised against *S. flexneri* O antigen. Lane 1, *E. coli* Sϕ874 containing pPM2213; lane 2, *E. coli* Sϕ874 containing pPM2716; lane 3, *E. coli* Sϕ874 containing pPM2716 and pNZ4110; lane 4, *E. coli* Sϕ874 containing pPM2716 and pK194.

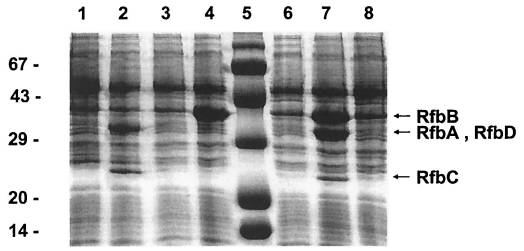

FIG. 3. Coomassie blue-stained SDS-PAGE gel of CE of *L. lactis* subsp. *cremoris* NZ9000 with pNZ8048 (lane 8), pNZ4116 (RfbAC) (lanes 1 and 2), pNZ4118 (RfbB) (lanes 3 and 4), or pNZ4117 (RfbACBD) (lanes 6 and 7) grown in the presence (lanes 2, 4, and 7) or in the absence (lanes 1, 3, and 6) of nisin. The arrows indicate the positions of the overproduced proteins.

that is introduced into pPM2716 when it is produced from pPM2213 is that the *S. flexneri rfbBDAC* genes are deleted (35). Cells of *E. coli* Sϕ874 harboring pPM2213, pPM2716, pPM2716 and pNZ4110, or pPM2716 and pK194 were subjected to Western blot analysis by using rabbit antiserum raised against *S. flexneri* O antigen (Fig. 2). Immunoreactive O antigen could be detected only in cells harboring pPM2213 or cells harboring pPM2716 and pNZ4110, indicating that the *L. lactis rfbACBD* operon was functional. Therefore, we concluded that the genes in the *rfbACBD* operon most likely encode a glucose-1-phosphate thymidylyl transferase, a dTDP-4-keto-L-rhamnose-3,5-epimerase, a dTDP-glucose-4,6-dehydratase, and a dTDP-L-rhamnose synthase, respectively.

Effects of *rfb* overexpression. To evaluate the control of Rfb activity in sugar nucleotide formation and EPS biosynthesis in *L. lactis*, pNZ8048 derivatives carrying the lactococcal *rfb* genes under control of the lactococcal *nisA* promoter were transformed into NZ9000, which allowed use of the nisin-controlled expression system (9, 26). Strain NZ9000 harboring the pNZ8048 derivatives carrying *rfbACBD* (pNZ4117), *rfbAC* (pNZ4116), or *rfbB* (pNZ4118) under control of the lactococcal *nisA* promoter (Table 1) were grown in the presence or absence of the inducer nisin, and CEs of these cultures were analyzed by SDS-PAGE (Fig. 3). Nisin-mediated induction of NZ9000 harboring pNZ4116 (*rfbAC*) resulted in the appearance of additional protein bands at molecular masses of approximately 32 and 22 kDa, which are the expected sizes of RfbA and RfbC, respectively (Fig. 3, lane 2). By analogy, nisin-mediated induction of strain NZ9000 harboring pNZ4118 (*rfbB*) resulted in the appearance of an additional protein band at a molecular mass of approximately 40 kDa, which is the expected size of RfbB (lane 4). Finally, nisin-mediated induction of NZ9000 harboring pNZ4117 (*rfbACBD*) resulted in the appearance of additional protein bands at molecular masses of approximately 40, 32, and 22 kDa (lane 7). The RfbA and RfbD proteins appeared to comigrate at an apparent molecular mass of 32 kDa, as deduced from the increase in the intensity of this band relative to the intensity of the RfbC (22-kDa) band when *rfbACBD* was expressed instead of *rfbAC*.

The RfbA enzyme activity level was determined in CEs of the wild-type strain (21 ± 0.5 U_{RfbA}) and the induced cultures of strains that overproduced RfbAC ($1{,}340 \pm 200$ U_{RfbA}) and RfbACBD (268 ± 28 U_{RfbA}); thus, the levels of overexpression of RfbA were 67- and 13-fold, respectively. The lower RfbA activity measured in the strain that overproduced RfbACBD than in the strain that overproduced RfbAC was probably due to the RfbA enzyme activity assay, in which RfbA and RfbB competed for the same substrate, dTDP-glucose. The level of RfbB enzyme activity increased 60- and 45-fold compared to the wild-type level (28 ± 1 U_{RfbB}) in the induced strains that overproduced RfbB ($1{,}570 \pm 150$ U_{RfbB}) and RfbACBD ($1{,}160 \pm 100$ U_{RfbB}), respectively. Moreover, the level of RfbBCD enzyme activity was 28-fold greater for the strain that overproduced RfbACBD (508 ± 70 U_{RfbBCD}) than for the wild type (18 ± 5 U_{RfbBCD}). Remarkably, in contrast to other bacteria, the lactococcal RfbD activity could be measured only by using NADH instead of NADPH in the RfbBCD enzymatic assay (data not shown), indicating that the lactococcal RfbD activity requires NADH as a cofactor. The increased enzyme activities demonstrate that controlled and functional overexpression of the *rfbACBD* genes was achieved.

The effect of Rfb activity on sugar nucleotide concentration and glucose 1-phosphate pool conversion was evaluated in the strains that overexpressed the *rfb* genes. We anticipated an effect on different sugar nucleotide levels since the substrate of the Rfb pathway, glucose 1-phosphate, is the central intermediate from which UDP-glucose and UDP-galactose are also formed, and modulation of the sugar nucleotide levels could affect growth. However, functional overexpression of the *rfb* genes resulted in a maximal growth rate that was not significantly different from that of the wild type (data not shown). Furthermore, overexpression of the *rfb* genes did not influence the absolute level of UDP-glucose or UDP-galactose (data not shown). In contrast, overexpression of *rfbAC* and *rfbACBD* resulted in a doubling of the intracellular dTDP-rhamnose levels (7.3 ± 0.6 μmol g of protein^{-1}, compared to 3.3 ± 0.5 μmol g of protein^{-1} in wild-type or noninduced cells), while the dTDP-glucose levels in these strains remained the same (data not shown). These results demonstrate that RfbAC activities exert control over the dTDP-rhamnose levels in wild-type cells.

To evaluate the effect of *rfb* overexpression on EPS production, the EPS-producing capacity was introduced into the *rfb*-overproducing strains since these strains do not natively produce EPS. This was done by transformation of these strains with pNZ4030, which contains the B40 *eps* gene cluster (56). Increased levels of Rfb activity had no effect on the level of EPS production (data not shown). Apparently, although increased levels of Rfb activity resulted in increased dTDP-rhamnose levels, the sugar nucleotide changes did not affect the growth rate or EPS production.

Effect of *rfb* mutation on growth and EPS production. To evaluate the effect of reduced Rfb activity on growth and EPS biosynthesis in *L. lactis*, we tried to inactivate the *rfb* genes (see Materials and Methods). Several attempts to disrupt the *rfbA* gene by single-crossover plasmid integration with pNZ4105 failed. Moreover, attempts to select mutants in which the *rfbB* gene was replaced by an erythromycin resistance gene cassette by direct double crossover, by using the nonreplicative plasmid pNZ4109, were unsuccessful. All erythromycin-resistant colonies obtained when the latter strategy was used appeared to be single-crossover integrants. Southern analysis of these strains revealed that in all cases the single-crossover plasmid integration had taken place downstream of the *rfbB* gene, leaving the *rfb* operon intact. These results strongly suggest that the *rfb* genes play an essential role in *L. lactis*. This suggestion was corroborated by the finding that the desired *rfbB*::*ery* strain could be obtained by transformation of pNZ4109 into *L. lactis* cells harboring an additional copy of the *rfbB* gene in *trans* in a replicating plasmid that harbors *rfbB* under control of the *nisA* promoter (pNZ4118). However, the strain obtained when this rescue strategy was used was still able to grow in the absence of nisin, suggesting that the RfbB activity level under these noninducing conditions was still sufficient to sustain growth, probably due to leakage of the *nisA* promoter in a high-copy system. Therefore, a conditional *rfbBD* mutant was constructed in which transcription of the chromosomal *rfbBD* genes was placed under control of the *nisA* promoter, while the *rfbAC* genes remained under control of the original *rfb* promoter (Fig. 1B). For this purpose, the nonreplicative plasmid pNZ4114 was transformed into strain NZ9000, and double-crossover mutants were selected based on tetracycline resistance and erythromycin sensitivity (see Materials and Methods). Southern blot analysis was used to confirm the anticipated genetic organization of the *rfb* locus, and a single mutant strain, designated *L. lactis* NZ4114, was used for further analysis. This nisin-controlled conditional *rfbBD* mutant did not grow in medium without nisin, while its growth in medium containing 1 ng of nisin ml^{-1} was similar to that observed for parental strain NZ9000, which confirmed that expression of the *rfb* genes is essential for growth of *L. lactis*.

To evaluate the effect of controlled limitation of *rfbBD* expression on growth, strain NZ4114 was grown overnight in media containing different levels of nisin and subsequently subcultured (2%, vol/vol) in medium lacking nisin, and the optical density was monitored over time (Fig. 4). Although the growth of each overnight culture was similar to the growth of the parent strain, nisin concentrations of 1.0, 0.5, and 0.3 ng ml^{-1} in the overnight cultures resulted in 22, 59, and 73% reductions in the specific growth rates of the subsequent cultures grown without nisin, respectively. Moreover, the stepwise

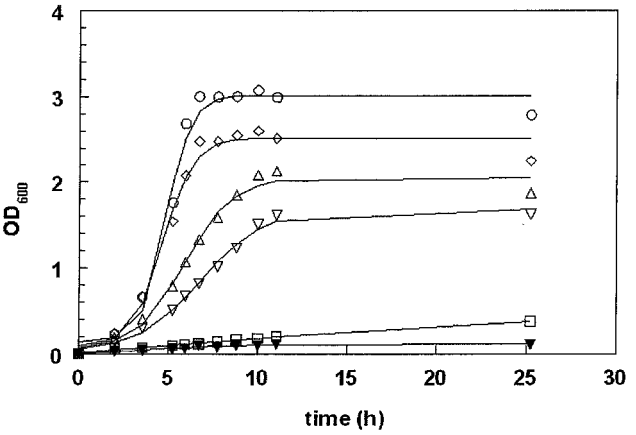

FIG. 4. Growth of *L. lactis* subsp. *cremoris* NZ4114 and dependence on nisin addition. Overnight cultures of NZ4114 grown in the presence of nisin at concentrations of 1 ng ml^{-1} (◇), 0.5 ng ml^{-1} (△), 0.3 ng ml^{-1} (▽), 0.05 ng ml^{-1} (□), and 0.01) ng ml^{-1} (▼) were subcultured in fresh medium without nisin, and growth was monitored for 25 h by measuring the optical density at 600 nm (OD_{600}). ○, control culture continuously grown in the presence of 1 ng of nisin ml^{-1}. The data are representative of the data from four independent experiments.

reductions in the final optical densities reached by these cultures corresponded to the levels of nisin induction used in the overnight cultures (Fig. 4). However, cells were not washed prior to subculturing, which could have resulted in delayed shutoff of dTDP-rhamnose synthesis and therefore a slower ceasing of growth. Finally, the dTDP-rhamnose levels in NZ4114 cells with reduced *rfbBD* expression appeared to be below the background level of the assay used, implying that the dTDP-rhamnose levels in these cells were significantly reduced (at least fivefold lower) compared to the level observed in wild-type cells (data not shown). These results validated the anticipated limitation of intracellular dTDP-rhamnose levels by controlled reduction of *rfbBD* expression and allowed evaluation of the effects of the limitations on the biosynthesis of rhamnose-containing sugar polymers.

The sugar compositions of the cell wall polysaccharides produced by RfbBD-limited cells appeared to be the same as those of wild-type cells (Table 2), suggesting that incorporation of rhamnose into the lactococcal cell wall is essential for growth. Besides cell wall polysaccharides (Table 2), rhamnose is known to be a constituent of several lactococcal EPS. To investigate the effect of dTDP-rhamnose limitation on EPS production (Table 3), the EPS-producing capacity was introduced into strain NZ4114, which is not a native EPS-producing strain, by transformation with pNZ4030 (56). No differences in growth rate and EPS yield between the mutant and the parental strain were observed after 24 h of fermentation when cells were grown in the presence of nisin (Table 3). However, the EPS production by the mutant strain was reduced to approximately 5% of the production by the wild type when the cells were grown under *rfbBD* expression-limiting conditions. These results primarily suggest that *rfb* expression is essential for both production of B40-EPS and growth. However, the observation that the final culture density decreased stepwise as the nisin concentration decreased while the levels of EPS production

TABLE 2. Sugar compositions of the polysaccharide fraction of cell walls of strain *L. lactis* subsp. *cremoris* NZ4114 and the parental strain grown in the presence or absence of nisin

Strain	Nisin concn (ng ml^{-1})		Sugar composition of polysaccharide (mol%)		
	Preculture	Subculture	Rhamnose	Galactose	Glucose
NZ9000	0	0	51.3 ± 0.1	15.5 ± 0.2	33.1 ± 0.0
NZ4114	1	1	53.5 ± 1.2	14.5 ± 1.3	32.4 ± 1.6
	1	0	54.1 ± 2.1	14.4 ± 2.3	31.9 ± 0.8
	0.5	0	48.1 ± 1.5	18.2 ± 0.2	34.7 ± 2.2
	0.3	0	48.2 ± 1.1	17.1 ± 0.0	34.7 ± 1.1
	0	0	NG[a]	NG	NG

[a] NG, no growth.

were reduced equally in all RfbBD-limited cultures indicates that EPS production and growth are not coupled. Interestingly, uncoupling of EPS production and growth has been reported previously for *L. lactis* strain NIZO B40 (32). Moreover, these results suggest that the precursor dTDP-rhamnose is preferentially used for the formation of cell wall polysaccharides rather than for EPS production, which may be due to differences in the kinetic properties of the different enzymes involved in the two pathways.

Characterization of EPS produced by the *rfb* mutant. The EPS produced by strain NZ4114 were analyzed by static light scattering after size exclusion chromatography. Estimation of the molecular masses revealed that the EPS produced by strain NZ4114 had an average molecular mass that was fourfold larger than that of the EPS produced by the parental strain, NZ9000 (Table 3; Fig. 5A). Furthermore, static light scattering measurements generated an average R_g that was twofold smaller than that measured for EPS produced by strain NZ9000 harboring pNZ4030 (Fig. 5B). Moreover, the viscosifying properties of the mutant EPS in chemically defined medium, which could be calculated from the molecular mass and the R_g (51), was drastically decreased (Table 3). In addition, monomer sugar analysis after hydrolysis of the purified EPS revealed that the EPS produced by the RfbBD conditional mutant strain consisted of the monosaccharides glucose, galactose, and rhamnose at a molar ratio of 1:0.3:0.2, which is clearly different from the ratio in the parental strain (1:0.6:0.4) (Table 3). These results indicate that the RfbBD conditional mutant produced EPS with an altered structure as a result of an at least partially altered repeating unit. Nevertheless, the polymerization and export machinery could still recognize and handle this altered repeating unit. Moreover, since the molecular mass was increased, the polymerization and export machinery may even have extended the polymer to a greater length. Finally, the decreased R_g indicated that there was a more compactly folded structure that filled less space, which probably was the consequence of the altered structure.

DISCUSSION

In this paper we describe identification and functional analysis of the chromosomal *L. lactis rfb* MG1363 operon that is involved in dTDP-rhamnose biosynthesis. Sequence analysis led to identification of four *rfb* genes whose predicted gene products exhibit high levels of homology with proteins involved in the biosynthesis of dTDP-rhamnose. Evidence for the *rfb* functionality of the *L. lactis rfb* genes was obtained by overproduction of the Rfb proteins, which led to increased Rfb activities. Moreover, expression of the *rfb* genes in *E. coli* complemented an O antigen production mutation that deleted the *S. flexneri* Rfb homologues.

In both gram-negative and gram-positive bacteria the *rfb* genes are often genetically linked to genes involved in CPS or O antigen production. Remarkably, even in *S. pneumoniae* serotypes that produce CPS that do not contain rhamnose, the *rfb* genes are linked to the *cps* locus (39). In contrast, analysis of the chromosomal localization of the *rfb* genes in the *L. lactis* IL-1403 genome sequence revealed that the lactococcal *rfb* gene cluster is not genetically linked to genes encoding related functions (3). This resembles the situation in *S. mutans*, although in this and various other streptococci the *rfbD* ortholog was found to be distant from the *rfbA*, *rfbB*, and *rfbC* genes (49, 50).

Functional overexpression of the *rfbACBD* or *rfbAC* genes

TABLE 3. EPS characteristics of *L. lactis* subsp. *cremoris* NZ4114 and parental strain NZ9000 harboring the EPS plasmid pNZ4030 grown in the presence or absence of nisin

Strain	Nisin concn (ng ml^{-1})		EPS concn (mg liter^{-1})	EPS concn (mg liter^{-1} OD_{600}^{-1})[a,b]	EPS Glc/Gal/Rha ratio[a]	Mol mass (10^{-6} g mol^{-1})[a]	R_g (nm)[a]	η (10^{-3} m^3 kg^{-1})
	Preculture	Subculture						
NZ9000	0	0	144 ± 1	55 ± 1	1:0.6:0.4	0.43 ± 0.01	44 ± 0.3	60 ± 1
NZ4114	1	1	114 ± 5	45 ± 1	1:0.6:0.4	0.42 ± 0.01	44 ± 0.0	58 ± 1
	1	0	7 ± 1	7 ± 1	1:0.3:0.2	1.7 ± 0.3	28 ± 0.6	5.2 ± 1
	0.5	0	6 ± 1	6 ± 1	1:0.4:0.2	1.9 ± 0.3	28 ± 0.7	3.6 ± 1
	0.3	0	8 ± 1	8 ± 1	1:0.3:0.1	1.2 ± 0.4	30 ± 0.2	3.2 ± 1
	0	0	NG[c]	NG	NG	NG	NG	NG

[a] The values are averages based on at least two independent experiments.
[b] OD_{600}, optical density at 600 nm.
[c] NG, no growth.

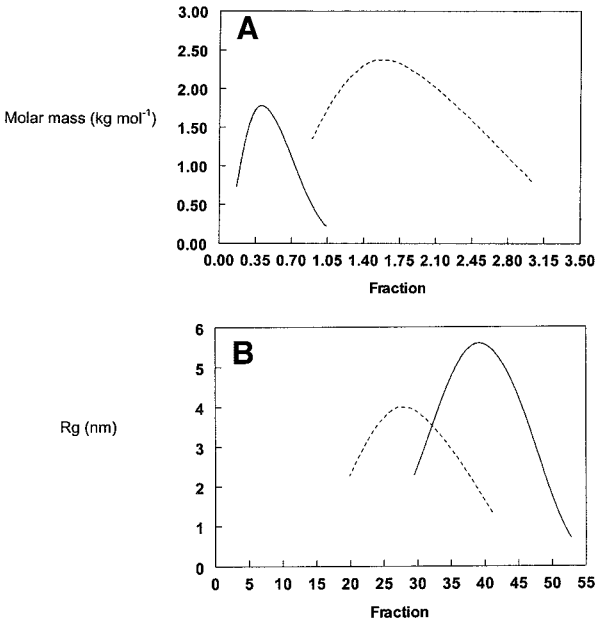

FIG. 5. Size distribution of molar mass (A) and R_g (B) of the EPS produced by strain NZ9000 harboring pNZ4030 (solid line) and the altered EPS produced by strain NZ4114 harboring pNZ4030 precultured in medium containing 1 ng of nisin ml^{-1} and subcultured in medium lacking nisin (dashed line). In panel A the fraction numbers should be multiplied by 10^6.

led to increased levels of Rfb proteins and a twofold increase in the dTDP-rhamnose level. However, the increased Rfb enzyme activities did not result in production of more B40-EPS. Similarly, GalU overproduction resulted in increased UDP-glucose and UDP-galactose levels but did not affect the level of B40-EPS production (2). These results indicate that there is no correlation between the levels of individual sugar nucleotides and the level of EPS in *L. lactis* harboring pNZ4000 derivatives. This apparently contradicts several reports that showed that there was a correlation between the activity level of enzymes involved in sugar nucleotide biosynthesis and the level of EPS produced (17, 28). However, this correlation seems to depend on the type of polysaccharide produced, as was clearly shown for GalU activity in *L. lactis* (2, 17). It is very possible that simultaneous increases in UDP-glucose, UDP-galactose, and dTDP-rhamnose levels could positively affect the B40-EPS level, since the repeating unit of this EPS contains two glucose moieties, two galactose moieties, and a rhamnose moiety. Alternatively, the level of B40-EPS produced could also be controlled by the activity of the specific EPS biosynthesis machinery encoded by the EPS plasmid rather than by the level of sugar nucleotides. This hypothesis is supported by the observation that overexpression of the priming glycosyltransferase *epsD* gene in *L. lactis* resulted in increased levels of B40-EPS (56, 57).

A nisin-controlled conditional *rfbBD* mutant was constructed by introduction of the *nisA* promoter upstream of the *rfbB* gene in the chromosome of *L. lactis*. This mutant, *L. lactis* NZ4114, was not able to grow in the absence of nisin, indicating that the *rfbB* and/or *rfbD* gene is essential for *L. lactis* growth. This finding explains our lack of success in construction of an *L. lactis rfbB* mutant by using conventional knockout strategies. In *L. lactis* NZ4114, lowering the nisin concentration resulted in a reduction in the growth rate and a lower final optical density. However, the sugar composition of the polysaccharide fraction of the cell wall in this strain appeared to be unaffected. Remarkably, although rhamnose is a major component of cell polysaccharides in *L. lactis*, as well as in *S. mutans* (45), inactivation of any of the four *S. mutans rml* genes led to viable cells lacking rhamnose in the cell wall polysaccharide (49, 50). In contrast to these findings for *S. mutans*, our results suggest that the rhamnose moieties in the lactococcal cell wall polysaccharides are essential for cell wall integrity in *L. lactis*. Therefore, limitation of dTDP-rhamnose precursor levels could interfere with wild-type cell wall polysaccharide production and result in a decrease in the growth of *L. lactis*. We used the nisin-controlled expression system to construct conditional mutations in essential genes like the *rfbBD* genes, which allowed us to study the corresponding mutant phenotypes. A similar strategy has recently been described for the (F_0F_1)-H^+-ATPase complex in *L. lactis* (24). However, since this expression system can be implemented in many other gram-positive hosts (13, 22), this approach has potential to be used in other bacteria.

The level of EPS production by the conditional *rfbBD* mutant *L. lactis* NZ4114 was only 5% of the parental level of EPS production when cells were grown under nisin limitation conditions. This low level of production could be complemented by addition of nisin to the medium. The effect of a lack of *rfbBD* expression on both EPS production and growth in *L. lactis* can probably be explained by the hypothesis that dTDP-rhamnose plays a crucial role in cell wall synthesis and an important role in the biosynthesis of the rhamnose-containing EPS. The enzymes in these pathways differ, and so may their kinetic properties, and we speculate that different affinities for dTDP-rhamnose may well explain the different effects on these processes. Data supporting this suggestion include recent observations reported by Cartee et al. (5) for reduction of capsule synthesis by *S. pneumoniae* due to reduction of the sugar nucleotide concentration. The sugars of the EPS produced by NZ4114 cells grown under RfbBD-limiting conditions were glucose, galactose, and rhamnose at a ratio that is different from the ratio for the polymer produced by the parental strain. These results indicate that the RfbBD conditional mutant produces EPS with an altered composition as the result of an at least partially altered repeating unit. This implies that it might be possible to change the EPS composition by reducing the availability of EPS precursors via inactivation of specific precursor-forming enzymes. Although the yield is limited, the EPS polymerization and export machinery is still capable of recognizing and processing EPS with an altered repeating unit and is apparently not exclusively specific for a single repeating unit. This is corroborated by the finding that expression of the *Streptococcus thermophilus eps* gene cluster in *L. lactis* resulted in production of very small amounts of EPS with a repeating unit that differs from the native structure due to a lack of lactococcal UDP-*N*-acetylglucosamine C_4-epimerase activity leading to incorporation of a galactose moiety instead of a GalNac moiety in the mutant EPS (47). However, it remains to be established what determines the low level of production (6 to 10 mg liter^{-1}) of EPS in these lactococci.

The global sugar of the altered EPS produced by strain NZ4114 includes fewer rhamnose and galactose moieties and was shown to have a fourfold-greater molecular mass than the EPS produced by the parental strain. Since the distributions of the molecular masses of both the B40-EPS and the altered EPS follow a typical symmetric Gaussian curve (Fig. 5A), we concluded that the altered EPS are homogeneous polymers and do not represent a mixture of altered and native EPS polymers. Besides the fourfold-greater molecular mass, the altered EPS had a twofold-smaller R_g than the native EPS. This finding suggests that there is a drastic decrease in the viscosifying properties of these EPS compared to the properties of wild-type B40-EPS (52). Moreover, the decreased R_g indicates that folding of the backbone of the altered EPS is much less hindered by side chains, resulting in greater chain flexibility and a more compactly folded structure. Hence, it is likely that the increased molecular mass of the altered EPS is due to increased chain length rather than an increase in the number or size of the side chains. Furthermore, these results suggest that the repeating unit of the altered EPS is a modification of that of the native EPS and partially lacks its side chains consisting of rhamnose and galactose phosphate. Finally, the production by strain NZ4114 of an EPS that is longer and more compact than the native EPS suggests that the chain length determination mechanism is dependent on the three-dimensional conformation of the polymer rather than on only the length of the chain itself.

Evaluation of the dTDP-rhamnose biosynthesis pathway described here allowed assessment of the role of the *rfbACBD* genes in *L. lactis* by overexpression and disruption studies of these genes. We could significantly influence the level of dTDP-rhamnose, which is a precursor for cell wall polysaccharides, as well as for EPS biosynthesis in *L. lactis*. We were also able to influence the levels of EPS production and even the repeating unit sugar composition by using a conditional *rfbBD* mutant. Various workers have previously established that it is possible to modulate polysaccharide biosynthesis by engineering at the level of specific *eps* genes (for a review see reference 57). To our knowledge, this is the first report showing that modulation of the household enzyme levels can lead to production of EPS with an altered composition. These results enlarge the knowledge base required for efficient targeting of bottlenecks in EPS biosynthesis and provide new opportunities for creating structural diversity by constructing polysaccharides with novel properties.

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