Identification and Molecular Characterization of the Chromosomal Exopolysaccharide Biosynthesis Gene Cluster from *Lactococcus lactis* subsp. *cremoris* SMQ-461

N. Dabour^{1,2} and G. LaPointe^{1*}

STELA Dairy Research Centre and Institute for Nutraceuticals and Functional Foods, Université Laval, Québec, QC, Canada G1K 7P4,¹ and Department of Dairy Science and Technology, Faculty of Agriculture, University of Alexandria, Alexandria, Egypt²

Received 9 March 2005/Accepted 20 July 2005

The exopolysaccharide (EPS) capsule-forming strain SMQ-461 of *Lactococcus lactis* subsp. *cremoris*, isolated from raw milk, produces EPS with an apparent molecular mass of $>1.6 \times 10^6$ Da. The EPS biosynthetic genes are located on the chromosome in a 13.2-kb region consisting of 15 open reading frames. This region is flanked by three IS1077-related *tnp* genes (*L. lactis*) at the 5' end and *orfY*, along with an IS981-related *tnp* gene, at the 3' end. The *eps* genes are organized in specific regions involved in regulation, chain length determination, biosynthesis of the repeat unit, polymerization, and export. Three (*epsGIK*) of the six predicted glycosyltransferase gene products showed low amino acid similarity with known glycosyltransferases. The structure of the repeat unit could thus be different from those known to date for *Lactococcus*. Reverse transcription-PCR analysis revealed that the *eps* locus is transcribed as a single mRNA. The function of the *eps* gene cluster was confirmed by disrupting the priming glycosyltransferase gene (*epsD*) in *Lactococcus cremoris* SMQ-461, generating non-EPS-producing reversible mutants. This is the first report of a chromosomal location for EPS genetic elements in *Lactococcus cremoris*, with novel glycosyltransferases not encountered before in lactic acid bacteria.

Lactic acid bacteria (LAB) are widely used in fermented dairy products, mainly for lactic acid formation but also for the production of minor flavor and preservation components. Some LAB are also able to produce exopolysaccharides (EPS), which are either excreted in the growth medium as slime (ropy form) or remain attached to the bacterial cell wall forming capsular EPS (6, 32). In the dairy industry, EPS-producing LAB, including the genera *Streptococcus*, *Lactobacillus*, and *Lactococcus*, are used in situ to improve the textural characteristics of fermented dairy products, especially low-fat yoghurt and cheese. LAB are food-grade bacteria that can produce a wide variety of structurally different EPS with potential uses for new applications, for example, in replacement of polysaccharides such as gellan, pullulan, xanthan, and bacterial alginates that are presently produced by non-food-grade bacteria (12, 25, 48).

EPS-producing LAB, including strains of *Lactococcus* spp., have been shown to express at least two distinct phenotypic forms of EPS, either ropy and/or capsular forms (32). Moreover, they produce EPS with considerable diversity in structure and composition (14, 54, 55, 58, 64). This diversity in EPS composition indicates that LAB contain a vast pool of glycosyltransferases with a wide range of sugar and linkage specificities. EPS-producing lactococci in particular have received growing attention in recent years, especially for the analysis of genes encoding EPS biosynthesis. The first lactococcal *eps* locus identified was that of *Lactococcus lactis* subsp. *cremoris*

NIZO B40; it comprises 14 plasmid-encoded genes (56). Since then, partial sequences of *eps* gene clusters have been identified in *L. lactis* subsp. *cremoris* NIZO B891 and NIZO B35 strains (58). Recently, a large cluster consisting of 23 putative EPS biosynthetic determinants has been identified on plasmid pCI658 in *L. lactis* subsp. *cremoris* HO2 (20).

Increased sequence information about the *eps* gene clusters of lactococci is important to identify strains that are able to produce novel EPS. The study of a new EPS is a fastidious process, requiring extraction, purification, and chemical analyses for determining the sugar composition and structure of the EPS produced (7, 14, 58). Furthermore, *Lactococcus* strains generally produce a rather low quantity of EPS (15, 16, 25, 61). Therefore, the identification of new wild-type *Lactococcus* strains producing unique EPS polymers is a considerable challenge that would benefit from rapid screening methods of the genetic elements involved. Restriction fragment length polymorphism has been used to classify lactococcal EPS-producing strains in relation to the monosaccharide composition of the repeat unit of the EPS into three major groups, along with a minor unique group (58). Recently, Deveau et al. (14) applied restriction fragment length polymorphism using two different enzymes (AclI and HindII) for grouping seven EPS-producing lactococcal strains. A novel group of EPS-producing lactococci was identified, including *L. lactis* subsp. *cremoris* SMQ-461. EPS biosynthesis by this strain has been shown to have an impact on reduced-fat Cheddar cheese production and physical characteristics, notably by increasing moisture retention and cheese yield (13).

The present study reveals the quantity and molecular mass of the EPS produced by *L. lactis* subsp. *cremoris* strain

* Corresponding author. Mailing address: STELA Dairy Research Centre, Room 1316, Pavillon Paul-Comtois, Université Laval, Québec, QC, Canada G1K 7P4. Phone: (418) 656-2131, ext. 3100. Fax: (418) 656-3353. E-mail: gisele.lapointe@fsaa.ulaval.ca.

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers used in the present study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence (5' to 3') ^a	Reference, source, or target
Strains		
<i>L. lactis</i> subsp. <i>cremoris</i> SMQ-461	Raw milk isolate, EPS ⁺ ; rosy white colonies on RRM17 medium	15
<i>L. lactis</i> subsp. <i>cremoris</i> ND461M	SMQ-461 derivative with 4.5-kb pND9 integrated into the chromosome; colonies with red phenotype on RRM17 medium with Em ^s	This study
<i>L. lactis</i> subsp. <i>cremoris</i> ND461R	ND461M derivative with pND9 excised from the chromosome; colonies reverted to wild-type rosy white on RRM17 medium; Em ^s	This study
<i>L. lactis</i> subsp. <i>cremoris</i> ND461D1	ND461M derivative with pND9 excised from the chromosome; red colonies on RRM17 medium; Em ^s	This study
<i>L. lactis</i> subsp. <i>cremoris</i> ND461D2	ND461M derivative with pND9 excised from the chromosome; red colonies on RRM17 medium; Em ^s	This study
<i>L. lactis</i> subsp. <i>cremoris</i> IL1403	Plasmid free	10
<i>E. coli</i> JM109	Cloning host (F' <i>proAB lacIqZΔM15</i>)	Promega, Inc.
<i>E. coli</i> TOP10	Cloning host (F' <i>mcrΔ Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK resL(Str^r) endA1 nupG</i>)	Invitrogen Life Technologies
Plasmids		
pCR4-TOPO	PCR cloning vector, Km ^r Am ^r	Invitrogen
pGEM-T Easy	PCR cloning vector, Am ^r	Invitrogen
pGh9	Thermosensitive shuttle vector, 4.6 kb; Em ^r	38
pND2	2.0-kb fragment (<i>epsBCD</i>) cloned into the EcoRI site of pUC18	This study
pND4	4.3-kb fragment (<i>imp</i> and <i>epsRXA</i>) cloned into pCR4-Topo; Am ^r	This study
pND5	3.5-kb fragment (<i>epsABCD</i>) cloned into pCR4-Topo; Am ^r	This study
pND6	3.5-kb fragment (<i>epsML</i>) cloned into pCR4-Topo; Am ^r	This study
pND7	9.7-kb fragment (<i>epsD</i> to <i>L</i>) cloned into pGEM-T; Am ^r	This study
pND8	Am ^r ; pCR4-Topo containing <i>epsDΔ</i>	This study
pND9	Em ^r ; 4.5 kb; pGh9 with <i>epsDΔ</i> cloned into the PstI-XhoI site	This study
Primers		
HD2	CGTACGATTTCGTACGACCAT	14; PCR for <i>epsB</i>
HD15	TGACCAGTGACACTTGAAGC	14; PCR for <i>epsB</i>
SMQ461ER	AATCCCTCCTAGATTAATCGC	PCR for <i>epsE</i>
AB40F	TTAAATGCTTCGGGGAATAAGGTTTGCTAGATTA	GenomeWalker-PCR
AB40FN	TGGAGAAGAAATGCAGGAAACACAGGAACAGACGA	GenomeWalker-PCR
AB40R	TCGTCTGTTCTGTGTTTCCTGCATTTCTTCTCCA	GenomeWalker-PCR
AB40RN	TAATCTAGCCAAACCTTATTCCTCGAAGCATTTAA	GenomeWalker-PCR
LB40R	TATTTTCATCACAATAATAATCCGGTACGGCTCGATCATCTT	GenomeWalker-PCR
LB40RN	GACTAGCAACAATCGTTTTACCATTTGACAGATAGT	GenomeWalker-PCR
wccf	ttctgcagTAACAGCTTCGAGTGTCACTGGTCA	PCR <i>epsDΔ</i>
wdnr	ctcgagGGCATGGTAGGCAGCTTTAATTTCTGGA	PCR <i>epsDΔ</i>
wdef	gctgctaccatgccGCGCTCTCTTACTTACTCATGTGT	PCR <i>epsDΔ</i>
wecr	ctcgagGGCTACCGCAGCACCACCTCG	PCR <i>epsDΔ</i>
SMQ461DF	CAGCTTTGAAGATTTGTATTGA	RT-PCR for <i>epsF</i>
SMQ461FR	GTGAGTTCCAACAGTTACAAAA	RT-PCR for <i>epsF</i>
SMQ461GR	CTTCACCAATATGTTAACTTC	RT-PCR for <i>epsG</i>
SMQ461HF	GGTATGATGTCAACTTTTCTT	RT-PCR for <i>epsH</i>
SMQ461HR	TGATCCTCCCATGACATTTTTT	RT-PCR for <i>epsH</i>
SMQ461JF	AGTGAATTAATAGGCAAGATA	RT-PCR for <i>epsI</i>
SMQ461JR	GACGCAAAATATCTTAATCATCA	RT-PCR for <i>epsI</i>
SMQ461MF	TATTGCAAGTATTTTATAGGAGC	RT-PCR for <i>epsM</i>
SMQ461MR	TAGTTCTAGAAATATATGGTGC	RT-PCR for <i>epsM</i>
SMQ461LR	CGAGCTGTTTGTGTTTTGTATAA	RT-PCR for <i>epsL</i>
SMQ461YR	CAACTGGTAAAAATAATTCT	RT-PCR for <i>orfY</i>

^a Restriction sites of primers are indicated in lowercase.

SMQ-461, as well as the organization and transcription of the novel chromosomal *eps* gene cluster. The function of the *eps* gene cluster in EPS biosynthesis is experimentally demonstrated by disrupting the priming glycosyltransferase gene (*epsD*), generating reversible non-EPS-producing mutants.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were maintained in 20% glycerol stock at -80°C .

L. lactis strains were grown at 30°C in M17 broth (Quelab, Montreal, Quebec, Canada) supplemented with 0.5% glucose (GM17) or lactose (LM17), unless specified otherwise. Media were solidified with 1.5% agar (Quelab). To distinguish between EPS-producing and nonproducing (mutant) cells, GM17 agar medium containing 0.08% ruthenium red (RRM17) was used. A stock solution of ruthenium red (Sigma Chemical Co, St. Louis, MO) at 10% (wt/vol) in water was sterilized through a 0.45- μm filter (Sartorius AG, Göttingen, Germany), and an appropriate volume was added to the molten GM17 agar just prior to pouring it into petri plates. The presence or absence of capsule on cells isolated from RRM17 was determined using India ink and crystal violet counterstaining according to the capsule staining method described by Collins and Lyne (11). Strains of *Escherichia coli* were routinely cultured in Luria-Bertani (LB; Quelab)

broth (40) and incubated at 37°C with aeration. When required, antibiotics were added at the following concentrations: chloramphenicol hydrochloride or erythromycin (Sigma) for *L. lactis* strains, 5 µg ml⁻¹, and ampicillin for *E. coli* strains, 100 µg ml⁻¹.

Exopolysaccharide extraction and purification. An overnight culture of *L. lactis* subsp. *cremoris* SMQ-461 in GM17 broth was standardized at an optical density at 650 nm of 0.5, used to inoculate 500 ml of sterilized skim milk (11% [wt/wt] total solids) at a level of 2% (vol/vol), and then incubated at 30°C or 25°C for 24 or 48 h. The EPS was extracted following the method of Cerning et al. (8), modified as follows. The fermented skim milk cultures were heated at 90°C for 15 min to inactivate enzymes potentially able to degrade the EPS polymer. The pH of milk samples was adjusted to 7.5 with 1 M NaOH and digested by pronase E (EC 3.4.24.31; Sigma) in sterilized distilled water at a final concentration of 1/50 (wt/wt) for 20 h at 40°C with shaking in the presence of 1/2,000 (wt/vol) merthiolate (Sigma) to inhibit bacterial growth. The bacterial cells were removed by centrifugation at 12,000 × g for 15 min at 4°C, and trichloroacetic acid was added to the supernatant at a final concentration of 12% (wt/vol). Precipitated proteins were removed by centrifugation at 12,000 × g for 20 min at 4°C. The EPS in the supernatant was precipitated with 1 volume of acetone. After being allowed to stand overnight at 4°C, the EPS was collected by centrifugation at 16,000 × g for 20 min at 4°C. The precipitated EPS was dissolved in deionized water, dialyzed for 5 days against deionized water at 4°C (two water changes per day), and freeze-dried. To purify the EPS, the lyophilized samples were dissolved in water, extracted two times with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 [vol/vol/vol]), and precipitated overnight with an equal volume of acetone. The precipitated EPS was dissolved in water, dialyzed, and lyophilized as described above. The EPS concentration in glucose equivalents was determined by the phenol-sulfuric acid method described by Dubois et al. (19). D,L-Glucose (Sigma) was used as a standard. For LM17 cultures, the same methods of EPS extraction and purification were applied, without the pronase E digestion step.

Size exclusion high-performance liquid chromatography (HPLC) analysis. The molecular mass of EPS was determined by gel permeation chromatography with a Waters HPLC system (600 controller with Waters TM 600 pump; Waters Limited, Mississauga, Ontario, Canada) at 25°C. Two columns were connected in a series: TSK 4000SW (600 mm by 7.5 mm) with a silica base (Beckman Coulter, Mississauga, Ontario, Canada) and TSK Gel G4000PWXL (300 mm by 7.8 mm) with a polymer base (Tosohas Keystone, Montgomeryville, PA). The mobile phase used was 0.1 M ammonium acetate (Sigma) at pH 7.2 with a flow rate of 0.5 ml min⁻¹. A volume of 50 µl of EPS sample (concentration, 1 mg ml⁻¹) was injected. The detection was performed with the SEDEX model 75 light-scattering detector (Scientific Products & Equipment, Concord, Ontario, Canada) at 45°C. The results were analyzed by HPLC system Millennium 32 software, version 3.25, with comparison to a dextran standard series of 5 × 10³, 1 × 10⁴, 2 × 10⁴, 5 × 10⁴, 1 × 10⁵, 2 × 10⁵, 4 × 10⁵, 8 × 10⁵, and 1.6 × 10⁶ Da (Showa Denko America, Inc., New York), which were migrated simultaneously.

DNA isolation and manipulation. Lactococcal genomic DNA was extracted as described by Hill et al. (26), with some modifications. Cells from 2 ml of late-log-phase culture were harvested; suspended in 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA (pH 8.0), 6.7% sucrose, and 50 µg of lysozyme (Sigma); and incubated 20 min on ice. Lysis was achieved by addition of 50 µl of 10% sodium dodecyl sulfate, after which the lysate was treated with 20 µl of a 20-mg ml⁻¹ stock solution of proteinase K (Sigma) at 65°C for 20 min. Two extractions with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) were carried out, followed by chloroform extraction. The DNA was precipitated with 2 volumes of 95% ethanol and a one-tenth volume of 3 M potassium acetate (pH 4.8) at -20°C. The pellet was washed twice with ethanol (70% [vol/vol]) and solubilized in 100 µl of 10 mM Tris-HCl (pH 8.0).

Plasmid extraction from lactococcal strains was performed according to O'Sullivan and Klaenhammer (44). Small-scale isolation of *E. coli* plasmids was performed with QIAGEN (Mississauga, Ontario, Canada) columns as instructed by the manufacturer. Restriction endonucleases (Roche Diagnostics, Laval, Québec, Québec, Canada) were used as recommended by the manufacturer. DNA hybridization was performed by transferring plasmid and chromosomal DNA onto positively charged nylon membranes (Roche) by capillary blotting (50). Probes were constructed by labeling with the Dig High-Prime labeling kit (Roche), the PstI/XhoI fragment of pGh9, and the internal PCR fragment of *epsB*, which was generated using primers HD2 and HD15 (Table 1). Prehybridization, hybridization, washes, and detection by chemiluminescence were performed as suggested by the manufacturer (Roche).

Chromosomal walking strategy for isolating *eps* genes. PCR was performed with primary and nested primers designed from the highly conserved regions (*epsA* and *epsL*) of lactococcal strains NIZO B40 and NIZO B891 (Table 1). Amplifications were obtained with the Universal Genome Walker kit (Clontech,

Inc., Palo Alto, CA) and Expand High Fidelity polymerase (Roche Diagnostics GmbH-Boehringer Mannheim, Mannheim, Germany), under standard conditions as recommended by the manufacturer. Reaction specificity was controlled using the first PCR amplifications as template DNA with nested primers. Nested amplifications which gave fragment sizes of 3 to 4 kb were purified with Microcon-PCR columns (Millipore, Microcon, and Milli-Q), ligated into pCR4-TOPO plasmid (Invitrogen Life Technologies, Carlsbad, Calif.) or pGEM-T Easy (Promega, Madison, Wisc.), and then transformed into *E. coli* strain TOP10 or JM109.

Nucleotide sequence analysis. Automated DNA sequence analysis was carried out on both strands by the DNA sequencing service of Laval University (Life and Health Sciences Pavillion, Québec, Québec, Canada) with an ABI Prism 3100 apparatus. Sequence data were assembled and analyzed with the Genetics Computer Group (Wisconsin) package 10 (Accelrys, San Diego, CA). Database similarity searches (46) were performed with the FASTA network service at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), National Institutes of Health, Bethesda, MD (2). The BLASTX program (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to translate the sequence of both DNA strands in all six open reading frames (ORFs) and to conduct similarity searches of the nucleotide and protein databases. Hydrophobicity plots were generated with the TMPred program at ch.EMBLnet.org (http://www.ch.emblnet.org/software/TMPRED_form.html), which allows a prediction of membrane-spanning regions and their orientation.

Transcription analysis of *eps* genes. RNA was extracted from 3-ml cultures of *L. lactis* subsp. *cremoris* SMQ-461 after 6, 12, and 24 h of incubation, corresponding to early, late exponential, and stationary growth phases, respectively, with the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. The extracted RNA was treated with RNase-free DNaseI (QIAGEN) at 25°C for 40 min, followed by a second purification step. Reverse transcription-PCR (RT-PCR) was performed on 100 ng, 1 µg, and 2 µg of RNA with primers (Table 1) derived from the *L. lactis* subsp. *cremoris* SMQ-461 *eps* cluster to cover the intergenic spaces of the *eps* locus. As controls, each fragment was amplified with the same primers by using chromosomal DNA from strain SMQ-461 as a template, and negative controls were performed under the same reaction conditions without the reverse transcription stage.

Insertional inactivation of *epsD*. For *epsD* inactivation, primers wccf and wdnr (Table 1) were used to amplify by PCR the first 330 bp of *epsD* and 302 bp of the adjacent upstream flanking sequence with the chromosomal DNA of strain SMQ-461 as a template. The wdcf and wecr primers were used to amplify the last 183 bp of *epsD* and 277 bp of downstream flanking DNA. The two purified PCR products were diluted 1/10, mixed, and amplified with primers wccf and wecr to create a final PCR product carrying a 165-bp internal deletion of *epsD* by overlap PCR. The resultant 1,092-bp PCR fragment was cloned into pCR4-TOPO (Invitrogen). After PstI-XhoI digestion, the fragment was ligated into the temperature-sensitive shuttle vector pGh9, also digested with PstI-XhoI, replacing the ISS1 sequence. This construct was designated pND9, which was verified by PstI-XhoI digestion and PCR using wccf and wecr as primers.

The pND9 construct was transformed into *L. lactis* subsp. *lactis* IL-1403 to produce supercoiled plasmid to facilitate its transfer to *L. lactis* subsp. *cremoris* SMQ-461 by electroporation. Transformants were selected by growth at 30°C on GM17 agar medium with 5 µg ml⁻¹ erythromycin (Sigma). A single erythromycin-resistant colony was then inoculated into liquid culture (GM17) containing erythromycin (5 µg ml⁻¹). After overnight growth, the saturated culture was diluted 100 fold in GM17 broth medium without erythromycin and incubated 150 min at 28°C to allow exponential growth to resume. The same culture was shifted to 38°C for 150 min to inhibit plasmid replication and allow integration. Serial dilutions were then performed with 0.1% (wt/vol) peptone water and plated on GM17 agar with added erythromycin (to detect the integrants) and on GM17 agar without antibiotic (to determine the viable cell count) with incubation at 38°C. Dilutions were also plated on RRM17 agar with erythromycin to select red colonies, indicating integration into the *eps* locus affecting the phenotype. In all cases, plates were incubated at 38°C for 24 h. To allow excision of the plasmid from the chromosome via a second recombination event, leaving either the deletion copy or reconstituting the wild-type genotype, integrants were serially passaged at least five times in GM17 broth at 30°C in the absence of erythromycin and plated on RRM17 after each passage. Erythromycin-sensitive colonies were screened for the expected deletion by PCR amplification using primers (HD2 and SMQ461ER) that anneal in the *eps* locus (*epsB* and *epsE*) outside the sequence region included in the pND9 construct.

Nucleotide sequence accession number. The sequence obtained in this study is available under GenBank accession no. AY741550.

FIG. 1. Phase contrast microscopy of India ink-stained cells of *Lactococcus lactis* subsp. *cremoris* SMQ-461, showing the cell-associated capsular exopolysaccharide layer. Arrows indicate cells surrounded by a clear zone representing the capsular layer.

RESULTS

Determination of EPS production and molecular mass. *L. lactis* subsp. *cremoris* SMQ-461 produced a capsular EPS (Fig. 1) and did not form any visibleropy strings longer than 5 mm when colonies on GM17 or LM17 were touched and pulled with sterilized toothpicks. The EPS production of 160

and 124 mg liter⁻¹ was measured from skim milk cultures at 30°C after 24 h and 48 h of incubation, respectively, under batch fermentation conditions. After incubation at 25°C for both 24 and 48 h, production of 115 mg liter⁻¹ EPS was measured. The production of EPS in LM17 (containing 2% lactose [wt/vol]) was found to be 152 and 100 mg liter⁻¹, respectively, after 24 and 48 h of incubation at 30°C. Stationary phase in LM17 was attained between 20 h and 24 h (optical density at 650 nm = 2.22). The apparent molecular mass of purified EPS from skim milk and LM17 cultures was $>1.6 \times 10^6$ Da, as determined by high-performance size exclusion chromatography.

Identification and cloning of the *eps* gene locus. To test whether EPS production by *L. lactis* subsp. *cremoris* SMQ-461 is linked to plasmid or chromosomal DNA, Southern hybridization was carried out on plasmid and total DNA with a probe from the internal gene fragment of *epsB*, which was generated by PCR. No hybridization signals were detected with digested and nondigested plasmid, whereas a strong signal was always associated with total DNA (Fig. 2). This result indicates that the *eps* locus of strain SMQ-461 is located on the chromosome.

Principal and nested primers in the most conserved regions of *epsRXABC* and *epsL* from known lactococcal *eps* operons were designed (Tables 1 and 2), and used in a genome walking PCR strategy with *L. lactis* subsp. *cremoris* SMQ-461 genomic DNA, resulting in cloned PCR fragments of 2 to 9.7 kb (Table 1). The inserts of these clones were completely sequenced on both strands for further analysis. The G+C moles percent content of the *eps* locus was 28%, which is identical to the reported *eps* gene cluster from *L. lactis* subsp. *cremoris* NIZO B40 (56) but below that of typical G+C mole percent content of 38 to 40% reported for *Lactococcus* spp. (27).

FIG. 2. Detection of the *eps* genes from *Lactococcus lactis* subsp. *cremoris* SMQ-461 by Southern hybridization. Digested and undigested plasmid extraction and total DNA (A); hybridization with the *epsB* probe (B). Lanes 1 and 2, plasmid extraction digested with SacI and HindIII, respectively; lane 3, undigested plasmid; lanes 4 and 5, total DNA digested with SacI and HindIII, respectively; and lane 6, undigested total DNA.

TABLE 2. Gene organization of identified ORFs, predicted physical properties of the hypothetical proteins encoded by the *eps* gene cluster from *Lactococcus lactis* subsp. *cremoris* SMQ-461, and percentage of identity of the predicted proteins with those involved in EPS biosynthesis in other bacteria

Gene	G+C mol%	Putative RBS ^a	No. of amino acids	Predicted pI	Highest % of identity with proteins from <i>L. lactis</i> subsp. <i>cremoris</i> or other bacteria					Predicted function
					HO2 (AF142639)	NIZO B40 (AF036485)	NIZO B891 ^b (AF100298)	NIZO B35 ^b (AF100297)	Others	
<i>epsR</i>	30	GAGGA	105	5.17	99 (EpsR)	98 (EpsR)				Transcription regulator
<i>epsX</i>	30	AGGGAG	255	5.41	91 (EpsX)	98 (EpsX)				Unknown protein
<i>epsA</i>	34	GGAG	259	9.52	93 (EpsA)	92 (EpsA)				Chain length determination
<i>epsB</i>	36	AGGAG	231	7.83	89 (EpsB)	89 (EpsB)	87 (EpsB)			Chain length determination
<i>epsC</i>	33	GGAG	254	5.65	97 (EpsC)	95 (EpsC)	96 (EpsC)	92 (EpsC)	50 (CapC) ^c	Phosphotyrosine-protein phosphatase
<i>epsD</i>	36	GGAG	228	9.06	97 (EpsD)	90 (EpsD)	89 (EpsD)	92 (EpsD)		Glycosyltransferase
<i>epsE</i>	32	GGA	149	9.66			97 (EpsE)			Glycosyltransferase
<i>epsF</i>	33	GGAGG	161	8.45	37 (EpsH)	40 (EpsF)	69 (EpsF)			Glycosyltransferase
<i>epsG</i>	29	GAGGA	187	6.91		22 (EpsG)				Galactosyltransferase
<i>epsH</i>	26	AAGA	384	9.49					22 (PBPA2679) ^d	Possible polymerase
<i>epsI</i>	24	AAG	232	9.31					23 (Eps6J) ^e	Glycosyltransferase
<i>epsJ</i>	26	GAA	357	9.30					24 (PppA) ^f	Protein involved in EPS biosynthesis
<i>epsK</i>	27	AGG	316	9.46		27 (EpsG)				Galactosyltransferase
<i>epsM</i>	27	AGG	482	9.78		38 (EpsK)				Repeat unit transporter
<i>epsL</i>	34	GGAGG	300	6.61	93 (EpsL)	90 (EpsL)		93 (EpsL)	22 (EpsL) ^g	Protein involved in EPS biosynthesis
<i>orfY</i>	32	GGAG	300	9.57	97 (OrfY)	96 (OrfY)		97 (OrfY)		Unknown protein

^a Sequence of the 3' end of the lactococcal 16S rRNA (3'-UCUUUCCUCC-5') (9).

^b Partial sequence only available in GenBank.

^c CapC from *Bacillus cereus* ATCC 14579 (GenBank accession no. AAP12140.1; locus tag BC5276).

^d PBPA2679 from *Photobacterium profundum* (GenBank accession no. CAG21057; predicted membrane protein).

^e Eps6J from *S. thermophilus* (accession no. AAN63722; putative glycosyltransferase).

^f PppA from *Thermoanaerobacter thermohydrosulfuricus* (GenBank accession no. CAB92958; putative pyruvyltransferase).

^g EpsL from *Streptococcus suis* (GenBank accession no. ZP_00331573; COG4632: exopolysaccharide biosynthesis protein).

Sequence analysis of the *eps* genes and gene products. The complete nucleotide sequence of 17.5 kb of chromosomal DNA was determined, revealing 19 open reading frames (Fig. 3). Within this region, the *eps* operon included 15 ORFs covering 13.2 kb and oriented in the same transcriptional sense. The upstream flanking region contained four features, of which the first was a partial ORF; the remaining three were oriented in the opposite transcriptional sense from the subsequent *eps* operon. These four ORFs are potentially involved in DNA recombination and mobility functions. The first partial ORF had only 11% amino acid identity with a putative integrase-recombinase from *Ralstonia eutropha* (GenBank accession no. AAP85809). The second and third ORFs were partial transposases revealing 70 to 82% identity with the transposase of IS1077E from *L. lactis* subsp. *lactis* IL-1403 (4). The fourth ORF (*tmp*) was a complete transposase sharing 27% identical amino acids with the transposon-related *ygcE* (GenBank accession no. AAK04736) from *L. lactis* subsp. *lactis* IL-1403 and 20% identity with the IS1253-like transposase protein

from *Streptococcus thermophilus* (GenBank accession no. AAN63779). As found for NIZO strain B40, *orfY* was found at the 3' end of the *eps* gene cluster and was oriented in the opposite transcriptional sense (Fig. 3). This gene is followed by a truncated ORF, whose product has 30% identity with transposase *yuiI* found in the genome of *L. lactis* subsp. *lactis* strain IL-1403 (GenBank accession no. AAK06125) and 29% identity with Orf1 of IS981 (GenBank accession no. M33933).

Based on predicted amino acid similarity, putative functions could be assigned to 11 of 15 ORFs identified (Table 2). The predicted functions of *eps* genes divide the *eps* operon into regions covering regulation (*epsR*), chain length determination (*epsABC*), biosynthesis of the repeating unit (*epsDEFG*, *epsI*, and *epsK*), polymerization (*epsH*), and export (*epsM*). No putative function can yet be assigned to EpsX, EpsJ, and EpsL. EpsL shares a highly significant identity of $\geq 90\%$ with related sequences from lactococcal strains (Table 2).

The genes encoding proteins involved in regulation and chain length determination are highly conserved among the

FIG. 3. Genetic organization of the *eps* gene cluster of *Lactococcus lactis* subsp. *cremoris* SMQ-461. Arrows represent potential ORFs, and gene designations are indicated under the arrows. P, polymerization; CLD, chain length determination; GTF, glycosyltransferase. The flag and hairpin indicate the putative promoter and terminator, respectively. The complete nucleotide sequence is available in GenBank under accession number AY741550.

EPS-producing lactococci. EpsR displays a high level of identity of 98% with the corresponding protein from *L. lactis* subsp. *cremoris* NIZO B40 and HO2 (20, 56). This gene product has 55% amino acid identity with *yqbH* (GenBank accession no. AAK05674) from the genome of *L. lactis* subsp. *lactis* strain IL-1403, which has five paralogs of this type of transcription regulator. Also, EpsR shows an amino acid identity of 21% with Xre, the transcription repressor of PBSX prophage from *Bacillus subtilis* (GenBank accession no. AAA22894) (63). These proteins contain a DNA-binding domain and belong to the LysR family of transcriptional regulators (51, 56). Hence, EpsR could be involved in the regulation of *eps* gene expression. The hydrophobicity plot for EpsR did not show any transmembrane segments. However, the hydrophobicity profile of EpsX shows two highly hydrophobic segments (residues 5 to 23 and 165 to 188), which could function as a membrane anchor. To date, there is no available biochemical or mutational experiments to assess the function of EpsX. OrfY located just downstream of the *eps* operon is also highly conserved among *L. lactis* *eps* operons and appears also to have been transferred to some *S. thermophilus* strains (98% identical to *cpsW* from strain MR-1C [GenBank accession no. AAM93404] and to *eps4Q* from the type IV *eps* operon [GenBank accession no. AAN63692]). The *orfY* gene product from strain SMQ-461 has 24% identity with the transcription regulator protein LytR from *Bacillus subtilis* (GenBank accession no. Q02115) (36). Regulatory proteins of the LytR group have been found in other LAB loci encoding EPS biosynthesis. They contain three putative transmembrane segments at the N terminus (31) and represent a different regulatory mechanism from EpsR that also has not been investigated to date.

The gene products of *epsA*, *epsB*, and *epsC* are predicted to be responsible for EPS chain length determination. They share a high identity (89 to 97%) with EpsA, EpsB, and EpsC from *L. lactis* subsp. *cremoris* NIZO B40 (GenBank accession no. NP_053033, NP_053032, and NP_053031) and HO2 (GenBank accession no. AAP32715, AAP32716, and AAP32717) (20, 56). EpsA also shows significant identity with EpsA (36%) from *Bacillus cereus* (GenBank accession no. BC5278) and with EpsC (26%) from *S. thermophilus* (GenBank accession no. AAC44010) (52), which have been classified as chain length-regulating proteins in capsular or exopolysaccharide biosynthesis, possessing two transmembrane domains. Prediction of membrane-spanning regions using the TMPred program shows that EpsA has two putative transmembrane helices (amino acids [aa] 24 [inside] to 44 [outside] and aa 175 [outside] to 194 [inside]). Moreover, a consensus sequence motif (SPKPKLYLAISVIAGLVLG) was identified at the C terminus (residues 170 to 188) of EpsA from strain SMQ-461. This motif (SPKX₁₁GX₃G) has been shown to be involved in determining O-antigen chain length in several gram-negative bacteria such as *Escherichia coli* (WzzB or *rol*) (3).

EpsB displays 35% identity with Cps19fD from *Streptococcus pneumoniae* (GenBank accession no. AAC44961) (24), which was recently identified as an autophosphorylating protein tyrosine kinase (42) and 47% with the tyrosine protein kinase BC5277 (GenBank accession no. AAP12141) from *Bacillus cereus* ATCC 14579 (29). The tyrosine protein kinase enzymes are a known family of prokaryotic proteins involved in EPS production that are essential for virulence (28). These

proteins are characterized by the presence of ATP-binding motifs and a tyrosine-rich region at the C terminus. Sequence analysis of EpsB revealed the presence of a Walker A ATP-binding site, AGKS (residues 56 to 59), and a Walker B-site, VVLID (residues 166 to 180), which may be required for functional phosphorylation of EpsB. Furthermore, the tyrosine-rich region at the C terminus was identified at residues 205 to 208. Sequence analysis supports the hypothesis that EpsB functions as the phosphotyrosine protein kinase and interacts with EpsA to form an EpsA-EpsB complex to facilitate EPS polymerization with the same mechanism as previously illustrated for the CpsC-CpsD complex from *S. pneumoniae*, which was experimentally demonstrated by mutation (42).

The predicted protein EpsC shows 50% amino acid identity with the phosphotyrosine protein phosphatase BC5276 (GenBank accession no. AAP12140) from *Bacillus cereus* ATCC 14579. Its N terminus has a DxHCH sequence, which is a highly conserved motif among other comparable proteins with similar function. Hence, EpsC may play an important role in EpsB dephosphorylation to facilitate EPS polymerization, as proposed for CpsB from *S. pneumoniae* (41). These results suggest that EpsA, EpsB, and EpsC from strain SMQ-461 all have a role in the control of polysaccharide chain length and, consequently, polysaccharide molecular weight.

Nucleotide sequence comparisons between the *eps* operon of strain SMQ-461 and other *eps* loci that were previously identified from *Lactococcus* spp. revealed that the 3,460-bp region including *epsRXABC* seems to be a highly conserved region and could come from a common ancestor of lactococcal strains. This region has the same organization in all lactococci and shows >95% identity (Fig. 4) with the corresponding sequence of *eps* loci from *L. lactis* subsp. *cremoris* HO2 (20) and NIZO B40 (56). However, there are some notable sequence variations (Fig. 4). The predicted protein product of *epsC* has an N terminus that is 24 aa shorter for strain B891 than for strains SMQ-461 and B40. Also, in comparison with the *eps* operon of SMQ-461, a deletion of 167 bp was detected in *epsX* from strain NIZO B40 (Fig. 4), leading to a shorter protein (139 aa compared to 255 aa for EpsX from SMQ-461). The nucleotide sequences of *epsR* and *epsX* from lactococci did not show any significant identity to any genes involved in polysaccharide biosynthesis reported for lactic acid bacteria, other than the lactococci (56). The first 226 bp of *epsR* and 115 bp of *epsX* from *L. lactis* subsp. *cremoris* strains share up to 97% identity with a related part of *orf4B*, the intergenic space and pseudogene (*orf4C*) from the type IV operon which was identified in *S. thermophilus* (GenBank accession no. AF454495.1). This result suggests a horizontal transfer between lactococci and streptococci. The *epsR* gene from lactococci is flanked by IS982 at its 5' end for strains HO2 and NIZO B40. This insertion sequence is lactococcal in origin and is known to be horizontally transferred from *L. lactis* to *S. thermophilus* (22, 23). Therefore, the transfer direction of *epsR* and *epsX* is suggested to be from lactococci to streptococci.

The 687-bp region encoding *epsD* shares a high identity of 90% to 91% between lactococcal strains. The 5' end of *epsD* from strain NIZO B40 contains a 6-bp deletion that does not affect the reading frame in comparison to the nucleotide sequences from strains SMQ-461, NIZO B891, and HO2. Downstream of *epsD*, the 913-bp region containing *epsEF* of SMQ-

FIG. 4. Nucleotide sequence comparison of the *eps* clusters of lactococci and streptococci. Only the sections of the *eps* locus of the *S. thermophilus* which share high identity with the related sequences of lactococci are indicated (GenBank accession no. AF454495 for the type IV *eps* operon and AF454498 for the type VII *eps* operon). White and gray boxes correspond to sequences that display identity of >91% and moderate identity of 78%, respectively. Black boxes correspond to sequences that did not show any significant identity levels of >20%. Size is indicated by numbers without parentheses. Dashed lines indicate the partial sequence available for strain NIZO B891. Black triangles show deletions and deletion sizes in base pairs are indicated below in parentheses. GenBank accession numbers are indicated in Table 2.

461 was similar only to the corresponding gene sequences from strain NIZO B891, sharing 95% identity with *epsE* and 91% with the first 144 bp of *EpsF* (Fig. 4). Overall nucleotide identity for *epsF* was 69% between strains SMQ-461 and B891, mainly due to the central and C-terminal regions of low similarity (Fig. 4). High-identity matches were found between *epsDEF* from strains SMQ-461 and NIZO B891 with *eps7LKJ* from the type VII operon identified in *S. thermophilus* (GenBank accession no. AF454498). The *eps7LKJ* genes were flanked at the 5' end by a complete *IS981*, which is known to be frequently transferred from lactococci to streptococci (23). This suggests that the *epsDEF* genes equivalent to those of strains NIZO B891 and SMQ-461 may have been horizontally transferred to certain streptococcal strains.

The predicted products of genes *epsGHIJKM* have little to no similarity with *eps* gene products from lactococci known to date. The putative proteins *EpsG* and *EpsK* are 18% identical, overall, and have moderate identity with *EpsG* from *L. lactis* subsp. *cremoris* strain NIZO B40 (Table 2). The most similar region is located in the first third of the proteins. This could suggest either significant divergence or a mosaic structure due to recombination events. While the putative products of *epsH*, *epsI*, and *epsJ* did not show any significant similarities with proteins encoded by *eps* genes from lactococci, they did show low levels of identity (22 to 29%) with proteins encoded by genes of the *eps* loci of streptococci and phylogenetically distant bacteria, such as *Photobacterium profundum* and *Thermoanaerobacter thermohydrosulfuricus* (Table 2).

The two genes *epsH* and *epsM* are predicted to be involved in polymerization and export of the EPS repeat units, based on sequence similarity and hydrophobicity analyses. The *epsH* gene encodes an estimated 44.4-kDa protein which did not display any significant similarity with protein sequences available for lactic acid bacteria. It shares low identity with a mem-

brane protein from *Photobacterium profundum* (Table 2). Analysis of its hydrophobicity plot by TMpred indicates that *EpsH* has 9 or 10 putative transmembrane segments (Fig. 5A), which resemble the plot of *EpsI* from *L. lactis* subsp. *cremoris* strain NIZO B40 (Fig. 5C). Similarly, *Wzy* of *E. coli* has nine predicted transmembrane segments and has been experimentally shown to be the polymerase implicated in the biosynthesis of a high-molecular-weight capsule (18). *EpsH* may thus function as the polysaccharide polymerase.

The *epsM* gene is located at the end of the cluster and encodes a large 55.3-kDa protein, which shares moderate identity (36 to 38%) with the transporter proteins *EpsK* from *L. lactis* subsp. *cremoris* NIZO B40 (56) and *EpsU*, *CpsU*, *EpsI*, and *Eps7M* from *S. thermophilus* strains (5, 21), as well as 28% with *Wzx* from *S. pneumoniae* (30). It is a predominantly hydrophobic protein with 14 putative transmembrane signals (Fig. 5B). A similar profile can be observed for the hydrophobicity plot of *EpsK* from *L. lactis* subsp. *cremoris* NIZO B40 (Fig. 5D) and *Wzx* from *E. coli*, which is sometimes referred to as a "flippase" (62). *Wzx* is believed to translocate the undecaprenyl-linked O-antigen subunits across the cytoplasmic membrane (37). From these characteristics, *EpsM* could be a membrane protein involved in the export of exopolysaccharide repeat subunits.

Transcription analysis of the *eps* gene cluster. A putative promoter with -35 and -10 motifs (TTGCAT-N₁₇-TATAAT) was found that is highly similar to the promoter sequence previously identified upstream of the transcription starts of the *eps* operon of *L. lactis* subsp. *cremoris* strain NIZO B40 (56). The end of the -10 sequence is located 124 bp upstream of the start codon of *epsR* for strain B40 and 122 bp upstream of *epsR* for SMQ-461. Over the 29 nucleotides covering the -35 and -10 motifs, only 3 nucleotides differ between the sequence from strain SMQ-461 and that of B40, while only 2 nucleotides

FIG. 5. Hydrophobicity plots of EpsH (A) and EpsM (B) from the *eps* gene cluster of *Lactococcus lactis* subsp. *cremoris* SMQ-461 compared to EpsI (C) and EpsK (D) from *L. lactis* subsp. *cremoris* strain NIZO B40 (GenBank accession no. AF036485; EpsI has protein identification no. AAC45236.1 and EpsK has protein identification no. AAC45238.1), indicating predicted transmembrane domains and their orientation using the TMpred program from ch.EMBLnet.org (http://www.ch.emblnet.org/software/TMPRED_form.html).

in this region differ between the sequence of SMQ-461 and that of strain HO2. Between the -35 motif and the start of *epsR*, there are 89% identical nucleotides out of 157 bp between SMQ-461 and B40, and 86% identical nucleotides out of 159 bp between SMQ-461 and HO2. Upstream of this region, however, identity decreases due to deletions. A potential Rho-independent transcription terminator was identified immediately downstream of the stop codon of *epsL* (-11.4 kcal mol $^{-1}$; nucleotides 15773 to 15804) and *orfY* (in the opposite orientation).

To confirm whether all downstream genes were expressed from this promoter as a single transcript, RNA was extracted from three different growth stages (early and late logarithmic phase and late stationary phase) and then analyzed by RT-PCR. Eight amplifications by RT-PCR were able to cover all the intergenic regions of the *eps* operon, except the region between *epsL* and *orfY*, indicating that the end of transcription is located after *epsL* (Fig. 6). These PCR products were obtained only with the RNA from late stationary phase, as no amplifications were detected with RNA isolated in the early and late logarithmic growth phases, even when higher amounts of RNA (up to 2 μ g) were used in the RT-PCRs. These results indicate that *L. lactis* subsp. *cremoris* strain SMQ-461 has an *eps* gene cluster comprising 15 genes organized in an operon transcribed from a single promoter.

Construction of the *epsD* deletion mutant. To investigate the function of the *eps* gene cluster in EPS biosynthesis by strain SMQ-461, *epsD* was targeted for inactivation by homologous recombination. This gene encodes a protein sharing a high level of identity with the priming glycosyltransferases of other lactococci, particularly *epsD* of strain NIZO B40, which was experimentally shown to link glucose-1-phosphate from UDP-

glucose to a lipid carrier. A deletion mutation of *epsD* was designed by PCR amplification of the DNA sequences representing the 5' and 3' ends of the target gene plus flanking sequences. The recombinant plasmid pND9 carrying the internally deleted *epsD* gene was introduced into the SMQ-461 strain by electroporation. The frequency of plasmid integration into the *eps* operon by homologous recombination was calculated as the number of antibiotic-resistant cells (obtained on GM17 medium with 5 μ g ml $^{-1}$ erythromycin at 38°C) divided by the total cell number (counted on GM17 without erythromycin at 38°C). A high integration frequency of 1.44×10^{-2} was reported with pND9, which is higher than the transposition

FIG. 6. Transcription analysis by RT-PCR of *eps* genes from *Lactococcus lactis* subsp. *cremoris* SMQ-461. Amplification of the *eps* locus was performed using specific primers (Table 1) of *epsR-epsA* (lane 1), *epsA-epsB* (lane 2), *epsB-epsD* (lane 3), *epsD-epsF* (lane 4), *epsF-epsH* (lane 5), *epsH-epsJ* (lane 6), *epsJ-epsM* (lane 7), *epsM-epsL* (lane 8), and *epsL-orfY* (lane 9). Lane M, 1-kb ladder (Life Technologies).

FIG. 7. Detection of the *epsD* mutants of *Lactococcus lactis* subsp. *cremoris* SMQ-461. (A) Ruthenium red plate assay showing two different cell phenotypes of *Lactococcus lactis* subsp. *cremoris* SMQ-461 after excision of the integrated plasmid. Small and large arrows show small red colonies (mutant type) and large rosy white colonies (colonies reverted to the wild type), respectively. (B) PCR assay confirming the different genotypes of cells using primers located in *epsB* and *epsE* (HD2 and SMQ461ER). Lane 1, wild-type strain; lane 2, strain ND461R (revertant with rosy white color); lane 3, 1-kb ladder (Life Technologies); and lane 4, strain ND461M (insertion mutant with red color).

frequency (4.9×10^{-3}) that was estimated for pGh9 containing the ISSI sequence and introduced into *L. lactis* subsp. *lactis* IL-1403 grown on GM17 (38).

Two colony types were obtained on RRM17 plates after provoking integration at the nonpermissive temperature of 38°C. Integrants were characterized as red and moderate rough phenotypes (small and dry colonies) (Fig. 7A). Rosy white colonies exhibited a phenotype identical to the wild-type strain (smooth, large, and shiny colonies). The two colony types were tested by PCR to identify the plasmid integration using a forward primer located in *epsB* and a reverse primer located in *epsE* (Table 1). The rosy white colonies produced the same size PCR fragment as the wild type (~2.6 kb), and the red colonies produced a ~7.1-kb fragment, which covered the same fragment size as the wild-type plus pND9 (Fig. 7B). Southern hybridization with the PstI/XhoI fragment of pGh9 as a probe indicated the presence of pND9 inserted into the ND461M chromosome (fragment size, >12.2 kb with XhoI-digested total DNA; there are no XhoI sites within the *eps* operon and one XhoI site in pND9).

Plasmid excision from the chromosome via a second recombination at the permissive temperature (28°C) led to either construction of the *epsD*Δ mutant or reconstituted the wild-type genotype. The number of red and rosy white colonies on RRM17 agar was calculated over four subcultures. After the subculture cycles, the rosy white colonies were dominant

(97.2% of the mixed culture) (Table 3), indicating that a high level of reversibility reconstructed the wild-type phenotype. About 2,000 red colonies were screened for erythromycin sensitivity to confirm plasmid excision for the construction of the stable *epsD*Δ mutant. Two red erythromycin-sensitive clones were identified (ND461D1 and ND461D2), indicating the low frequency with which the *L. lactis* subsp. *cremoris* SMQ-461 deletion mutants were obtained (0.1%). No hybridization with the PstI/XhoI fragment of pGh9 as probe was found for DNA isolated from strains ND461R, ND461D1, and ND461D2, indicating the absence of pND9 plasmid DNA in these mutants. Production of EPS by the three disrupted mutants was further investigated by EPS extraction; the absence of capsule formation was tested via light microscopy with India ink (data not shown). The level of extracted EPS was similar to the negative control (EPS-nonproducing lactococcal strain *L. lactis* subsp. *lactis* IL-1403), and the capsular layer was not present, indicating the loss of extracellular polysaccharide production.

DISCUSSION

Many slime-forming thermophilic and mesophilic strains of LAB can produce EPS, which increase viscosity and improve the texture of fermented milk products (7, 49). In this study, we demonstrate the production of EPS by capsule-forming mesophilic *L. lactis* subsp. *cremoris* SMQ-461 grown in skim milk and LM17 media. Previous studies have shown that production of EPS by LAB is medium dependent, and the yield of EPS varies considerably among different strains (17). The EPS production level of strain SMQ-461 is similar to the value previously estimated for strain *L. lactis* subsp. *cremoris* B30 (64) and higher than that estimated for lactococcal strains NIZO B35, NIZO B39, NIZO B40, and NIZO B891, which were grown under similar conditions (49). Also, it was clearly shown that the amount of EPS produced declined in both growth media upon prolonged fermentation at 30°C. The decrease of EPS

TABLE 3. Evaluation of the phenotype reversibility of *epsD*Δ mutants during subculturing for 160 generations

Generation no.	No. of colonies, CFU ml ⁻¹ (%)	
	Red	White
40	1.32×10^8 (55)	1.09×10^8 (45)
80	1.03×10^8 (30)	2.40×10^8 (70)
120	0.53×10^8 (14)	3.31×10^8 (86)
160	0.13×10^8 (3)	4.53×10^8 (97)

concentration may be due to hydrolysis of EPS by glycohydrolases, as previously reported for *Lactobacillus rhamnosus* R (47). Strain SMQ-461 was selected for further studies of the impact of EPS on the production of reduced-fat Cheddar cheese (13).

Chemical analysis of the purified EPS from skim milk culture of SMQ-461 has shown a molar ratio of 2:1 (glucose to galactose) (15). This ratio has not been reported for any of the EPS produced by lactococcal strains NIZO B891, NIZO B39, NIZO B1137, MLT1, MLT2, and SMQ-420 (15, 58). The apparent molecular mass is the same for the purified EPS from skim milk and LM17 cultures. This high molecular mass is similar to the reported sizes ($>1 \times 10^6$ and $\sim 1.7 \times 10^6$ Da) of EPS from *L. lactis* subsp. *cremoris* strains LC330 and SBT 0495, respectively (39, 43).

The gene cluster involved in the biosynthesis of exopolysaccharide by *L. lactis* subsp. *cremoris* SMQ-461 is located on the chromosome, although all *eps* genes characterized to date for lactococci are plasmid encoded (20, 56, 59). In other LAB such as streptococci and lactobacilli, genes responsible for EPS biosynthesis are chromosomally located (35, 45), with the exception of *Lactobacillus casei* subsp. *casei*, where EPS biosynthesis has been associated with the presence of a plasmid (60). However, there is no evidence that such a chromosomal location results in a higher stability of the phenotype over multiple subculturing and fermentation processes.

The function of the *eps* genes in EPS biosynthesis was confirmed by disruption of the priming glycosyltransferase gene *epsD*, which generated non-EPS-producing mutants that were reversible. The *eps* genes are transcribed as a single polycistronic mRNA which may be driven by a promoter upstream of *epsR*. Similar large transcripts covering all genes involved in polysaccharide production were reported for the *eps* gene clusters from *L. lactis* subsp. *cremoris* NIZO B40 and HO2 (20, 56). Moreover, the transcription signal was detected by RT-PCR only with the RNA extracted from stationary-phase cells, which could indicate either transcript levels below detection level or RNA processing during the logarithmic growth phase. Further experiments are necessary to investigate transcript stability.

On the basis of the similarities of the deduced amino acid sequences of the *eps* operon of SMQ-461 strain with the related proteins from *eps/cps* clusters of other EPS-producing bacteria, potential functions were assigned to the genes, which were grouped into regions involved in regulation, chain length determination, biosynthesis of the repeating unit, polymerization, and export. The low similarity of the predicted proteins coded by *epsGHIJKM* with other LAB *eps* operons indicates that new genes encoding EPS biosynthesis, including potential glycosyltransferases, have been identified in the SMQ-461 strain.

The central region (*epsD*, *-E*, *-F*, *-G*, *-I*, and *-K*) of the *eps* cluster encodes six proteins predicted to be glycosyltransferases, while the chemical composition of the purified EPS indicated a molar ratio of 2:1 (glucose to galactose) (15). However, this ratio may be associated with either a trisaccharide or a hexasaccharide repeat unit, composed of 4 units of glucose and 2 units of galactose. EpsD, EpsE, and EpsF display different degrees of identity (40 to 97%) at the amino acid level with the corresponding proteins from *L. lactis* subsp. *cremoris*

NIZO B40, NIZO B891, and HO2 (20, 56, 58). The functions of the gene products from *L. lactis* subsp. *cremoris* NIZO B40 have been studied by heterologous expression (57). EpsD acts as undecaprenyl-phosphate glycosyl-1-phosphate transferase, which catalyzes the first step in polysaccharide biosynthesis by transferring the first glucose unit from UDP-glucose to the lipid carrier. EpsE and EpsF from strain NIZO B40 interact together to link the second unit of glucose from UDP-glucose to lipid-linked glucose. The same function may hold for EpsD from *L. lactis* subsp. *cremoris* SMQ-461, while EpsE and EpsF may act together to link the second and the third glucose units to lipid-linked glucose to form a trisaccharide.

The EpsG and EpsK gene products of strain SMQ-461 both share moderate identity (35%) with EpsG from *L. lactis* subsp. *cremoris* strain NIZO B40 (56). EpsG of NIZO B40 has been shown experimentally to possess galactosyltransferase activity and links galactose from UDP-galactose to lipid-linked cellobiose (57). For strain SMQ-461, EpsG may also encode a galactosyltransferase that acts to transfer a galactose unit to the trisaccharide to form the lipid-linked tetrasaccharide. The order of monosaccharides in the repeat unit structure for the EPS from strain NIZO B40 follows the order of the genes in the corresponding *eps* operon. For strain SMQ-461, the gene encoding EpsI is located between *epsG* and *epsK*; thus, it may transfer the fourth glucose unit to form the lipid-linked pentasaccharide. As EpsK shares similarity with EpsG, it could be the galactosyltransferase that adds the final galactose to form the hexasaccharide repeat unit. The roles of these putative glycosyltransferases are thus predicted, but mutational and biochemical analyses will be required to experimentally establish their functions, while the final linkage structure of the repeat unit remains to be determined by nuclear magnetic resonance analysis.

The organization of the *eps* clusters from *L. lactis* strains is conserved and similar to that of the gene clusters encoding EPS biosynthesis in *S. thermophilus* and CPS biosynthesis in other streptococci (1, 24, 33, 52). Recent reports investigated the functional analysis of genes involved in polysaccharide biosynthesis and the chain length determination mechanism in certain pathogens (28, 34, 42). To date, most of the available knowledge about the gene functions of the *eps/cps* clusters from food-grade LAB is focused on the region encoding glycosyltransferases (1, 53, 56, 58). The mechanisms of repeat unit exportation from the cell and subsequent assembly into high-molecular-weight polymers are still unclear. A better insight into these processes might contribute to optimizing EPS production. Alterations in the repeat unit by engineering the EPS composition or the molecular mass will consequently modify the rheological properties of the native EPS. In the current study, a complete chromosomal *eps* operon, transcribed as a single mRNA, is presented. New potential glycosyltransferases have been identified in the *eps* operon of strain SMQ-461. Therefore, *L. lactis* subsp. *cremoris* SMQ-461 would be a good model for mutational study to investigate the complexity of EPS polymerization and transport, as well as to be able to engineer tailor-made exopolysaccharides with desired properties for which the genes may be maintained in the chromosome.

ACKNOWLEDGMENTS

This research was made possible with the financial assistance of the Canadian Research Network on Lactic Acid Bacteria, supported by the Natural Sciences and Engineering Council of Canada; Agriculture and Agri-Food Canada; Novalait, Inc.; Dairy Farmers of Canada; and Rosell-Lallemand, Inc. This work was partially supported by the Egyptian Ministry of Higher Education through a Ph.D. scholarship awarded to N.D.

We acknowledge E. Emond for advice at the initial stages of this work and D. Lacroix for all his advice, stimulating discussions, and supplemental materials during this research. We are indebted to S. Moineau and H. Deveau for classification and providing the EPS-producing lactococcal strain. We are grateful to A. Hassan for the photograph of lactococcal capsules.

REFERENCES

- Almiron-Roig, E., F. Mulholland, M. J. Gasson, and A. M. Griffin. 2000. The complete *cps* gene cluster from *Streptococcus thermophilus* NCFB 2393 involved in the biosynthesis of a new exopolysaccharide. *Microbiology* **146**: 2793–2802.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Batchelor, R., P. Alifano, E. Biffali, S. I. Hull, and R. A. Hull. 1992. Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (*rol*) from *Escherichia coli* and *Salmonella typhimurium*: protein homology and functional complementation. *J. Bacteriol.* **174**: 5228–5236.
- Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarne, J. Weissenbach, S. D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* **11**:731–753.
- Broadbent, J. R., D. J. McMahon, D. L. Welker, C. J. Oberg, and S. Moineau. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. *J. Dairy Sci.* **86**: 407–423.
- Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **87**:113–130.
- Cerning, J. 1995. Production of exopolysaccharides by lactic acid bacteria and dairy propionibacteria. *Lait* **75**:463–472.
- Cerning, J., C. Bouillanne, M. Desmazeaud, and M. Landon. 1986. Isolation and characterization of exocellular polysaccharide produced by *Lactobacillus bulgaricus*. *Biotechnol. Lett.* **8**:625–628.
- Chiaruttini, C., and M. Milet. 1993. Gene organization, primary structure and RNA processing analysis of a ribosomal RNA operon in *Lactococcus lactis*. *J. Mol. Biol.* **230**:57–76.
- Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* **11**:260–263.
- Collins, C. H., and P. M. Lyne. 1970. *Microbial methods*, 3rd ed. Butterworth & Co., Ltd., London, United Kingdom.
- Crescenzi, V. 1995. Microbial polysaccharides of applied interest: ongoing research activities in Europe. *Biotechnol. Prog.* **11**:251–259.
- Dabour, N., E. E. Kheadr, I. Fliss, and G. LaPointe. 2005. Impact of ropy and capsular exopolysaccharide-producing strains of *Lactococcus lactis* subsp. *cremoris* on reduced-fat Cheddar cheese production and whey composition. *Int. Dairy J.* **15**:459–471.
- Deveau, H., and S. Moineau. 2003. Use of RFLP to characterize *Lactococcus lactis* strains producing exopolysaccharides. *J. Dairy Sci.* **86**:1472–1475.
- Deveau, H., M. R. Van Calsteren, and S. Moineau. 2002. Effect of exopolysaccharides on phage-host interactions in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **68**:4364–4369.
- De Vuyst, L., F. De Vin, F. Vaningelgem, and B. Degeest. 2001. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* **11**:687–707.
- De Vuyst, L., F. Vanderveken, S. Van de Ven, and B. Degeest. 1998. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. *J. Appl. Microbiol.* **84**:1059–1068.
- Drummlsmith, J., and C. Whitfield. 1999. Gene products required for surface expression of the capsular form of the group 1 K antigen in *Escherichia coli* (O9a:K30). *Mol. Microbiol.* **31**:1321–1332.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350–356.
- Forde, A., and G. F. Fitzgerald. 2003. Molecular organization of exopolysaccharide (EPS) encoding genes on the lactococcal bacteriophage adsorption blocking plasmid, pCI658. *Plasmid* **49**:130–142.
- Germond, J. E., M. Delley, N. D'Amico, and S. J. Vincent. 2001. Heterologous expression and characterization of the exopolysaccharide from *Streptococcus thermophilus* Sfi39. *Eur. J. Biochem.* **268**:5149–5156.
- Guédon, G., F. Bourgoïn, M. Pébay, Y. Roussel, C. Colmin, J. M. Simonet, and B. Decaris. 1995. Characterization and distribution of two insertion sequences, IS1191 and iso-IS981, in *Streptococcus thermophilus*: does intergeneric transfer of insertion sequences occur in lactic acid bacteria co-cultures? *Mol. Microbiol.* **16**:69–78.
- Guédon, G., F. Bourgoïn, and B. Decaris. 1998. Does gene horizontal transfer occur in lactic acid bacteria co-cultures? *Lait* **78**:53–58.
- Guidolin, A., J. K. Morona, R. Morona, D. Hansman, and J. C. Paton. 1994. Nucleotide sequence analysis of genes essential for capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 19F. *Infect. Immun.* **62**: 5384–5396.
- Higashimura, M., B. W. Mulder-Bosman, R. Reich, T. Iwasaki, and G. W. Robijn. 2000. Solution properties of villan, the exopolysaccharide from *Lactococcus lactis* subsp. *cremoris* SBT 0495. *Biopolymers* **54**:143–158.
- Hill, C., J. Massey, and T. R. Klaenhammer. 1991. Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57**: 283–288.
- Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. Group 17: gram-positive cocci, p. 527–558. In W. R. Hensyl (ed.), *Bergey's manual of determinative bacteriology*, 9th ed. Williams and Wilkins, Baltimore, Md.
- Ilan, O., Y. Bloch, G. Frankel, H. Ullrich, K. Geider, and I. Rosenshine. 1999. Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharide. *EMBO J.* **18**:3241–3248.
- Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltzman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, S. D. Ehrlich, R. Overbeek, and N. Kyrpides. 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature* **423**:87–91.
- Jiang, S.-M., L. Wang, and P. R. Reeves. 2001. Molecular characterization of *Streptococcus pneumoniae* type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. *Infect. Immun.* **69**:1244–1255.
- Jolly, L., and F. Stingle. 2001. Molecular organization and functionality of exopolysaccharide gene clusters in lactic acid bacteria. *Int. Dairy J.* **11**:733–745.
- Knoshaug, E. P., J. A. Ahlgren, and J. E. Trempey. 2000. Growth associated exopolysaccharide expression in *Lactococcus lactis* subspecies *cremoris* Ropy352. *J. Dairy Sci.* **83**:633–640.
- Kolkman, M. A. B., D. A. Morrison, B. A. M. van der Zeijst, and P. J. M. Nuijten. 1996. The capsule polysaccharide synthesis locus of *Streptococcus pneumoniae* serotype 14: identification of the glycosyl transferase gene *cps14E*. *J. Bacteriol.* **178**:3736–3741.
- Kolkman, M. A., W. Wakarchuk, P. J. Nuijten, and B. A. van der Zeijst. 1997. Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete *cps* locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit. *Mol. Microbiol.* **26**:197–208.
- Lamothe, G. T., L. Jolly, B. Mollet, and F. Stingle. 2002. Genetic and biochemical characterization of exopolysaccharide biosynthesis by *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Arch. Microbiol.* **178**:218–228.
- Lazarevic, V., P. Margot, B. Soldo, and D. Karamata. 1992. Sequencing and analysis of the *Bacillus subtilis* *lytRABC* divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier. *J. Gen. Microbiol.* **138**:1949–1961.
- Liu, D., R. A. Cole, and P. R. Reeves. 1996. An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. *J. Bacteriol.* **178**:2102–2107.
- Maguin, E., H. Prévost, S. D. Ehrlich, and A. Gruss. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* **178**:931–935.
- Marshall, V. M., E. N. Cowie, and R. S. Moreton. 1995. Analysis and production of two exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* LC330. *J. Dairy Res.* **62**:621–628.
- Miller, J. H. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Morona, J. K., R. Morona, D. C. Miller, and J. C. Paton. 2002. *Streptococcus pneumoniae* capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase. *J. Bacteriol.* **184**:577–583.
- Morona, J. K., J. C. Paton, D. C. Miller, and R. Morona. 2000. Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. *Mol. Microbiol.* **35**:1431–1442.
- Nakajima, H., S. Toyoda, T. Toba, T. Itoh, T. Mukai, H. Kitazawa, and S. Adachi. 1990. A novel phosphopolysaccharide from slime-forming *Lactococcus lactis* subspecies *cremoris* SBT 0495. *J. Dairy Sci.* **73**:1472–1477.
- O'Sullivan, D., and T. Klaenhammer. 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59**:2730–2733.

45. Péant, B., G. LaPointe, C. Gilbert, D. Atlan, P. Ward, and D. Roy. 2005. Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. *Microbiology* **151**:1839–1851.
46. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
47. Pham, P. L., I. Dupont, D. Roy, G. LaPointe, and J. Cerning. 2000. Production of exopolysaccharide by *Lactobacillus rhamnosus* R and analysis of its enzymatic degradation during prolonged fermentation. *Appl. Environ. Microbiol.* **66**:2302–2310.
48. Roller, S., and I. C. M. Dea. 1992. Biotechnology in the production and modification of biopolymers for foods. *Crit. Rev. Biotechnol.* **12**:261–277.
49. Ruas-Madiedo, P., R. Tuinier, M. Kanning, and P. Zoon. 2002. Role of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* on the viscosity of fermented milks. *Int. Dairy J.* **12**:689–695.
50. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**:597–626.
52. Stinglele, F., J. R. Neeser, and B. Mollet. 1996. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. *J. Bacteriol.* **178**:1680–1690.
53. Stinglele, F., J. W. Newell, and J.-R. Neeser. 1999. Unraveling the function of glycosyltransferases in *Streptococcus thermophilus* Sfi6. *J. Bacteriol.* **181**:6354–6360.
54. van Casteren, W. H., P. de Waard, C. Dijkema, H. A. Schols, and A. G. Voragen. 2000. Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B891. *Carbohydr. Res.* **327**:411–422.
55. van Casteren, W. H., C. Dijkema, H. A. Schols, G. Beldman, and A. G. Voragen. 2000. Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39. *Carbohydr. Res.* **324**:170–181.
56. van Kranenburg, R., J. D. Marugg, I. I. van Swam, N. J. Willem, and W. M. de Vos. 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. *Mol. Microbiol.* **24**:387–397.
57. van Kranenburg, R., I. I. van Swam, J. D. Marugg, M. Kleerebezem, and W. M. de Vos. 1999. Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. *J. Bacteriol.* **181**:338–340.
58. van Kranenburg, R., H. R. Vos, I. I. van Swam, M. Kleerebezem, and W. M. de Vos. 1999. Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other gram-positive cocci: complementation, expression, and diversity. *J. Bacteriol.* **181**:6347–6353.
59. Vedamuthu, E. R., and J. M. Neville. 1986. Involvement of a plasmid in production of ropiness (mucoidness) in milk cultures by *Streptococcus cremoris* MS. *Appl. Environ. Microbiol.* **51**:677–682.
60. Vescovo, M., G. L. Scolari, and V. Bottazzi. 1989. Plasmid-encoded ropiness production in *Lactobacillus casei* ssp. *casei*. *Biotechnol. Lett.* **2**:709–712.
61. Welman, A. D., and I. S. Maddox. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends Biotechnol.* **21**:269–274.
62. Whitfield, C., P. A. Amor, and R. Koplín. 1997. Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer: lipid A-core ligase and by determinants of polymer chain length. *Mol. Microbiol.* **23**:629–638.
63. Wood, H. E., K. M. Devine, and D. J. McConnell. 1990. Characterisation of a repressor gene (*xre*) and a temperature-sensitive allele from the *Bacillus subtilis* prophage, PBSX. *Gene* **96**:83–88.
64. Yang, Z., E. Huttunen, M. Staaf, G. Widmalm, and H. Tenhu. 1999. Separation, purification and characterisation of extracellular polysaccharides produced by slime-forming *Lactococcus lactis* ssp. *cremoris* strains. *Int. Dairy J.* **9**:631–638.