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Production of Exopolysaccharides by *Lactococcus lactis* subsp. *cremoris* MG1363 Expressing the *eps* Gene Clusters from Two Strains of *Lactobacillus rhamnosus*

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The aim of this study was to transfer the 18.5 kb gene clusters coding for 17 genes from *Lactobacillus rhamnosus* to *Lactococcus lactis* subsp. *cremoris* MG1363 in order to determine the effect of host on exopolysaccharide (EPS) production and to provide a model for studying the phosphorylation of proteins which are proposed to be involved in EPS polymerization. *Lactobacillus rhamnosus* RW-9595M and ATCC 9595 have 99% identical operons coding for EPS biosynthesis, produced different amounts of EPS (543 vs 108 mg/l). *L. lactis* subsp. *cremoris* MG1363 transformed with the operons from RW-9595M and ATCC 9595 respectively, produced 326 and 302 mg/l EPS in M17 containing 0.5% glucose. The tyrosine protein kinase transmembrane modulator (Wzd) was proposed to participate in regulating chain elongation of EPS polymers by interacting with the tyrosine protein kinase Wze. While Wzd was found in phosphorylated form in the presence of the phosphorylated kinase (Wze), no phosphorylated proteins were detected when all nine tyrosines of Wzd were mutated to phenylalanine. *Lactococcus lactis* subsp. *cremoris* could produce higher amounts of EPS than other EPS-producing lactococci when expressing genes from *L. rhamnosus*. Phosphorylated Wzd was essential for the phosphorylation of Wze when expressed *in vivo*.

Keywords: Exopolysaccharide, gene transfer, tyrosine phosphorylation, transmembrane modulator, protein tyrosine kinase, protein tyrosine phosphatase

Introduction

Exopolysaccharides (EPS) secreted into the surrounding environment by lactic acid bacteria (LAB) may have beneficial effects on human health through immunomodulation [1], antitumour [2] and cholesterol-lowering activity [3]. In the food industry, EPS produced by LAB function as viscosifiers, stabilizers, emulsifiers and gelling agents [4]. EPS from LAB display a great variety of structures and different sugar moieties [5] and the genetic diversity of EPS gene clusters has been shown

with many different bacterial strains. The molar mass of EPS is an important determinant of intrinsic viscosity, and may be the key physicochemical parameter to determining their capacity for immunomodulation, along with their charge [6–8].

Biosynthesis of heteropolysaccharides (HePs) is the result of polymerization of repeating units composed of more than one type of monosaccharide. The sugars transported into the cytoplasm are converted to nucleotide sugars to be used as EPS precursors for the biosynthesis of the repeating unit. The repeating units are exported outside the cell membrane and polymerized, then either attached to the cell surface or released into the medium [9]. EPS production thus requires genes coding for repeat unit assembly, regulation, polymeriza-

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tion, chain length determination and export [10, 11]. In *Streptococcus pneumoniae*, polysaccharides attached to the cell wall are termed capsular polysaccharides (CPS). CpsB, CpsC and CpsD were shown to regulate CPS production by reversible phosphorylation events. CpsD is an autophosphorylating protein tyrosine kinase (PTK) and CpsC (TKM: tyrosine protein kinase transmembrane modulator) is required for the phosphorylation of CpsD [12, 13]. Similar systems have been proposed for EPS production by *S. thermophilus*. EpsC (TKM) is necessary for EpsD (PTK) phosphorylation while both EpsC and EpsD are required for the activity of EpsE (initiating glycosyltransferase), thus enabling EPS repeat unit biosynthesis [14]. A single point mutation in the *eps*C gene encoding the PTK of *L. johnsonii* has been shown to convert rough colonies to smooth mucoid morphology with increased EPS production [15]. However, protein phosphorylation was not shown in this case.

Lactobacillus rhamnosus RW-9595M is one of the highest HePS-producing strains of LAB, as up to 2350 mg/l can be produced under pH controlled conditions in supplemented whey permeate (SWP) medium [16]. The EPS of this strain has high rhamnose content (57%), composed of L-rhamnose, D-glucose and pyruvate substituted D-galactose in a ratio of 4 : 2 : 1 [17]. The closely-related strain *L. rhamnosus* ATCC 9595 produces only 116 mg/l compared to the 1611 mg/l produced by RW-9595M in BMM medium (pH controlled at 6.0) with 40 g/l glucose [18]. Strain ATCC 9595 can stimulate pro-inflammatory cytokines but strain RW-9595M induces anti-inflammatory cytokine production, especially when the EPS is hydrolyzed [19, 20]. Thus, the cytokine stimulation profile differs between ATCC 9595 and RW-9595M, and further depends on EPS molecular mass [20]. The highly similar chromosomal EPS biosynthesis gene clusters of the two strains code for 17 ORFs (18.5 kb). Six putative glycosyltransferases are responsible for biosynthesis of the repeating unit and four *rml* genes are involved in the anabolism of dTDP-L-rhamnose. The proposed functions of three genes are related to chain length determination. The first two (TKM and PTK) are located in the 5' region of the gene cluster while in the 3' region, *wzb* (protein tyrosine phosphatase, PTP) has been shown to have phosphatase activity [21]. The TKM (Wzd) and PTP (Wzb) proteins are identical in amino acid sequence between strains ATCC 9595

and RW-9595M, while the PTK differs by one amino acid (T72 in ATCC 9595 is K72 in RW-9595M PTK). This mutation is not positioned in a proposed phosphorylation site. There are five promoters responsible for initiating transcription of adjacent EPS biosynthesis genes [18].

In this study, the genes from *L. rhamnosus* ATCC 9595 and RW-9595M were transferred to *Lactococcus lactis* subsp. *cremoris* MG1363 in order to determine how the host species affects EPS yield. We also determine the phosphorylation state of the two proteins that are proposed to be involved in regulating chain elongation and show the impact of tyrosine mutation of Wzd on phosphorylation in *L. lactis* subsp. *cremoris* MG1363.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids for this study are listed in Table 1. *L. rhamnosus* strains ATCC 9595 and RW-9595M were grown without agitation at 37℃ in MRS (De Man Rogosa Sharpe Broth; EMD chemicals Inc., Darmstadt, Germany) medium supplemented with 0.5% (w/v) glucose. *L. lactis* subsp. *cremoris* MG1363 was grown in M17 broth (Quelab, Montreal, QC) supplemented with either 0.5% or 2% (w/v) glucose (GM17) incubated at 30℃. GM17 agar medium containing 0.08% ruthenium red (Sigma, USA) was designated RGM17 and was used to confirm EPS production, as non EPS-producing *L. lactis* subsp. *cremoris* MG1363 colonies are colored red on this medium [22]. *Escherichia coli* strain TOP10 was grown at 37℃ in low salt Luria Bertani (LB) medium with agitation or on LB solidified with 1.5% agar (EMD chemicals Inc., Germany). Antibiotics were added when necessary at the following concentrations: 5 μg/ml erythromycin and chloramphenicol for *L. lactis* strains, and 50 μg/ml kanamycin for *E. coli*.

DNA manipulation and transformation

Genomic DNA was extracted from *L. rhamnosus* with the method of Audy *et al.* [23]. The Phase Lock Gel Heavy tube (Eppendorf, Germany) was utilized for the phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) extraction. Plasmid DNA extraction from *L. lactis* was performed with the method of O'Sullivan and Klaenhammer [24]. Plasmid DNA extraction from *E.*

Table 1. Strains and plasmids used in this study.

Strain and plasmid	Relevant characteristic(s)	Source or reference
Strain		
L. rhamnosus ATCC 9595	Low EPS-producing strain (116 mg/l)	ATCC[1]
L. rhamnosus RW-9595M	High EPS-producing strain (1611 mg/l)	Denis Roy[2]
E. coli Top 10	Cloning host (F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC)φ80lacZ ΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara- leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-)*	Invitrogen
L. lactis subsp. *cremoris* MG1363	Plasmid free (Lac⁻ Prt⁻)	[40]
Plasmid		
pIL253	Em, 4.9 kb, pAMβ1 replicon	[29]
pNZ124	Cm, 2.8 kb, pSH71 replicon	[41]
pMG36CT	Cm, 3.9 kb, pWV01 replicon	[42]
pCR-XL-topo	Km, T- overhangs for ligation of long PCR products	Invitrogen
pEPS1R and pEPS1A	pIL253 + 10 kb BamHI-SalI PCR amplicon (genes *wzd* to *wzm*) from either RW9595M (R) or ATCC 9595 (A)	This study
pEPS2R and pEPS2A	pNZ124 + 3.7 kb PvuII-PstI PCR amplicon (gene *welE* to *wzb*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pDEBR and pDEBA	pMG36CT + 2.6 kb SacI-AatII PCR amplicon (*wzd, wze* and *wzb*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pDER and pDEA	pMG36CT + 1.8 kb SacI-AatII PCR amplicon (*wzd* and *wze*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pDBR and pDBA	pMG36CT + 1.8 kb SacI-AatII PCR amplicon (*wzd* and *wzb*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pEBR and pEBA	pMG36CT + 1.6 kb SacI-AatII PCR amplicon (*wze* and *wzb*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pDR and pDA	pMG36CT + 923 bp SacI-AatII PCR amplicon (*wzd*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pER and pEA	pMG36CT + 856 bp SacI-AatII PCR amplicon (*wze*) from either RW-9595M (R) or ATCC 9595 (A)	This study
pBR and pBA	pMG36CT + 835 bp SacI-AatII PCR amplicon (*wzb*) from either RW-9595M (R) or ATCC 9595 (A)	This study
p9YFDEBR	pMG36CT + 2.6 kb SacI-AatII PCR amplicon (*wzd* 9 mutated tyrosines, *wze* and *wzb*) from RW-9595M	This study

[1]ATCC (American Type Culture Collection, Manassas, VA, USA) ; Qiagen S.A. (Courtaboeuf, France) ; Stratagene (LaJolla, CA, USA); Amersham Biosciences (Orsay, France).
[2]Original source: Denis Roy, INAF, Université Laval. (Dupont *et al.*, 2000).

coli was carried out using the plasmid mini prep kit (Millipore Corporation, USA). PCR was performed with the primers listed in Table 2 by Biometra T-Gradient thermocycler in a total volume of 50 µl containing 1 U Taq DNA polymerase (Feldan-bio, Canada), 1× PCR buffer, 0.2 µM of each primer, 0.4 mM of dNTP Mix and 50 ng DNA. The initial denaturation was 2 min at 94℃. Amplification consisted of a 15 s denaturation step at 94℃, a 1 min annealing step at primer-specific Tm and a 1 min/kb extension step at 72℃. After 30 cycles of amplification, there was a final extension step for 5 min at 72℃. LongAmp *Taq* DNA polymerase (Feldan) was used for amplifying gene fragments longer than 4 kb. This long range PCR was performed using conditions as above, but the elongation step was performed at 68℃. Negative control reactions were performed for all amplifications. Target genes were amplified using genomic DNA of each *L. rhamnosus* strain as template and the

Table 2. Oligonucleotide primers used in this study.

Name	Sequence[1]
P1wzdBamHI	CCG GAT CCG GGT ATT ATG CTT GGG AGT G
WzmXhoI	GGC TCG AGC AAC AAA GCC ATC ATT ACC AG
P3welEcoRV	CCG ATA TCT GAG ATT TCA CAC TGG TTA GTG
WzbNsiI	GCA TGC ATA TGA AAT TAG CAC TCG CAC AAC C
WzdRSacI	CGG AGC TCA AGA GCA AAT GGA CCT TGC AC
WzdFAatII	CGG ACG TCT CTC CTC AAT ACT TAA ACG CGT C
WzdFXbaI	CGT CTA GAT ACT AAA ACG CGT CTC CGG CTT CG
WzdF-Wzb	ATA CTA TCT AAG CTC AAT ACT TAA ACG CGT CTC CGG
WzeRSacI	CGG AGC TCA ATT TTC ATT AGA GAA AAT TTT GC
WzeRXbaI	GCG TCT AGA TTG AGG AGA AAA AAC ATG AAT TTT TC
WzeFAatII	CGG ACG TCT CGT ACT TAT CAC CAT TCG GTT GAC
WzeF-Wzb	AAT ACT ATC TAA GGC ATG CTA TTT ATT AGA TTC GTT CCC
WzbRsacI	CGG AGC TCT TGA TGT GCA TTG CCA TAT GTT ACC
WzbFAatII	CGG ACG TCA TGA AAT TAG CAC TCG CAC AAC C
WzbR-Wzd	TTA AGT ATT GAG CTT AGA TAG TAT TGG AAG GGG AAC
WzbR-Wze	ATA AAT AGC ATG CCT TAG ATA GTA TTG GAA GGG GAA C
pMG36R	AAA TGG CAA TCG TTT CAG CAG
pMG36F	CGT CGC CTT TAC CAA CTG TC

[1]Restriction sites in primers are indicated with double underlined letters.

amplicons were digested and purified by Qiaquick PCR purification kit or Qiaquick gel purification kit (Qiagen, Mississauga, ON, Canada). The digested products were ligated with linearized vector using T4 DNA ligase (New England Biolab, Pickering, ON, Canada), and a molar ratio of vector and insert was 1 : 2 or 1 : 3. *L. lactis* subsp. *cremoris* MG1363 was transformed with the recombinant plasmids by electroporation [25] and extracted plasmid DNA from transformants was verified by digestion with restriction enzymes as well as by DNA sequencing with an ABI Prism 3100 apparatus.

Construction of plasmids

Long range PCR was carried out to amplify a 10,565 bp fragment coding for genes *wzd* to *wzm* (primers P1wzdBamHI and wzmXhoI) and a 3,747 bp fragment coding for genes *welE* to *wzb* (primers P3welEEcoRV and wzbNsiI), using as template the chromosomal DNA from each strain ATCC 9595 (NCBI GenBank Acc. AY659976) and RW-9595M (NCBI GenBank Acc. AY659979). The PCR products were purified after gel electrophoresis from agarose gel stained with crystal violet and the four products were cloned into the pCR-XL-TOPO vector. *E. coli* TOP10 was transformed with the two recombinant plasmids according to the manufacturer's instructions. The restriction enzymes BglII and HindIII were utilized for digestion to confirm
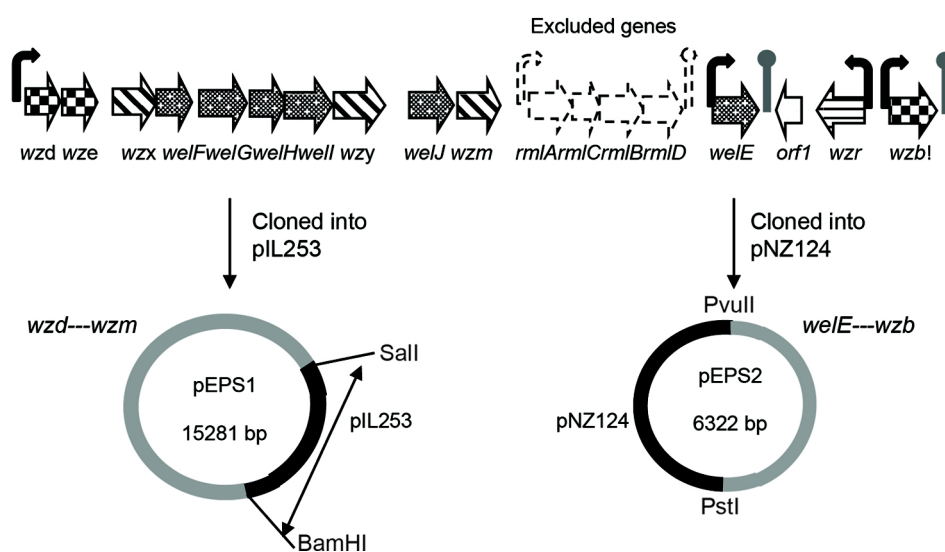

Fig. 1. Construction of plasmids pEPS1 and pEPS2 carrying EPS gene clusters in two fragments and deleting ORFs *rmlA, rmlC, rmlB, rmlD*. Plasmids pEPS1 and pEPS2 contain all the genes required for the biosynthesis of EPS: 10.5 kb and 3.7 kb *eps* genes (gray) were ligated to vectors pIL253 and pNZ124 (black) shuttle vectors, respectively, as described in "Materials and methods".

the inserts with genes *wzd* to *wzm* and genes *welE* to *wzb,* respectively (Fig. 1). The plasmids with genes *wzd* to *wzm* were verified by digestion into two fragments (3,790 bp and 10,295 bp) and the plasmids with genes *welE* to *wzb* were verified by digestion into three fragments 157 bp, 1924 bp and 5185 bp. Recombinant plasmid pCR-XL-TOPO with *wzd* to *wzm* was digested using BamHI/XhoI, then purified and ligated to pIL253 linearized with BamHI/SalI, resulting in plasmids pEPS1A (*L. rhamnosus* strain ATCC 9595) and pEPS1R (*L. rhamnosus* strain RW-9595M) coding for genes *wzd* to *wzm*. These recombinant plasmids (15,281 bp) include the endogenous P1 promoter from each strain. Plasmid pEPS2 (6322 bp) was constructed with *welE* to *wzb* digested by NsiI/EcoRV and ligated to pNZ124 linearized by PstI/PvuII. pEPS2A (encoding *welE* to *wzb* from *L. rhamnosus* strain ATCC 9595) and pEPS2R (encoding *welE* to *wzb* from *L. rhamnosus* strain RW-9595M) include the endogenous P3 promoter from each strain. *L. lactis* subsp. *cremoris* MG1363 was transformed by electroporation with plasmids pEPS1A and pEPS2A or pEPS1R and pEPS2R. To verify the inserts of pEPS1, PCR was performed to amplify *wzd* to *wze* (1,795 bp). EcoRI digestion was utilized for plasmid pEPS2 (6322 bp) to confirm insert size by the presence of three fragments at 2,496 bp, 3,765 bp and 61 bp.

The genes *wzd, wze* and *wzb* were cloned into pMG36CT, in combinations designed to eliminate each of the genes in turn, and to be able to replace the wild type *wzd* gene with genes containing 9 point mutations (Fig. 2). PCR was carried out to amplify genes for cloning into pMG36CT and the chromosomal DNA from each strain ATCC 9595 and RW-9595M was utilized for the PCR reaction. Genes *wzd, wze* or *wzb* were amplified with primers wzdRSacI and wzdFAatII, wzeRSacI and wzeFAatII, or wzbRSacI and wzbFAatII, respectively and then each PCR product was cloned into pMG36CT linearized with SacI/AatII for pD, pE, or pB, respectively. Genes *wzd* and *wze* were amplified together using wzdRSacI and wzeFAatII and cloned into pMG36CT linearized with SacI/AatII for the deletion of *wzb* (pDE). For the deletion of *wzd*, two genes, *wze* and *wzb,* were amplified separately with primers wzeRSacI/wzeF-wzb and wzbR-wze/wzbFAatII, respectively. The second step consisted of diluting both amplicons 1:50 and mixing them in equimolar ratio in order to amplify the combined
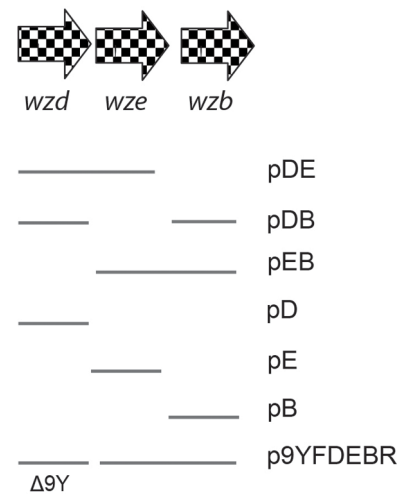

Fig. 2. Overview of plasmid construction leading to different combinations of three genes, *wzd*, *wze* and *wzb* from *Lactobacillus rhamnosus* RW-9595M or ATCC 9595, as well as *in vivo* expression of Wzd carrying nine tyrosine to phenylalanine mutations.

two genes using primers wzeRSacI and wzbFAatII. The amplification product of the combined genes was cloned into pMG36CT digested with SacI/AatII (pEB). For the deletion of *wze*, primers wzdRSacI/wzdF-wzb and wzbR-wzd/wzbFAatII were utilized for the first PCR. Two amplicons, wzd and wzb, were combined by PCR using wzeRSacI as reverse primer and wzbFAatII as forward primer, and the combined product was cloned into pMG36CT (pDB). For pMG36CT containing the 2.6 kb SacI-AatII PCR amplicon (*wzd, wze* and *wzb*) from either RW-9595M or ATCC 9595 (pDEB), *wze* and *wzb* were cloned into the plasmid first. Two genes were amplified with primers wzeRXbaI/wzeF-wzb and wzbR-wze/wzbFAatII for the first PCR, and two amplicons, wze and wzb, were combined by PCR using wzeRXbaI as reverse primer and wzbFAatII as forward primer. The combined genes were cloned into pMG36CT digested with XbaI/AatII (pMG36CT-wzewzb). The *wzd* gene was amplified using primers wzdRSacI and wzdFXbaI, and then cloned into pMG36CT-wzewzb digested with SacI/XbaI. A synthetic *wzd* gene carrying nine tyrosine mutations to phenylalanine was constructed by gene synthesis (GenScript, USA) and cloned into pMG36CT-wzewzb as was carried out with the original *wzd* gene, giving plasmid p9YFDEBR. The sequences of all constructs were verified by Sanger sequencing reactions

using the ABI Prism 3100 apparatus.

EPS isolation and quantification

L. lactis subsp. *cremoris* MG1363 (pEPS1A and pEPS2A or pEPS1R and pEPS2R) was cultured twice for 20 h in GM17 before inoculation at 2% (v/v) in 250 ml M17 broth supplemented with 0.5% glucose and 5 μg/ml erythromycin and chloramphenicol, respectively, then incubated for 24 h at 30℃. Following the method of Cerning [9] with some modifications, EPS was extracted from 50 ml of culture supernatant after 0 h and 24 h incubation. Bacterial cells were first removed by centrifugation at 12,000 ×*g* for 30 min at 4℃ then the EPS in supernatant was precipitated with 3 volumes of ethanol. The precipitated EPS was dialyzed for a total of five days, three days against distilled water and two days against demineralized water. EPS quantity is expressed in glucose equivalent units as determined by the phenol/sulfuric acid method [26]. Data are presented as the means of three independent extractions.

Western blotting

A volume of 15 ml of bacterial cells grown to 5×10^8 CFU/ml (A_{600} of 0.6 to 0.7) in GM17 were centrifuged at 15,000 ×*g* for 10 min at 4℃. The pellets were suspended in TE buffer (10 mM Tris-HCl (pH 8) and 0.1 mM EDTA) and mechanically disrupted by mini beadbeater (BioSpec, USA) with 1 g glass beads by two 30 s treatments with 1 min cooling on ice between two treatments. Twenty microliters were separated by SDS-PAGE and transferred by semi-dry electroblotting onto polyvinylidene difluoride membrane with the Trans-Blot SD semi-Dry transfer cell (Bio-Rad Laboratoires, Ltd., Canada) under constant current of $5.5\,\text{mA cm}^{-2}$ for 30 min. After blocking the membranes for 1 h with Block-PO Noise cancelling reagents (Millipore), mouse monoclonal IgG anti phosphotyrosine 4G10 (Millipore) was added at 1 : 1,000 dilution overnight at 4℃ to detect phosphotyrosine proteins. After six 5 min washes with Tris buffered saline (TBS) (100 mM Tris, pH 7.4, 0.9% NaCl) with 0.05% Tween 20 (TBST), membranes were incubated for 1 h with 1:20,000 dilution of secondary antibody (mouse anti-IgG) coupled with horseradish peroxidase (HRP) (Millipore). After six washes as above, $0.12\,\text{ml cm}^{-2}$ of Supersignal solution (Thermo scientific, Rockford, IL, USA) was used for HRP detection and visualized by

exposure to Amersham Hyperfilm ECL (GE Life Science, Buckingham, UK).

Results

Growth of recombinant *L. lactis* subsp. *cremoris* strains

Equivalent initial viable cell counts of 5×10^7 CFU/ml were inoculated for growth curves of *L. lactis* subsp. *cremoris* MG1363 carrying only vector plasmids (pIL253 and pNZ124) or carrying two recombinant plasmids. *L. lactis* subsp. *cremoris* MG1363 (pEPS1R and pEPS2R) and MG1363 (pEPS1A and pEPS2A) have identical growth (Fig. 3). The two recombinant strains reached stationary phase at 8 h, when the cell density was about 3×10^9 CFU/ml. In contrast, the stationary phase was attained sooner for strains of MG1363 carrying only the vector plasmids (pIL253 and pNZ124), at 6 h of incubation and at a slightly higher viable count of 4×10^9 CFU/ml.

EPS production by recombinant *L. lactis* subsp. *cremoris*

The lower glucose concentration of 0.5% was routinely used to limit carbon and acid production, while 2% provided sugar in excess during plate or batch culture. Two types of colonies were obtained on RGM17 solid medium, depending on the glucose concentration of either 0.5% or 2% (Fig. 4). On the plates containing 2% glucose, only
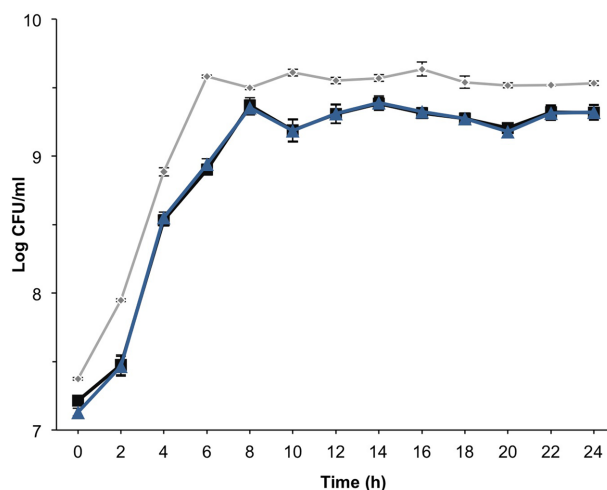

Fig. 3. Growth of *L. lactis* subsp. *cremoris* MG1363 (pIL253 and pNZ124) (gray diamonds, ◆), MG1363 (pEPS1R and pEPS2R) (black squares, ■) and MG1363 (pEPS1Aand pEPS2A) (triangles, ▲) in GM17 at 30℃. Data are the means of three independent growth curves.
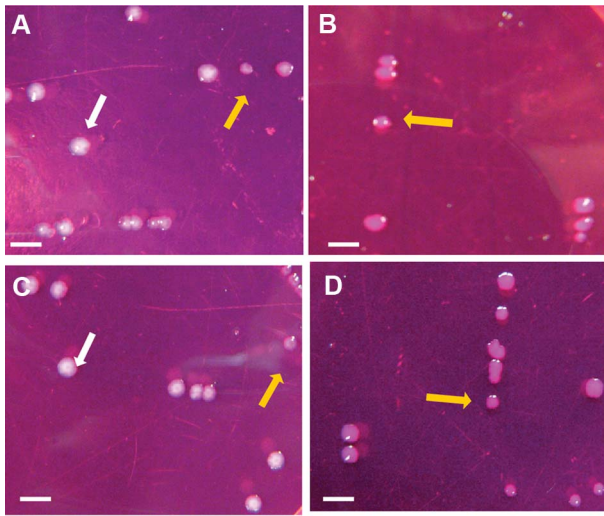
Fig. 4. Detection of the EPS producing derivatives of *L. lactis* subsp. *cremoris* MG1363 on RGM17 plates containing two concentrations of glucose and ruthenium red, showing two different colony types of *L. lactis* subsp. *cremoris* MG1363. Orange arrows show small red colonies and white arrows show large rosy white colonies. Scale bar = 5 mm. *L. lactis* subsp. *cremoris* MG1363 (pEPS1R and pEPS2R) with 0.5% (a) and 2% (b) glucose. *L. lactis* subsp. *cremoris* MG1363 (pEPS1A and pEPS2A) with 0.5% (c) and 2% (d) glucose.

Table 3. EPS production by recombinant strains of *L. lactis* subsp. *cremoris* MG1363 after 24 h incubation in GM17 medium containing 0.5% glucose.

Strain	EPS production (mg/l)
L. lactis subsp. *cremoris* MG1363 (pIL253 and pNZ124)	30.8 ± 9.0
L. lactis subsp. *cremoris* MG1363 (pEPS1R and pEPS2R)	302.7 ± 6.2
L. lactis subsp. *cremoris* MG1363 (pEPS1A and pEPS2A)	326.8 ± 26.4

as the values remained similar to *L. lactis* subsp. *cremoris* MG1363 (pIL253 and pNZ124). *L. lactis* subsp. *cremoris* MG1363 (pEPS1A and pEPS2A) and MG1363 (pEPS1R and pEPS2R) produced respectively 302 or 326 mg/l (Table 3) in GM17 with 0.5% glucose, which is not significantly different (Student's t test, $p = 0.46$).

Determination of protein tyrosine phosphorylation state

As negative controls, there was no phosphorylated protein when extracts from *L. lactis* subsp. *cremoris* MG1363 with or without pMG36CT were probed with the anti-phosphotyrosine antibody (Fig. 5). No difference was observed between the recombinant strains carrying genes from ATCC 9595 (data not shown) or from RW-9595M (Fig. 5). When the three proteins were expressed separately in *L. lactis* subsp. *cremoris* MG1363 carrying pD, pE or pB, no tyrosine phosphorylated proteins were detected. Two tyrosine-phosphorylated proteins were detected in extracts of *L. lactis* subsp. *cremoris* MG1363

red colonies were present for both recombinant strains *L. lactis* subsp. *cremoris* MG1363 (pEPS1R and pEPS2R) and MG1363 (pEPS1A and pEPS2A). On RGM17 plates with 0.5% glucose, both red and rosy white colonies were present for both strains. Ruthenium red can bind with the charged polysaccharide pellicle of *L. lactis* [27]. Red and moderately rough colonies exhibited a phenotype similar to the wild-type strain *L. lactis* subsp. *cremoris* MG1363. Rosy white colonies were smooth, large and shiny and this mucoid colony phenotype resembles that of the strain of origin for the genes, *L. rhamnosus* RW-9595M. There was no visible difference between the colony types of *L. lactis* subsp. *cremoris* MG1363 (pEPS1A and pEPS2A) and MG1363 (pEPS1R and pEPS2R).

The white colonies were chosen for quantifying EPS production in GM17 broth containing either 0.5% or 2% glucose after 24 h incubation without pH control. A red colony of *L. lactis* subsp. *cremoris* MG1363 (pIL253 and pNZ124) was used as the EPS-negative control strain. In GM17 with 2% glucose, all recombinant strains produced a non-significant level of EPS around 30 mg/l,
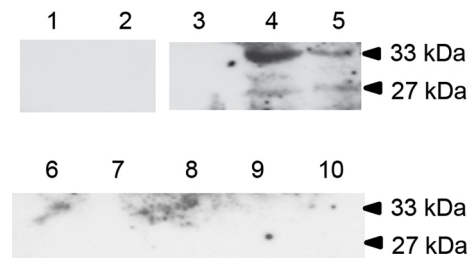

Fig. 5. Western blotting of the phosphorylation state of Wzd and Wze proteins expressed in *L. lactis* subsp. *cremoris*, probed with mouse monoclonal anti phosphotyrosine antibody. The proteins were separated by 12% SDS-PAGE. Detection of tyrosine phosphorylated Wzd (33 kDa) and Wze (27 kDa) expressed in *L. lactis* subsp. *cremoris* MG1363 (lane 1) with pMG36CT (lane 2), p9YFDEBR (lane 3), pDER (lane 4), pDEBR (lane 5), pDBR (lane 6), pEBR (lane 7), pDR (lane 8), pER (lane 9) and pBR (lane 10).

(pDER), one was the size of Wzd (33 kDa), and the other was the size of Wze (27 kDa). On the other hand, the antibody did not detect either the 33 kDa or the 27 kDa protein in extracts of the Wze or Wzd deletion mutants, *L. lactis* subsp. *cremoris* MG1363 (pDBR) or (pEBR) respectively. *L. lactis* subsp. *cremoris* MG1363 (pDEBR) gave the same two bands of the expected sizes of 33 kDa and 27 kDa as with *L. lactis* subsp. *cremoris* MG1363 carrying pDER. The 27 kDa anti-phosphotyrosine antibody signals of Wze were equivalent, although weak, for *L. lactis* subsp. *cremoris* MG1363 pDER and pDEBR (Lanes 4 and 5 of Fig. 5). However, the 33 kDa Wzd signal was stronger for *L. lactis* subsp. *cremoris* MG1363 (pDER) than in the extract from the strain carrying pDEBR. When all 9 tyrosines were mutated in the Wzd sequence, no phosphorylated proteins were detected, while the proteins were detected by Commassie staining.

Discussion

L. rhamnosus RW-9595M and ATCC 9595 produce different quantities of EPS, although their ORFs are 99% identical [18]. The 13 out of 17 genes of the *eps* gene clusters from two strains were successfully cloned and transferred to *L. lactis* subsp. *cremoris* MG1363, a host that does not produce significant amounts of liberated EPS (around 15 to 30 mg/l). Four genes (*rmlA, B, C* and *D*) for synthesis of dTDP-rhamnose were excluded, because the MG1363 genome already codes for these functions. Both *L. lactis* subsp. *cremoris* MG1363 (pEPS1R and pEPS2R) and MG1363 (pEPS1A and pEPS2A) produced more EPS than other EPS-producing *L. lactis* subsp. *cremoris* strains such as SMQ-461 (152 mg/l in M17 with 2% lactose) [22]. The EPS biosynthetic genes are located on the chromosome in strain SMQ-461. The NIZO B40 *eps* gene cluster from *L. lactis* NZ9000 (pNZ4030) produced only 93 mg/l with 6% glucose when transferred to strain MG1363 [28]. However, the identical *eps* gene cluster transferred to the high copy number plasmid pIL253, estimated at 45-80 copies per chromosome equivalent [29], led to increased EPS production at 343 mg/l in the same strain. In this study, the *eps* genes were also cloned into high copy number plasmids pIL253 and pNZ124. Therefore, the transformed *L. lactis* subsp. *cremoris* MG1363 strains may produce more EPS than SMQ-461 because of the copy number of *L. rhamnosus*

eps genes provided by the recombinant plasmids.

EPS production requires sugar transportation into the cytoplasm in order to form precursors for the biosynthesis of repeating units, so sugar concentration affects EPS production [9]. Increased carbon source generally leads to raised EPS production in lactic acid bacteria. EPS production is also associated with growth as *L. rhamnosus* R started the production of EPS at the end of the exponential phase [30]. The synthesis was carried on beyond the growth decline phase with glucose. In our study, EPS producing mutants reached stationary phase later than the non EPS-producing strain, *L. lactis* subsp. *cremoris* MG1363 (pIL253 and pZN124). The higher EPS production obtained with 0.5% than with 2% glucose might be due to catabolite repression, analogous to the glucose catabolite repression of the *gal* operon, suggested in a previous study [31]. In Gram positive bacteria, catabolite repression could be regulated by CcpA binding to DNA target sites, which are catabolite-responsive elements (*cre*). While binding of CcpA to *cre* upstream of the promoter activates transcription, when *cre* is located downstream, CcpA down regulates the activity of the promoter. Repression also occurs when CcpA binds to a *cre* site that overlaps the promoter [32–34]. Thus, the CcpA protein of *L. lactis* subsp. *cremoris* MG1363 may recognize a potential CRP site overlapping the -35 sequence of the P1 promoter [18]. In the original parental strains of *L. rhamnosus*, DNA upstream of the *eps* cluster, which was not included in the cloned constructs in this study, may counter catabolite repression by CcpA.

L. rhamnosus RW-9595M produces 1161 mg/l EPS while ATCC 9595 produces about 60 mg/l EPS after 24 h fermentation controlled at pH 6.0 with 4% glucose. However, EPS production by the two recombinant *L. lactis* subsp. *cremoris* strains did not differ significantly. Thus, *L. lactis* subsp. *cremoris* MG1363 produces the same amount of EPS irrespective of gene source. In the present study, only 13 genes were transferred to *L. lactis* subsp. *cremoris* MG1363. The whole genome sequences of these *L. rhamnosus* strains have not been determined, while 17 ORFs have been identified as putative EPS biosynthesis genes. Therefore, it is also possible that these two *L. rhamnosus* strains have another system to regulate or increase EPS production. However, *L. lactis* expressing the genes from ATCC 9595 is able to synthesize over twice the amount of EPS as the original strain.

This means that either ATCC 9595 metabolism may not be able to provide enough precursors to allow the same level of production as RW-9595M, or that gene regulation in RW-9595M provides more precursors. Previous studies showed that *L. lactis* subsp. *cremoris* MG1363 expressing *eps* genes from *S. thermophilus* produced an EPS of a different structure and quantity [35]. They suggest that these differences are due to the lack of a precursor, and the difficulty of certain glycosyltransferases to recognize the subunit. The polymerization machinery has a wide specificity, as it can still produce EPS even though the subunit is different.

The auto- and trans-phosphorylation of the CpsD (PTK) requires the presence of the CpsC (TKM), and appears to negatively affect CPS polymerization in *S. pneumoniae* strain Rx1-19F [12]. A similar protein complex formation has been proposed in *L. lactis* [36] and *S. thermophilus* [14]. The proteins Wzd, Wze and Wzb predicted to be the modulator, the tyrosine kinase and the tyrosine phosphatase, may play a role in the control of EPS elongation [37]. In the present study, these proteins were expressed in *L. lactis* subsp. *cremoris* MG1363 using the genes *wzd, wze* and *wzb* from *L. rhamnosus* ATCC 9595 and RW-9595M. The phosphorylation states of Wzd, Wze and Wzb proteins did not differ between *L. rhamnosus* ATCC 9595 and RW-9595M. When they were expressed individually, none of the three proteins were tyrosine phosphorylated. This suggests that the proteins are not phosphorylated by any protein of *L. lactis* nor can they auto-phosphorylate. When expressed together, Wzd and Wze were both found in phosphorylated form. There were no phosphorylated proteins in the absence of Wzd or Wze. This means that the Wzd modulator is needed for auto- or trans-phosphorylation of the Wze kinase in *L. lactis*, as described for *S. pneumoniae* [38], but does not have kinase activity itself. Our study shows that Wze is also required for tyrosine phosphorylation of Wzd. The Wzd-P signal conferred by pDEBR was consistently low compared to the signal in the *wzb* mutant strain. This finding indicates that dephosphorylation of Wzd-P involves the Wzb phosphatase. Moreover, the mutant Wzd protein showed identical results to that of the Wzd deletion. No proteins were phosphorylated when all nine tyrosines in Wzd were mutated to phenylalanine. This result suggests that tyrosine phosphorylation of Wzd is necessary for the phosphorylation of Wze, but also that the presence and phosphorylation of Wze is required for the phosphorylation of Wzd.

Mechanisms for polysaccharide biosynthesis seem to vary with species and with the diverse types of polysaccharide such as CPS or EPS. Many studies show mainly the phosphorylation of the kinase regardless of the type of polysaccharide. In *S. pneumoniae*, deletion of the modulator and the kinase leads to a decrease in the amount of CPS attached to the cell wall [39]. The modulator and kinase are also essential for EPS production in *L. lactis* NIZO B40 [36]. A phosphorylated protein of approximately 25 kDa was detected in this strain. That study suggested that the protein kinase (EpsB) is phosphorylated when the modulator (EpsA) is present. However, this protein could instead have been phosphorylated EpsA, which is similar in size (28 kDa) to the kinase (25 kDa). Therefore, it is possible that the modulator is also phosphorylated in this species.

The properties of EPS as food additives and their beneficial effects on human health such as immunomodulation are related to the chemical and physical properties of EPS. Increasing knowledge on biosynthesis of EPS will make it possible to enhance their production under defined conditions and to develop their applications by studying the relationship between structure and function. This study shows that *L. lactis* subsp. *cremoris* MG1363 could produce EPS with the *eps* gene clusters of *L. rhamnosus* under conditions of limited quantities of glucose and provides a model of EPS production for both strains. While the phosphorylation state of Wze is well known, our study contributes that phosphorylation of Wzd occurs in *L. lactis* subsp. *cremoris* MG1363 and in *L. rhamnosus*. Future experiments will use the *L. lactis* expression system to determine the role of Wzd and the impact of tyrosine phosphorylation on EPS biosynthesis, in order to determine their function in modulating the biosynthesis of exopolysaccharides.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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