Supporting information

Biosynthesis of cell wall polysaccharide in *Lactococcus lactis*: a dual chain assembly pathway to generate high structural diversity

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Table S3. Strains, plasmids, bacteriophages used and developed in this study

Strain, plasmid, or phage	Feature(s)	Source
Bacterial strains *L. lactis* **subsp.** ***cremoris***		
NZ9000	*L. lactis* MG1363 derivative containing *nisRK*, host to phages jj50, p2, and sk1	[1]
VES5751	*L. lactis* MG1363 derivative exhibiting a deficient PSP phenotype due to a spontaneous mutation in *llmg_0226* (CCAA duplication)	[2]
NZ9000-*wpsJ*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01120* resulting in a TGA and a TAA stop codon in-frame insertion	This work
NZ9000-*wpsA*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01135* resulting in a TGA and a TAA stop codon in-frame insertion	This work
NZ9000- *wpsB*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01140* resulting in a TGA and a TAA stop codon in-frame insertion	This work
NZ9000-*wpsD*	NZ9000 with a GAATTC inserted in the locus tag *llnz_01145* resulting in a TGA stop codon in-frame insertion.	[3]
NZ9000-*wpsE*	NZ9000 with a TAATGACCC inserted in the locus tag *llnz_01150* resulting in a TAA and a TGA stop codon in-frame insertion	This work
NZ9000-*wpsF*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01155* resulting in a TGA and a TAA stop codon in-frame insertion	This work
NZ9000-*wpsG*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01160* resulting in a TGA and a TAA stop codon in-frame insertion	This work
NZ9000-*wpsH*	NZ9000 with a GATATCG inserted in the locus tag *llnz_01175* resulting in a TGA stop codon insertion	This work
NZ9000-*wpsI*	NZ9000 with a TGATAACCC inserted in the locus tag *llnz_01160* resulting in a TGA and a TAA stop codon in-frame insertion	This work
Plasmids		
pCNR	Recombineering-facilitating vector containing *recT*, P*nisA,* Cm^r derived from the low-copy vector pPTPi	This work
pVPL3004	Low-copy vector expressing *cas9* along with tracRNA, Ery^r	[4]
pCRISPR	High-copy vector carrying CRISPR repeats and used for integrating targeting spacer sequences, Tet^r	[4]
pPTPL	*E. coli-L. lactis* promoter-probe vector, Tet^r	[5]
pPTPL::NZProm1-8	pPTPL containing one of the 9 intergenic regions found within the NZ9000 CWPS gene cluster	This work

Bacteriophages		
jj50	936 species, propagated on NZ9000	[6]
sk1	936 species, propagated on NZ9000	[7]
p2	936 species, propagated on NZ9000	[8]
MCC1	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
MCC5	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
MCC17	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
IT1	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
IT2	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
IT3	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
IT4	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work
IT5	936 species, derivative of Φsk1, propagated on *L. lactis* VES5751	This work

Table S2. Primers used in the development of the promoter-probe pPTPL and pCRISPR constructs, for the screening of the CWPS mutants, and the primer extension analysis of the identified CWPS promoter regions.

Purpose	**Sequence 5'-3'**	**Oligo Name**	**pPTPL Construct**
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01075* into pPTPL	gcgcgcggatcccagtaacgattttctctgg	oIT95	*NZProm1*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01075* into pPTPL	gcgcgctctagacagttgttttgacgcagc	oIT96	*NZProm1*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01085* into pPTPL	gcgcgcggatcctggtcttcaatgggaagt	oIT97	*NZProm4*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01085* into pPTPL	gcgcgctctagattcgacacgtccgctgtca	oIT98	*NZProm4*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01090* into pPTPL	gcgcgcggatcccagaacttggttggactc	oIT99	*NZProm5*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01090* into pPTPL	gcgcgctctagagacgcaactctgttccaa	oIT100	*NZProm5*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01095* into pPTPL	gcgcgcggatccaagcgacaggttttgtca	oIT101	*NZProm6*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01095* into pPTPL	gcgcgctctagacaaaccctccatatttcgc	oIT102	*NZProm6*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01135* into pPTPL	gcgcgcggatccaacggacctaacacaaacg	oIT103	*NZProm2*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01135* into pPTPL	gcgcgctctagattgataaggacaactggc	oIT104	*NZProm2*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01145* into pPTPL	gcgcgcggatccatttgggtacaagtattgc	oIT105	*NZProm7*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01145* into pPTPL	gcgcgctctagaccacttctgatattccttc	oIT106	*NZProm7*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01160* into pPTPL	gcgcgcggatccctaagagctactggtaagt	oIT107	*NZProm8*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01160* into pPTPL	gcgcgctctagatgtctgaatatggtgtgag	oIT108	*NZProm8*
Fwd primer for cloning intergenic region upstream of locus tag *llnz_01175* into pPTPL	gcgcgcggatccatctggtgcagtgatgtt	oIT109	*NZProm3*
Rev primer for cloning intergenic region upstream of locus tag *llnz_01175* into pPTPL	gcgcgctctagagccccatcactccaataa	oIT110	*NZProm3*
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01120*	aaacacgtctttttttaatgattgctcttgttgcg	oIT45	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01120*	aaaacgcaacaagagcaatcattaaaaaaagacgt	oIT46	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01120*	gcagcacaagaagatagcag	oIT47	N/A

Rev primer for sequencing and screening for mutated variants of *llnz_01120*	gcagtcatagcttaaacttgtagc	oIT48	N/A
Fwd primer annealing to the inserted sequence in *llnz_01120* used for screening mutant variants	gctcttgtttgataaccc	oIT49	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_1135*	aaacagggaatattagaaaatctaaaaattgtagg	oIT50	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01135*	aaaacctacaatttttagattttctaatattccct	oIT51	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01135*	acaatgatttggtatcgtccac	oIT52	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01135*	cacccacctctttgacacg	oIT53	N/A
Rev primer annealing to the inserted sequence in *llnz_01135* used for screening mutant variants	ggcttgatagggttatca	oIT54	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01140*	aaaccagctttcttctttatctatacgattcgttg	oIT55	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01140*	aaaacaacgaatcgtatagataaagaagaaagctg	oIT56	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01140*	ccttaccaaacttgattgaacc	oIT57	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01140*	caatgcctccaccttcac	oIT58	N/A
Fwd primer annealing to the inserted sequence in *llnz_01140* used for screening mutant variants	cgattcgttgataaccc	oIT59	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01150*	aaacattcaattttagaacaaacatacaaaaactg	oIT60	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01150*	aaaacagtttttgtatgtttgttctaaaattgaat	oIT61	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01150*	ttatgaaattgatgaatactctaaacctag	oIT62	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01150*	cagcaatagataggacttgaag	oIT63	N/A
Fwd primer annealing to the inserted sequence in *llnz_01150* used for screening mutant variants	catacaaaaactaatgaccc	oIT64	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01155*	aaacgtaacatacttggatagcgaagatacagatg	oIT65	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01155*	aaaacatctgtatcttcgctatccaagtatgttac	oIT66	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01155*	gatatatcaatgtatgaagatgc	oIT67	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01155*	cttcaagactatttagaacc	oIT68	N/A

Fwd primer annealing to the inserted sequence in *llnz_01155* used for screening mutant variants	gaagatacatgataaccc	oIT69	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01160*	aaacaatagactcagacactatatttaaggatagg	oIT70	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01160*	aaaacctatccttaaatatagtgtctgagtctatt	oIT71	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01160*	acaaatttatgggcttttacc	oIT72	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01160*	cctggtccataaagattg	oIT73	N/A
Fwd primer annealing to the inserted sequence in *llnz_01160* used for screening mutant variants	tatttaaggattgataaccc	oIT74	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01175*	cagtaccttcttccattaaatcgg	oIT75	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01175*	actttaacttcacccatatctgg	oIT76	N/A
Fwd primer annealing to the inserted sequence in *llnz_01175* used for screening mutant variants	tgtaagtgcttgatatcg	oIT77	N/A
Fwd oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01180*	aaactgctcttttctttggaattatcctatccatg	oIT78	N/A
Rev oligonucleotide for cloning CRISPR spacer into pCRISPR targeting the mutated region of *llnz_01180*	aaaacatggataggataattccaaagaaaagagca	oIT79	N/A
Fwd primer for sequencing and screening for mutated variants of *llnz_01180*	ggtttgggagaaggaaaag	oIT80	N/A
Rev primer for sequencing and screening for mutated variants of *llnz_01180*	cacagctacaagaaaagc	oIT81	N/A
Fwd primer annealing to the inserted sequence in *llnz_01160* used for screening mutant variants	atcctatcctgataaccc	oIT82	N/A
Amplification of *NZProm1* promoter fragments with IRD700-labelled oligonucleotides	catgaaagggattattttagcg	oIT83	N/A
Amplification of *NZProm1* promoter fragments with IRD700-labelled oligonucleotides	ctttacccactgacacgtgc	oIT84	N/A
Fwd primer, amplification of region containing *NZProm1* promoter region for sequencing ladders	aaacgtcaatttaacatccaatc	oIT85	N/A
Rev primer, amplification of region containing *NZProm1* promoter region for sequencing ladders	gcacgtgtcagtgggtaaag	oIT86	N/A
Amplification of *NZProm2* promoter fragments with IRD700-labelled oligonucleotides	cgatgacaaaaaaatgaaaattttac	oIT87	N/A
Amplification of *NZProm2* promoter fragments with IRD700-labelled oligonucleotides	gaaaatctaaaaattgtagagga	oIT88	N/A
Fwd primer, amplification of region containing *NZProm2* promoter region for sequencing ladders	gtaattatcgcctaccgtttg	oIT89	N/A
Rev primer, amplification of region containing *NZProm2* promoter region for sequencing ladders	tcctctacaatttttagattttc	oIT90	N/A
Amplification of *NZProm3* promoter fragments with IRD700-labelled oligonucleotides	gtataaaaaggtaaccttcttag	oIT91	N/A

Amplification of *NZProm3* promoter fragments with IRD700-labelled oligonucleotides	cttagttacattggagtgatgg	oIT92	N/A
Fwd primer, amplification of region containing *NZProm3* promoter region for sequencing ladders	caattctttgaagatgaatatgc	oIT93	N/A
Rev primer, amplification of region containing *NZProm3* promoter region for sequencing ladders	ccatcactccaatgtaactaag	oIT94	N/A

Table S3. Mutational oligonucleotides used to generate the CWPS gene knock-out derivatives using CRISPR-Recombineering.

Purpose	Sequence 5'-3'	Oligo Name
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01120*	t*c*t*g*g*ttcaataaagagtggttgagcaactgtaaaaattaaac cgggttatcaaacaagagcaatcattaaaaaaagacgttctggtttg	oIT129
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01135*	t*t*t*a*t*cactacatagtctaattgataaggacaactggcttgata gggttatcaaatttttagattttctaatattccctcaccttcattg	oIT130
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01140*	a*a*c*c*a*ttttaacatatgtcttatttctgctctatctttacggggtt atcaacgaatcgtatagataaagaagaaagctgccaaaataatt	oIT131
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01150*	g*g*t*a*g*aaccatcatcaataagtataatttccaagtttttgtcga attcatgtttgttctaaaattgaatttatacactcttccaaatata	oIT132
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01155*	c*g*c*c*t*caactacatcactaccatcattttcagctttagcttttatg ggttatcatgtatcttcgctatccaagtatgttactataaagtc	oIT133
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01160*	t*t*t*a*t*ttgtctgaatatggtgtgagaataacgatttatagggtta tcaatccttaaatatagtgtctgagtctattttagctttggcccc	oIT134
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01175*	g*a*a*g*t*aactttgattgataaattcgtagtcaaagttttatccgcg atatcaagcacttacagtatgtccttcatagaaagtaaagagtaa	oIT135
Recombineering Oligo used in the stop codon insertion into the locus tag *llnz_01175*	a*c*a*a*a*atgataaacaccatctggttcgttaaagaacggcatgg gttatcaggataggataattccaaagaaaagagcaattgacaaataa	oIT124

*=5' phosphorthioate nucleotide modifications

Table S4. Genomic sequence analysis of the eight sk1 escape mutants.

Phage Name	**Size (bp)**	**Open Reading Frames**	**Total #SNPs**	**Baseplate-encoding region (*orf15-orf18*) #SNPs**
sk1	28451	54	N.A.	N.A.
IT1	28452	54	5	5
IT2	28452	54	4	4
IT3	28452	54	5	5
IT4	28452	54	6	5
IT5	28452	54	7	7
MCC1	28452	54	5	5
MCC5	28452	54	5	5
MCC17	28452	54	6	5

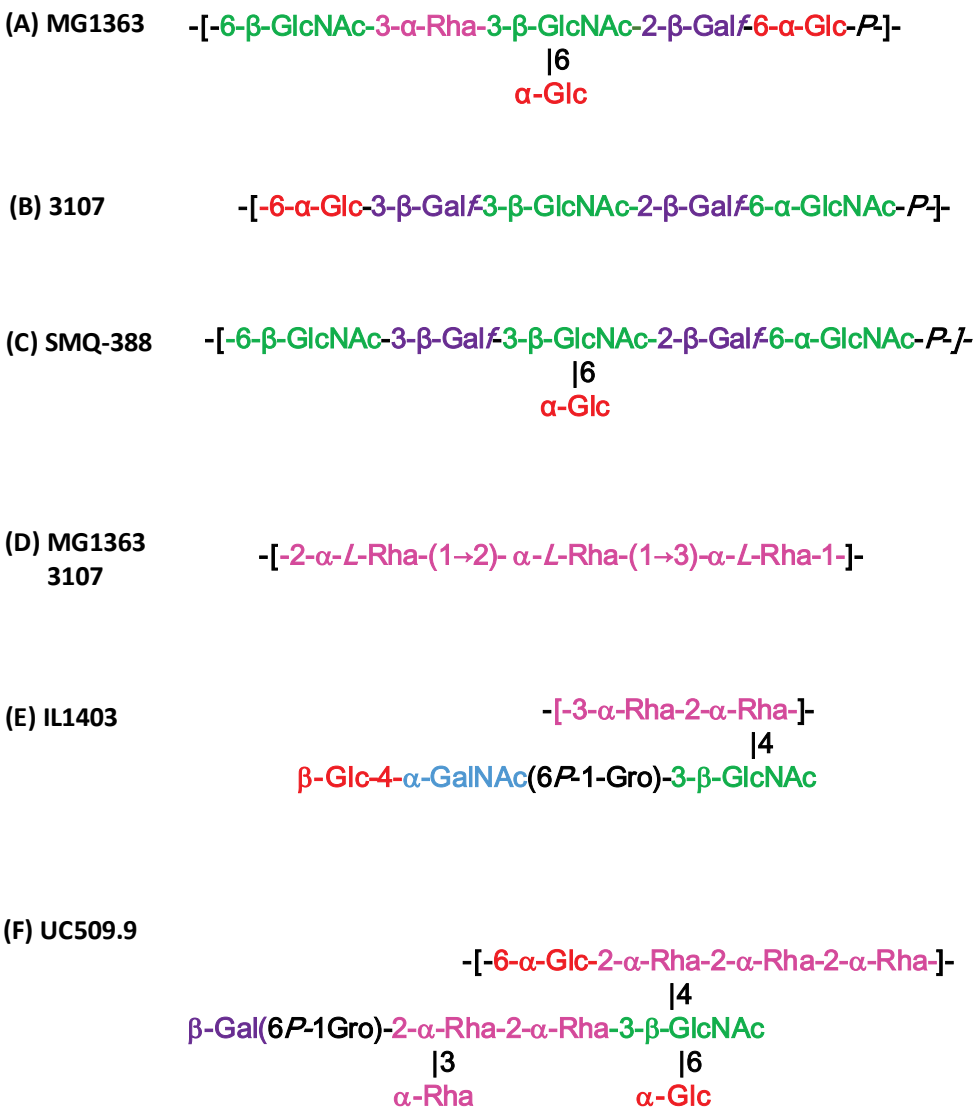

Figure S1. Chemical structures of *L. lactis* CWPS previously established by NMR.
(A, B, C) PSP structures found in MG1363, 3107 and SMQ-388 (C-type strains) (2, 3, 9);
(D) Rhamnan structure found in MG1363 and 3107 (C-type strains) (10);
(E, F) One chain CWPS structures found in IL1403 and UC509.9 (B and A type strains, respectively) (11, 12). Gro, glycerol; *P*, phosphate.

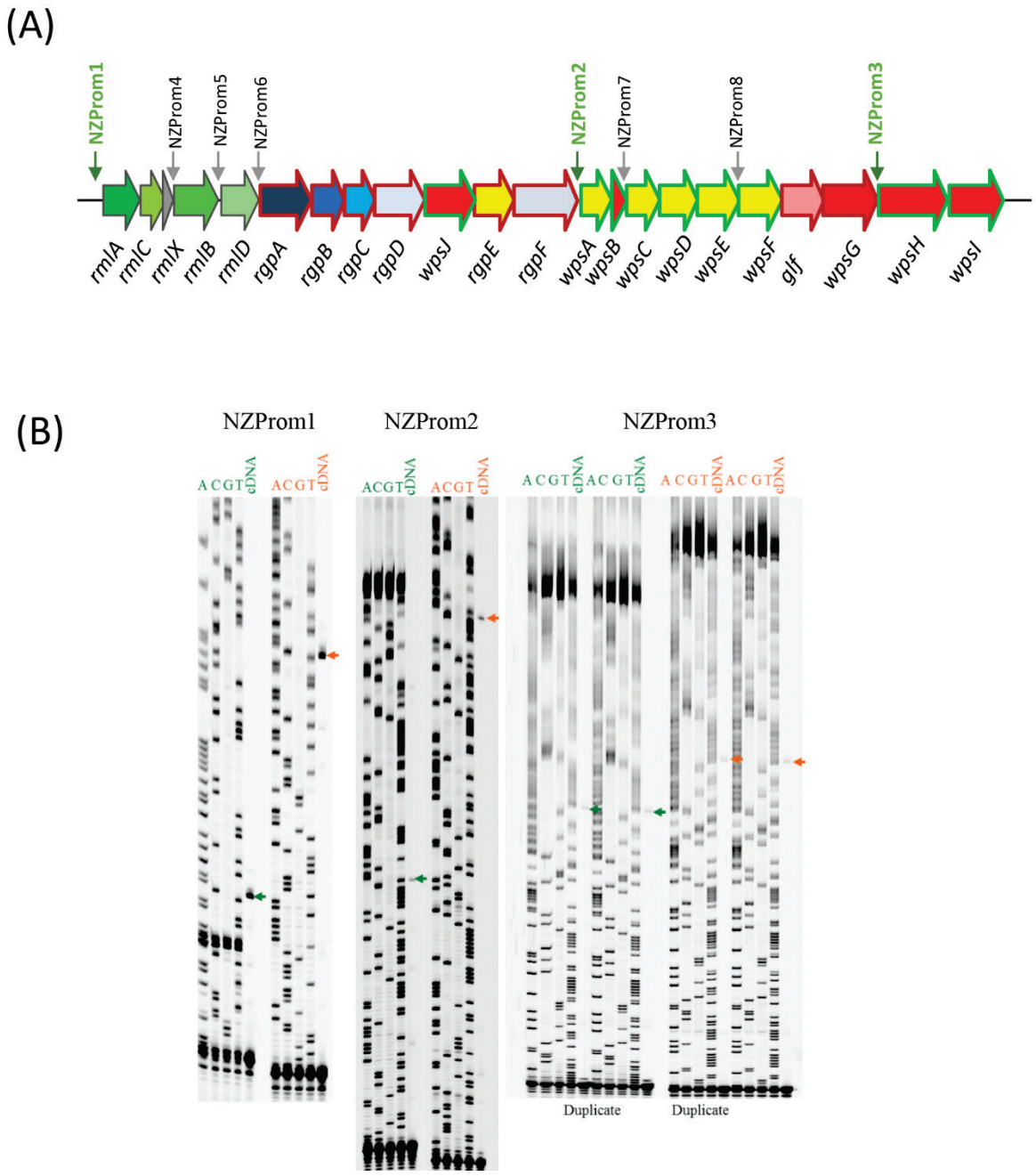

Figure S2. (A) Schematic representation of the *L. lactis* NZ9000 *cwps* gene cluster encoding rhamnan and PSP biosynthesis. The intergenic regions that were cloned into pPTPL vector for promoter probing are highlighted over the operon (*NZProm1-8*) (Table S2). Intergenic regions highlighted with a green arrow contain a putative promoter based on X-gal blue/white colony assays and primer extension analysis. Genes with successfully introduced non-sense mutations are highlighted with a green border while genes for which non-sense mutations could not be obtained are highlighted by a dark-red border. (B) 10 % Li-Cor Matrix KB Plus acrylamide gel including the sequence ladders and primer extension reactions for the promoter containing regions *NZProm1*, *NZProm2*, and *NZProm3*.

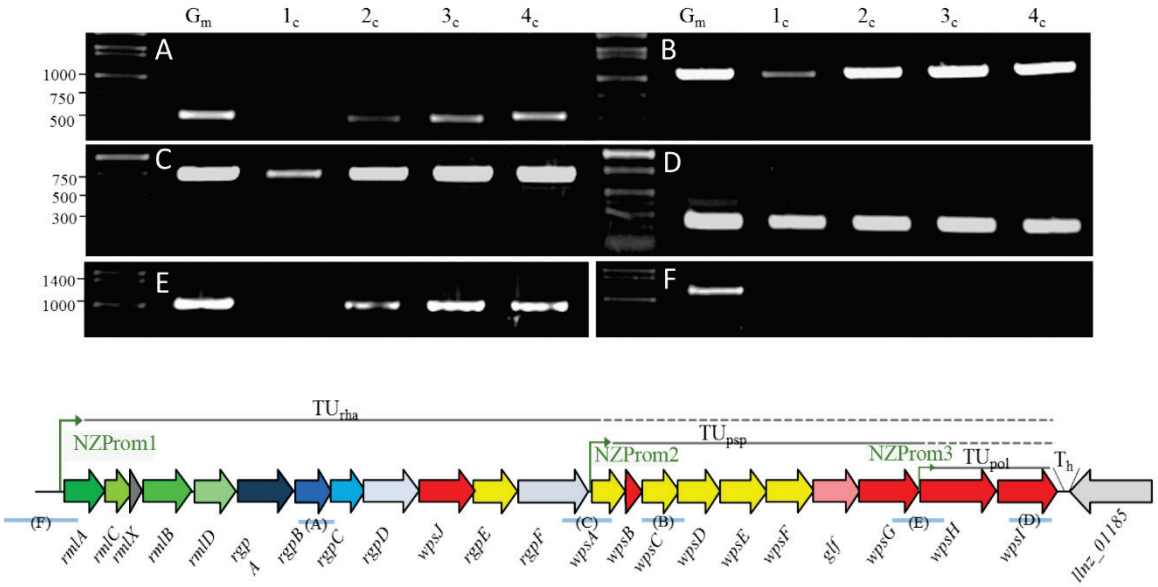

Figure S3. (*Top panel*) Gel electrophoresis summarizing the RT-PCR results for the transcriptional organization of the *cwps* gene cluster. Following total mRNA isolation at mid-exponential growth from four biological independent cultures of *L. lactis* NZ9000 (1_c-4_c) and conversion into cDNA, the transcriptional organization of the gene cluster was examined through PCR amplification of five regions within the cluster as well as one flanking *NZProm1* region as negative control. Genomic DNA (G_m) was also included for every set of primers as a positive control. PCR amplifying regions within or directly flanking (A) *rgpB*, (B) *wpsC*, (C) *NZProm5*, (D) *wpsI*, (E) *NZProm8*, (F) *NZProm1* (see bottom panel). Numbers on the left-hand side of the figure indicate the predicted size of the ladder's DNA fragments. Amplifications from cDNA of fragments C and E overlapping *NZProm2* and *NZProm3*, respectively, indicate transcriptional read-through, while the results for both the positive (A, B, D) and negative control (F) amplifications were according to expectations

(~~*Down*~~ *Bottom panel*) Schematic representation of the *L. lactis* NZ9000 *cwps* gene cluster highlighting its deduced transcriptional organization. The three identified promoter regions are shown by green arrows ahead of *rmlA*, *wpsA*, and *wpsH*. Three transcriptional units based on these promoter regions are also identified with grey lines (dashed lines indicate potential transcriptional read-through identified by the RT-PCR above). The regions amplified during RT-PCR are also highlighted with light blue bands below the cluster. Finally, the end of the gene cluster is highlighted by the inclusion of the first ORF (*llnz_01185*) downstream of it as well as the deduced terminator hairpin (T_h) as deduced by the online platform ARNold. TU_{rha}, rhamnan transcriptional unit; TU_{psp}, PSP transcriptional unit; TU_{pol}, polymerization module transcriptional unit.

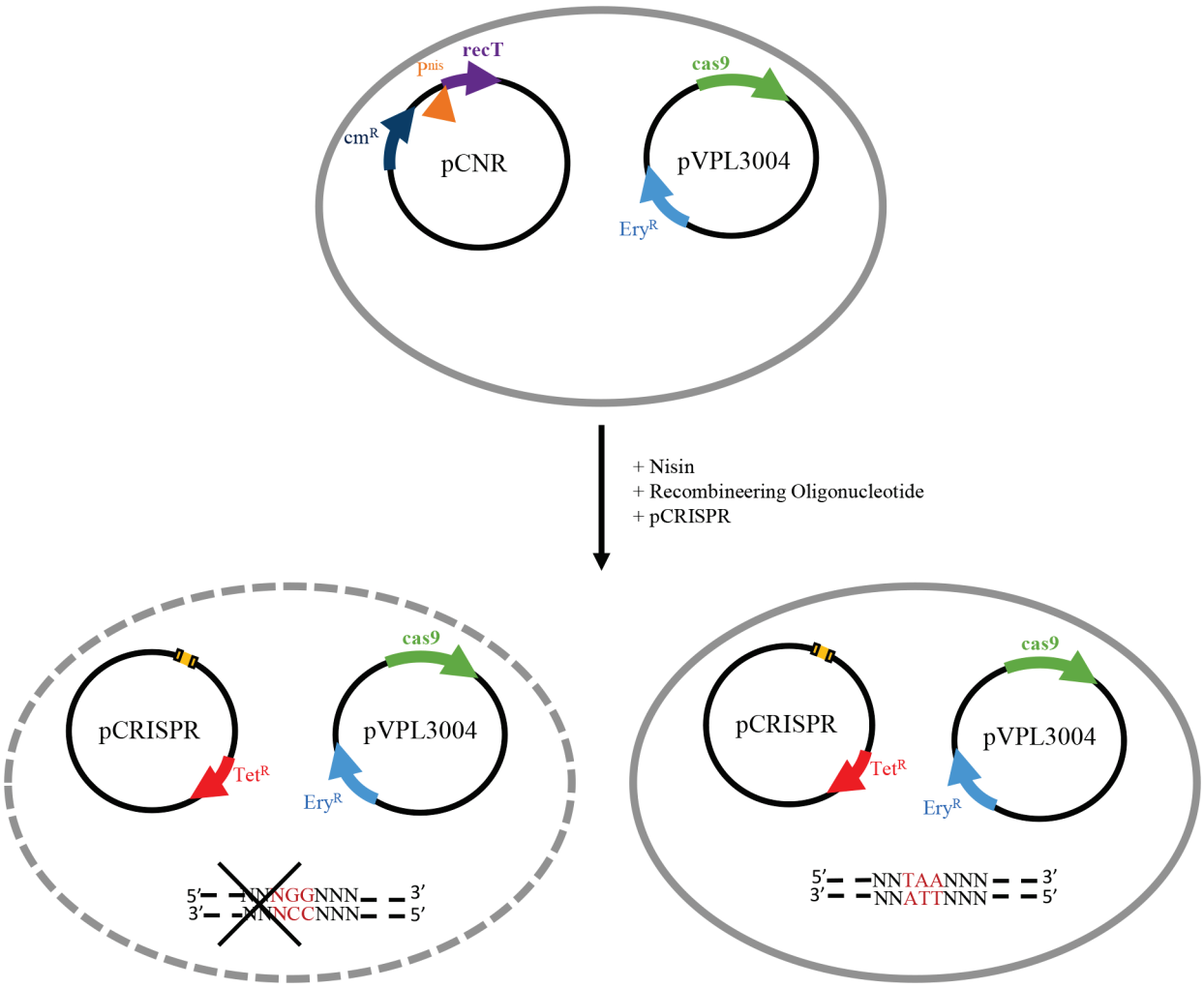

Figure S4. Schematic representation of the CRISPR-Cas9-assisted recombineering employed in the current study. *L. lactis* NZ9000 carrying pCNR and pVPL3004 is made electrocompetent and the *recT* gene in pCNR is induced by the addition of the nisin peptide. The recombineering oligonucleotide carrying a nonsense mutation for the gene of interest that also removes the NGG protospacer adjacent motif needed for Cas9 recognition and cleavage. The pCRISPR carrying the targeting sequence matching that of the nonsense mutation is simultaneously introduced into the *L. lactis* strain. Any strains that successfully incorporate the recombineering oligonucleotide into their genomes will bypass the CRISPR-Cas9 nuclease while any cells retaining the wild-type sequence will be eliminated from the total population through the action of Cas9.

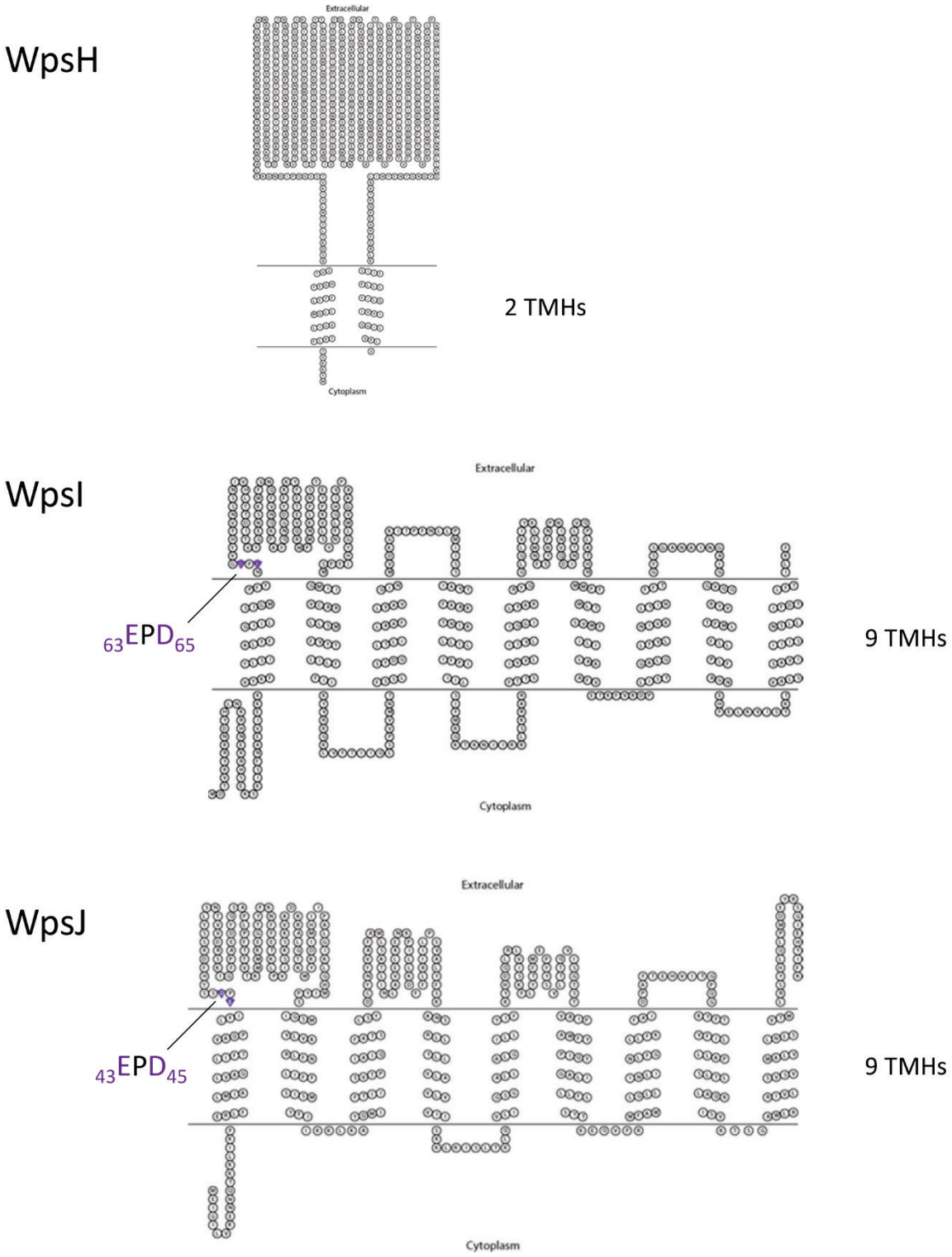

Fig. S5: Predicted topology of WpsH, WpsI and WpsJ. Transmembrane helices were predicted by TMHMM 2.0 and graphical representation were made with TOPO2. The modified catalytic motif "DXD" present in the first extracellular loop of WpsI and WpsJ is indicated.

References

1. Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. Journal of Biotechnology 64:15-21.
2. Chapot-Chartier MP, Vinogradov E, Sadovskaya I, Andre G, Mistou MY, Trieu-Cuot P, Furlan S, Bidnenko E, Courtin P, Pechoux C, Hols P, Dufrene YF, Kulakauskas S. 2010. Cell surface of *Lactococcus lactis* is covered by a protective polysaccharide pellicle. Journal of Biological Chemistry 285:10464-71.
3. Ainsworth S, Sadovskaya I, Vinogradov E, Courtin P, Guerardel Y, Mahony J, Grard T, Cambillau C, Chapot-Chartier M-P, van Sinderen D. 2014. Differences in lactococcal cell wall polysaccharide structure are major determining factors in bacteriophage sensitivity. mBio 5:e00880-14.
4. Oh JH, van Pijkeren JP. 2014. CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*. Nucleic Acids Research 42:e131.
5. O'Driscoll J, Glynn F, Cahalane O, O'Connell-Motherway M, Fitzgerald GF, van Sinderen D. 2004. Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. Applied and Environmental Microbiology 70:5546-5556.
6. Mahony J, Deveau H, Mc Grath S, Ventura M, Canchaya C, Moineau S, Fitzgerald GF, van Sinderen D. 2006. Sequence and comparative genomic analysis of lactococcal bacteriophages jj50, 712 and P008: evolutionary insights into the 936 phage species. FEMS Microbiology Letters 261:253-61.
7. Chandry PS, Moore SC, Boyce JD, Davidson BE, Hillier AJ. 1997. Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. Molecular Microbiology 26:49-64.
8. Higgins DL, Sanozky-Dawes RB, Klaenhammer TR. 1988. Restriction and modification activities from *Streptococcus lactis* ME2 are encoded by a self-transmissible plasmid, pTN20, that forms cointegrates during mobilization of lactose-fermenting ability. Journal of Bacteriology 170:3435-3442.