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Three distinct glycosylation pathways are involved in the decoration of *Lactococcus lactis* cell wall glycopolymers

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Extracytoplasmic sugar decoration of glycopolymer components of the bacterial cell wall contributes to their structural diversity. Typically, the molecular mechanism that underpins such a decoration process involves a three-component glycosylation system (TGS) represented by an undecaprenyl-phosphate (Und-P) sugar-activating glycosyltransferase (Und-P GT), a flippase, and a polytopic glycosyltransferase (PolM GT) dedicated to attaching sugar residues to a specific glycopolymer. Here, using bioinformatic analyses, CRISPR-assisted recombineering, structural analysis of cell wall-associated polysaccharides (CWPS) through MALDI-TOF MS and methylation analysis, we report on three such systems in the bacterium *Lactococcus lactis*. On the basis of sequence similarities, we first identified three gene pairs, *csdAB*, *csdCD*, and *csdEF*, each encoding an Und-P GT and a PolM GT, as potential TGS component candidates. Our experimental results show that *csdAB* and *csdCD* are involved in Glc side-chain addition on the CWPS components rhamnan and polysaccharide pellicle (PSP), respectively, whereas *csdEF* plays a role in galactosylation of lipoteichoic acid (LTA). We also identified a potential flippase encoded in the *L. lactis* genome (*Inz_02975*, *cflA*) and confirmed that it participates in the glycosylation of the three cell wall glycopolymers rhamnan, PSP, and LTA, thus indicating that its function is shared by the three TGSs. Finally, we observed that galactosylation of both rhamnan and PSP can increase resistance to bacteriophage predation and that LTA galactosylation alters *L. lactis* resistance to bacteriocin.

The cell wall of Gram-positive bacteria plays a protective role in maintaining cell integrity, yet also makes the bacterial cell vulnerable because it mediates interactions with phage predators

or host immune defenses. Substantial knowledge has been accumulated regarding the molecular and biochemical steps involved in the assembly of various cell wall constituents, including the major one, peptidoglycan (1), as well as other constituting glycopolymers, such as lipoteichoic acids (LTAs)⁵ (2), wall teichoic acids (WTA) (3), and cell wall polysaccharides (CWPS) (4). Bacterial glycopolymers are characterized by their astonishing structural diversity. A three-component glycosylation system (TGS) has previously been proposed for the extracytoplasmic addition of sugar residues on bacterial glycoconjugates, such as teichoic acids (TAs) in Gram-positive bacteria, thus contributing to generate structural diversity (5). A TGS is characterized by the initial synthesis of an undecaprenyl-phosphate (Und-P)-sugar intermediate, catalyzed by a membrane-anchored glycosyltransferase (GT) at the inner face of the cytoplasmic membrane. Following this, a so-called flippase reorients the above-mentioned membrane-associated sugar intermediate from the cytoplasmic to the periplasmic side (or outer side) of the membrane. The final attachment of the sugar onto the glycoconjugate is catalyzed by an integral membrane GT, which contains between eight and 13 transmembrane helices (TMHs) and possesses a GT-C fold (5). In particular, the three functions involved in this process have been attributed to specific genes in *Listeria monocytogenes* (6, 7): GtcA (a flippase enzyme), GtcB (Und-P GT), and GtcC (a polytopic transmembrane (PolM) GT) were shown to be involved in the galactosylation of WTA in *L. monocytogenes* serotype 4nonb (7). Additionally, GtlA (Und-P GT) and GtlB (PolM GT) were shown to attach galactose (Gal) onto the LTA moiety of *L. monocytogenes* 10403S (6).

Various *Lactococcus lactis* strains produce an apparently unique dual-component CWPS structure (8): a rhamnose-rich and unexposed (*i.e.* peptidoglycan-embedded) polysaccharide chain, known as the rhamnan (9), and a surface-exposed phosphopolysaccharide or so-called polysaccharide pellicle (PSP)

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This article contains Tables S1 and S2 and Figs. S1–S9.

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⁵ The abbreviations used are: LTA, lipoteichoic acid; CWPS, cell wall polysaccharide(s); GT, glycosyltransferase; PolM GT, polytopic glycosyltransferase; PSP, polysaccharide pellicle; RBP, receptor-binding protein; SEC, size-exclusion chromatography; TGS, three-component glycosylation system; TMH, transmembrane helix; Und-P, undecaprenyl-phosphate; WTA, wall teichoic acid; HF, hydrogen fluoride; TA, teichoic acid; Gal, galactose; Glc, glucose; t-Glc, terminal Glc; t-Gal, terminal Gal; Hex, hexose; Rha, rhamnose; ABC, ATP-binding cassette; Ino, *m*-inositol.

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Glycosylation of *L. lactis* glycopolymers

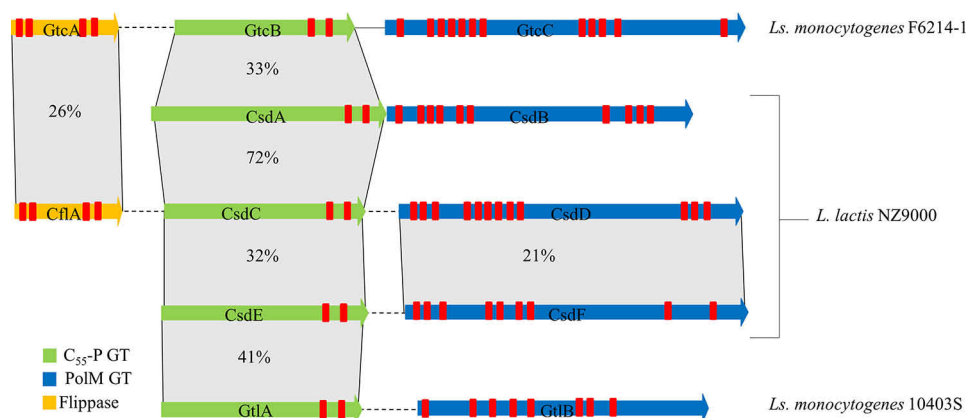


Figure 1. Schematic representation of a locus map showing three of the genes, *gtcABC*, associated with WTA galactosylation in *L. monocytogenes* F6214-1 and two genes, *gtlAB*, associated with LTA galactosylation in *L. monocytogenes* 10403S along with seven genes identified in *L. lactis* NZ9000 by sequence homology search. Genes are color-coded according to predicted functional domains: green, Und-P GT; blue, PolM GT; yellow, flippase. Percentage similarity based on BLASTP alignment (of the encoded protein products) is also indicated. Finally, sequences representing the predicted TMHMs (predicted with TMHMM server version 2.0) are indicated by red rectangular bands. Solid lines, adjoining genes; dashed lines, order/orientation of genes altered for visualization purposes.

(10, 11). The biosynthetic machinery responsible for the production of these two components is encoded by a large (25–30-kb) gene cluster (the *cwps* gene cluster) (10). Through the application of transposon-mediated, spontaneous, or directed mutagenesis strategies, the importance of the PSP for bacteriophage infection has been defined (10–13). The PSP subunits exhibit a certain degree of structural diversity between *L. lactis* strains. For example, the PSP of both *L. lactis* MG1363 and *L. lactis* SMQ-388 is a polymer of phosphohexasaccharide subunits containing a Glc side chain (11, 14), whereas the PSP of *L. lactis* 3107 is composed of phosphopentasaccharide linear repeating units (12). Interestingly, the PSP subunit structures of *L. lactis* SMQ-388 and *L. lactis* 3107 are virtually identical with the exception of the presence of the Glc side chain in the structure of the former. The genetic divergence of the *cwps* gene cluster of the two strains does not appear to account for the emergence of such a structural discrepancy in their PSP, prompting an investigation into the existence of a potential TGS in *L. lactis* similar to those discovered in *L. monocytogenes*.

In the current study, we demonstrate the existence of three sets of gene pairs in *L. lactis* NZ9000 (*llnz_00690* and *llnz_00695*, here termed *csdA* and *csdB*; *llnz_03080* and *llnz_03075*, here termed *csdC* and *csdD*; and *llnz_07820* and *llnz_07825*, here termed *csdE* and *csdF*), whose products exhibit sequence and topological identity to the two GTs of the previously characterized *L. monocytogenes* TGS (6, 7). The function of each gene set was characterized in *L. lactis* NZ9000, in which they were shown to be required for the glycosylation of a distinct glycopolymer within the cell envelope. *CsdAB* was shown to glucosylate the rhamnan, *CsdCD* was shown to glucosylate the PSP, and, finally, *CsdEF* is involved in LTA galactosylation. Additionally, a candidate flippase-encoding gene (*llnz_02975*, here renamed *cflA*), was identified, and its functionality was characterized. Our results show that its product, *CflA*, forms part of the three glycosylation pathways, thus completing the putative TGSs. Finally, the effect on fitness and bacteriophage resistance

conferred by the sugar substitutions of the above-mentioned glycopolymers is reported.

Results

Identifying lactococcal homologues of *L. monocytogenes* *gtcABC* and *gtlAB*

Previous NMR structural analysis of the CWPS in certain *L. lactis* strains identified the presence of a Glc side chain on the PSP subunit produced by *L. lactis* MG1363 (11) and *L. lactis* SMQ-388 (14). This feature is absent in the PSP subunit of *L. lactis* 3107, which was unexpected given the high sequence identity between the 3'-region of the *cwps* gene clusters of strains 3107 and SMQ-388 (12), whereas all three strains also harbor the same number of putative glycosyltransferase-encoding genes in their respective *cwps* clusters. This observation suggests that additional genes located outside the *cwps* gene cluster contribute to the biosynthesis of the final PSP structure of certain strains.

Recently, genes had been identified in *L. monocytogenes* that are responsible for the attachment of sugar side chains on LTA (i.e. *gtlA* and *gtlB*) (6), or on WTA (i.e. *gtcA*, *gtcB*, and *gtcC*) (7). We used the corresponding protein sequences to identify genes that may be responsible for the Glc substitution of the PSP of *L. lactis* NZ9000. The *L. lactis* NZ9000 genome contains three gene pairs whose products exhibit sequence similarity to and share transmembrane topology properties with the *L. monocytogenes* proteins: the putative Und-P GT-encoding genes *llnz_00690* (*csdA*), *llnz_03080* (*csdC*), and *llnz_07820* (*csdE*), as well as their adjacent and putative PolM GT-encoding genes *llnz_00695* (*csdB*), *llnz_03075* (*csdD*), and *llnz_07825* (*csdF*), respectively (Fig. 1). At protein level, the three predicted Und-P GTs encoded by *L. lactis* NZ9000 exhibit varying levels of similarity to each other and to the *L. monocytogenes* *GtcB* (33–72% amino acid identity), whereas the putative PolM GT proteins do not exhibit such a high degree of identity (Fig. 1). *CsdA*, *CsdC*, and *CsdE* belong to the GT2 family (CAZY; RRID: SCR_012909) that contains enzymes transferring sugar from NDP-sugar to various substrates, including Und-P. They con-

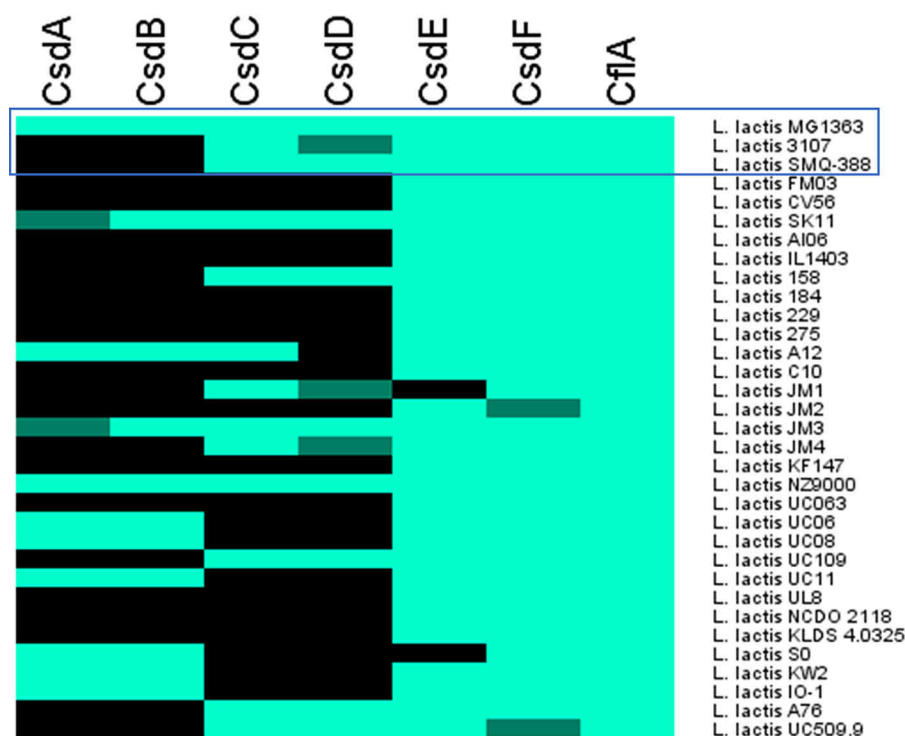


Figure 2. Heat map representing the presence (light green), absence (black), or nonfunctionality (dark green) of each of the six proteins encoded by the three glycosyltransferase-associated gene pairs and the flippase (CflA) in a total of 32 *L. lactis* publicly available genomes as well as *L. lactis* SMQ-388. The heat map was generated in Mev (V4_9_0). The top three strains, for which PSP structure was previously established by NMR, are highlighted by a blue frame.

tain two TMHs at their C terminus and the typical DXD conserved motif (⁹²DVD⁹⁴ in CsdA, ⁹⁵DAD⁹⁷ in CsdC, and ¹⁰²DAD¹⁰⁴ in CsdE) of GT-A fold enzymes, which interacts with the phosphate group of nucleotide donors. The three proteins CsdB, CsdD, and CsdF contain 10, 12, and 9 predicted TMHs (TMHMM analysis), respectively. In addition, HHpred analysis predicts structural similarity of these three proteins with ArnT, a typical GT with a GT-C-fold that is found in modifying GTs of TGSs. Interestingly, a gene encoding a protein with sequence similarity to the third protein involved in the glycosylation of *L. monocytogenes* WTA (GtcA), which is presumed to encode a flippase, is present in the *L. lactis* NZ9000 genome (*llnz_02975*, designated here as *cflA* (carbohydrate flippase)). Similar to GtcA, CflA is a predicted small (148 amino acids) integral membrane protein containing a total of four TMHs, as found previously in polyprenyl-monophosphate sugar flippase candidates of TGSs (7).

The presence of the three identified *L. lactis* NZ9000 gene pairs within the genomes of *L. lactis* 3107 and SMQ-388 was investigated through sequence alignments (Fig. 2), revealing that only *csdEF* is shared by all three strains. *L. lactis* SMQ-388 also contains homologues of *csdCD*, but appears to lack *csdAB*, whereas *L. lactis* 3107 lacks both *csdAB* and a functional copy of *csdCD*, due to a transposase insertion within *csdD*.

Prevalence of the genes encoding putative sugar decoration systems

A genome-wide search of 32 publicly available genomes of *L. lactis*, in addition to *L. lactis* SMQ-388, failed to identify any

additional candidate gene pairs that may encode components of TGSs other than those presented in Fig. 1. Sequence comparisons at protein level indicated the almost ubiquitous presence of *csdEF* in *L. lactis* genomes (29 of 33 strains), whereas *csdAB* and *csdCD* are much less widely distributed (9 and 10 strains of a total of 33 examined strains, respectively) (Fig. 2). A small subset of the genomes appears to contain a functional copy of one of the two genes in a given gene pair, whereas the other is predicted to be nonfunctional due to sequence frameshifts or inserted transposon elements. Furthermore, a protein of high sequence similarity to CflA, with a predicted flippase functionality, is encoded by all assessed strains. Interestingly, the genomic region surrounding *csdCD* is either immediately flanked or interrupted by transposase-encoding genes, or it forms part of a predicted prophage region. Based on these results and in association with the structural data regarding the glucose side chain found in the PSP of both *L. lactis* MG1363 and *L. lactis* SMQ-388 (and its absence in *L. lactis* 3107), the *csdCD* gene pair appeared to be the most likely candidate responsible for the attachment of the glucose side chain to PSP.

CsdCD is involved in PSP side-chain glycosylation

To ascertain whether the enzymes encoded by *csdCD* contribute to the final PSP structure, several mutants/derivatives of *L. lactis* NZ9000 and 3107 were generated. *L. lactis* NZ9000 derivatives carrying nonsense mutations in either *csdC* or *csdD* or both were generated (Table 1). In parallel, the *csdCD* gene pair was expressed in *L. lactis* strain 3107, which lacks functional *csdCD* homologs (Table 1).

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Table 1
Strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Feature(s)	Source
Bacterial strains		
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000	<i>L. lactis</i> MG1363 derivative containing <i>nisRK</i> , host to phages jj50, p2, and sk1	Ref. 46
<i>L. lactis</i> subsp. <i>cremoris</i> 3107	Host to phages LC3, TP901-1, 66901, 66902, 66903, 62604, 62605, and 62601	Ref. 47
<i>L. lactis</i> subsp. <i>cremoris</i> VES5751	<i>L. lactis</i> MG1363 derivative exhibiting a deficient PSP phenotype due to a mutation in <i>llmg_0226</i>	Ref. 11
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>csdAB</i>	NZ9000 with GAATTCG insert in <i>llnz_00690</i> (<i>csdA</i>) and <i>llnz_00695</i> (<i>csdB</i>), resulting in a TGA stop codon insertion in each gene	This work
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>csdC</i>	NZ9000 with GAATTCG insert in <i>llnz_03080</i> (<i>csdC</i>) resulting in a TGA stop codon in <i>csdC</i>	This work
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>csdD</i>	NZ9000 with GAATTCG insert in <i>llnz_03075</i> (<i>csdD</i>) resulting in a TGA stop codon in <i>csdC</i>	This work
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>csdCD</i>	NZ9000 with GAATTCG insert in <i>llnz_03075</i> (<i>csdD</i>) and <i>llnz_03080</i> (<i>csdC</i>) resulting in a TGA stop codon insertion in both genes	This work
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>csdEF</i>	NZ9000 with GATAACCC insert in <i>llnz_07820</i> (<i>csdE</i>) and <i>llnz_07825</i> (<i>csdF</i>), resulting in a TGA and TAA stop codon insertion, respectively	This work
<i>L. lactis</i> subsp. <i>cremoris</i> NZ9000- <i>cflA</i>	NZ9000 with TAATAGGGG insert in <i>llnz_02975</i> (<i>cflA</i>) resulting in a TAA and TAG double stop codon in <i>cflA</i>	This work
Plasmids		
pJP005	Recombineering-facilitating vector containing <i>recT</i> , P ^{<i>nisA</i>} , Cm ^r	Ref. 39
pNZ44	High-copy expression vector, contains P44 constitutive promoter, Cm ^r	Ref. 37
pNZ44str	Derivative vector of pNZ44, Str ^r	Developed by Andrea Garzon, UCC Collection
pCNR	Recombineering-facilitating vector containing <i>recT</i> , P ^{<i>nisA</i>} , Cm ^r derived from the low-copy vector pJP005	This work
pVPL3004	Low-copy vector expressing <i>cas9</i> along with <i>tracrNA</i> , Ery ^r	Ref. 41
pCRISPR	High-copy vector carrying CRISPR repeats and used for inserting target spacer sequences, Tet ^r	Ref. 41
pCRISPR:: <i>cflA</i>	pCRISPR plasmid carrying a CRISPR repeat targeting the recombineered sequence of gene <i>cflA</i> , Tet ^r	This work
pNZ44:: <i>csdCD</i>	pNZ44 containing genes <i>csdC</i> and <i>csdD</i>	This work
pNZ44:: <i>csdA</i>	pNZ44 containing gene <i>csdA</i>	This work
pNZ44:: <i>csdB</i>	pNZ44 containing gene <i>csdB</i>	This work
pNZ44:: <i>csdAB</i>	pNZ44 containing genes <i>csdA</i> and <i>csdB</i>	This work
pNZ44str:: <i>csdAB</i>	pNZ44str containing genes <i>csdA</i> and <i>csdB</i>	This work
pNZ44:: <i>csdE</i>	pNZ44 containing gene <i>csdE</i>	This work
pNZ44:: <i>csdF</i>	pNZ44 containing gene <i>csdF</i>	This work
pNZ44:: <i>csdEF</i>	pNZ44 containing genes <i>csdE</i> and <i>csdF</i>	This work
Bacteriophages		
jj50 ^a	Spontaneously acquired derivative of jj50 (NC_008371.1), propagated on NZ9000	This work
p2	936 species, propagated on NZ9000	Ref. 48
sk1	936 species, propagated on NZ9000	Ref. 49
MCC1	Derivative of sk1, propagated on NZ9000	Ref. 8
LC3	P335 species, propagated on 3107	Ref. 50
TP901-1	P335 species, propagated on 3107	Ref. 47
66901	936 species, propagated on 3107	Ref. 51
66902	936 species, propagated on 3107	UCC Culture Collection
66903	936 species, propagated on 3107	UCC Culture Collection
62604	936 species, propagated on 3107	Ref. 51
62605	936 species, propagated on 3107	Ref. 51
62601	936 species, propagated on 3107	Ref. 51
63301	P335 species, propagated on 3107	Ref. 52
50101	P335 species, propagated on 3107	Ref. 52
07501	P335 species, propagated on 3107	Ref. 53
58601	P335 species, propagated on 3107	Ref. 53
86501	P335 species, propagated on 3107	Ref. 52

CWPS produced by each of these constructed strains was extracted by HF treatment, and the obtained rhamnan and PSP oligosaccharides were separated by size-exclusion chromatography (SEC-HPLC) (9). The purified components were hydrolyzed, and their monosaccharide composition was determined, whereas they were also analyzed by MALDI-TOF MS. Compositional analysis of the PSP of the *L. lactis* NZ9000-*csdCD* double mutant revealed a clear decrease of Glc/Gal ratio compared with that of *L. lactis* NZ9000 (Table 2). Structural modification of the PSP from *L. lactis* NZ9000-*csdCD* was confirmed by MALDI-TOF MS analysis. The obtained mass spectrum of the WT *L. lactis* NZ9000 purified PSP oligosaccharides exhibits a major peak corresponding to the hexasaccharide subunit in agreement with the structure previously established by NMR (11) (Fig. 3A), whereas this peak was absent in the spectrum of

Table 2
Relative monosaccharide composition of PSP oligosaccharides purified by HP-SEC after HF extraction from the different *L. lactis* strains

Strain	Rha	GlcNAc	Gal ^a	Glc
<i>L. lactis</i> NZ9000	0.8	2.6	1	2.4
<i>L. lactis</i> NZ9000- <i>csdCD</i>	0.6	2.1	1	1
<i>L. lactis</i> NZ9000- <i>csdC</i>	0.5	2.1	1	1.1
<i>L. lactis</i> NZ9000- <i>csdD</i>	0.6	2.5	1	1.1
<i>L. lactis</i> NZ9000- <i>cflA</i>	0.7	2	1	1.3
<i>L. lactis</i> 3107 pNZ44	0	1.1	1	0.4
<i>L. lactis</i> 3107 pNZ44:: <i>csdCD</i>	0.3 ^b	1.0	1	0.9

^a Values are standardized relative to Gal.

^b Presumed contamination of PSP by short rhamnan chains during HP-SEC purification.

the PSP extracted from the *L. lactis* NZ9000-*csdCD* mutant. The latter exhibits a major peak that corresponds to a pentasaccharide subunit resulting from the loss of one hexose (Hex)

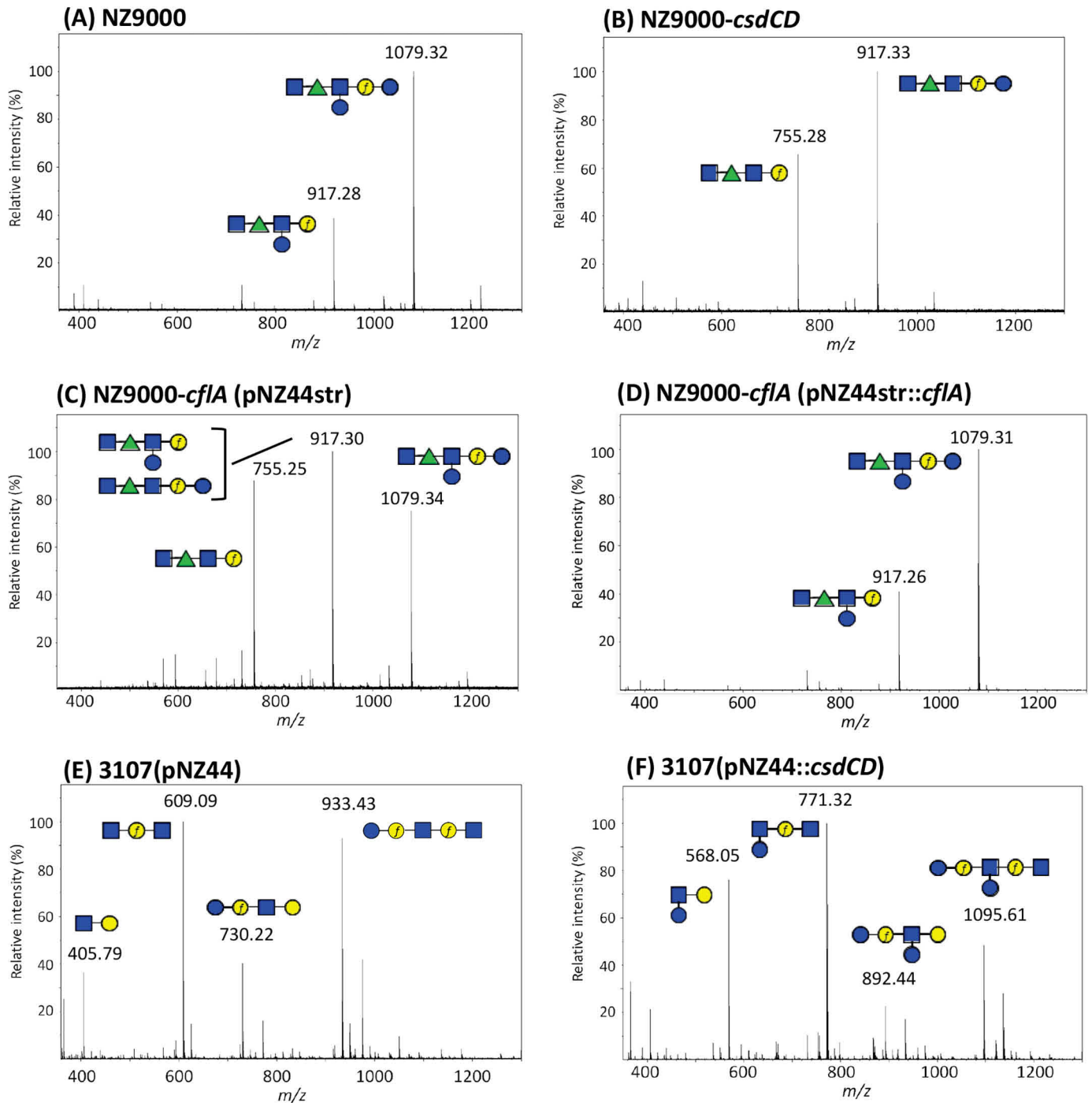


Figure 3. MALDI-TOF MS spectra obtained on purified PSP oligosaccharides extracted by HF treatment from cell walls of WT *L. lactis* NZ9000 (A), *L. lactis* NZ9000-*csdCD* mutant (B), *L. lactis* NZ9000-*cfIA*(pNZ44str) flippase mutant (C), *L. lactis* NZ9000-*cfIA*(pNZ44str::*cfIA*) complemented flippase mutant (D), *L. lactis* 3107 with empty pNZ44 (E), and *L. lactis* 3107(pNZ44::*csdCD*) (F). m/z values on spectra correspond to $[M + Na]^+$ ion adducts. During HF extraction, the polymeric PSP of *L. lactis* NZ9000 is cleaved at the level of phosphodiester bonds, leading to hexasaccharide (M_{calc} 1079.37) and also partially cleaved after Gal f , leading to a pentasaccharide (M_{calc} 917.32), as shown previously by NMR (9). Similarly, the polymeric PSP of *L. lactis* 3107 is cleaved during HF extraction at the level of phosphodiester bonds, leading to pentasaccharide (M_{calc} 933.32), and also partially after the two Gal f present in the structure, leading to fragments with M_{calc} of 730.24, 609.21, and 406.13 (for Na^+ adducts) (10). Calculated and observed m/z values of the different oligosaccharide structures tentatively assigned to major peaks of the spectra are listed in Table S2. Structures are drawn using the symbol nomenclature for graphical representation of glycans (SNFG). Blue square, GlcNAc; green triangle, Rha; blue circle, Glc; yellow circle, Gal. *f*, furanose.

(Fig. 3B). Methylation analysis showed that terminal Glc (t-Glc) detected in the WT PSP was absent in the PSP of mutant NZ9000-*csdCD*, thus confirming that the residue absent in the PSP of NZ9000-*csdCD* was the side-chain Glc. To examine the involvement of the individual *csdC* and *csdD* genes, the struc-

ture of PSP purified from the single *csdC* and *csdD* mutants was also analyzed. Monosaccharide composition analysis revealed a clear decrease of Glc/Gal ratio in both mutants compared with the PSP of *L. lactis* NZ9000 (Table 2). MALDI-TOF MS analysis and methylation analysis of the PSP obtained from the corre-

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sponding *csdD* mutant revealed the complete loss of the side-chain Glc, similar to the effect observed in the double *csdCD* mutant (Fig. S1). However, in the case of the *csdC* mutant, MS and methylation analyses indicated that a portion of the PSP (estimated at 20% based on methylation analysis) still contained the side-chain Glc despite *csdC* inactivation (Fig. S1). These results suggest that another enzyme with undecaprenyl-phosphate-glucose (Und-P-Glc) synthase activity partially complements the *csdC* deficiency by producing the sugar-lipid intermediate that is then available for CsdD. Altogether, these results demonstrate that the *csdCD* gene pair is involved in Glc side-chain addition on the PSP subunit.

In the *L. lactis* 3107 strain expressing functional *csdCD*, compositional analysis of the purified PSP revealed an increase of Glc relative to Gal compared with the value in the PSP of WT *L. lactis* 3107 (Table 2). MALDI-TOF MS analysis confirmed the presence of PSP with an additional hexose in *L. lactis* 3107(pNZ44::*csdCD*) compared with WT 3107 (Fig. 3, E and F). The presence of the Glc side chain in *L. lactis* 3107(pNZ44::*csdCD*) was confirmed by methylation analysis.

CsdAB is required for rhamnan glucosylation

To assess the function of CsdAB, a similar strategy to the one employed for CsdCD was followed, consisting of mutant/derivative construction in *L. lactis* NZ9000 and 3107 followed by CWPS analysis. First, in *L. lactis* NZ9000-*csdAB*, PSP monosaccharide composition was unchanged compared with WT (data not shown). Because the rhamnan component purified from *L. lactis* NZ9000 contains mainly Rha and only trace amounts of Glc, Gal, and GlcNAc, the influence of *csdAB* inactivation on rhamnan composition could not be clearly assessed. Therefore, the *csdAB* genes were overexpressed in *L. lactis* NZ9000 to investigate their role.

We observed that *csdAB* overexpression in *L. lactis* NZ9000 caused a substantial increase of Glc in the rhamnan component (Glc/Rha ratio around 1:3), whereas just a low amount of Glc, relative to Rha, was detected in the WT rhamnan. MALDI-TOF MS analysis of rhamnan showed a complex pattern, with higher *m/z* value peaks in *csdAB*-overexpressing NZ9000 (pNZ44::*csdAB*) compared with NZ9000 (WT) (Fig. 4). In the spectrum of WT rhamnan, a major family of signals were separated by 438 mass units (Fig. 4A), which can be attributed to a difference of one rhamnan subunit constituted of 3 Rha (9). In contrast, in the spectrum of the rhamnan from *csdAB*-overexpressing NZ9000, a major family of signals was detected with 600-mass unit increments that can be assigned to a difference of 3 Rha plus 1 Hex, corresponding to one rhamnan subunit bearing a Glc side chain (Fig. 4B). Methylation analysis confirmed a substantial increase of t-Glc in the rhamnan purified from *L. lactis* NZ9000 (pNZ44::*csdAB*), whereas a 2,3-disubstituted Rha appeared with a concomitant decrease in the level of 3-Rha, compared with the control NZ9000 rhamnan (Fig. S2). These results confirmed the presence of Glc substitutions on rhamnan chains and identified the site of branching. From the methylation analysis, we deduced the following structure for the subunits substituted with Glc: $\rightarrow 2)-\alpha$ -Rha-(1 \rightarrow 2)- α -Rha-(1 \rightarrow 3)-[Glc-(1 \rightarrow 2)]- α -Rha-. No effect of *csdAB* overexpression was

detected on PSP structure either by composition or MS analysis (Fig. S3).

Overexpression of *csdA* alone in NZ9000 did not modify the rhamnan or PSP structures (Figs. S3B and S4B). In contrast, overexpression of *csdB* alone in NZ9000 was shown to result in an increase of Glc in the rhamnan component (Glc/Rha ratio around 1:4) and an MS spectrum showing higher molecular mass species (Fig. S4C) similar to that observed for *csdAB* overexpression. Interestingly, the MS spectrum of PSP from NZ9000 overexpressing only *csdB* also appeared modified, with an increased intensity of the peak corresponding to the pentasaccharide *versus* the hexasaccharide peak (Fig. S3C). This result was corroborated by methylation analysis indicating a reduction of Glc side-chain addition in PSP in this latter strain. These results can be explained by assuming that part of the Und-P-Glc intermediate synthesized by CsdC, which possesses the same Und-P-Glc synthase activity as CsdA and is involved in PSP glucosylation, is titrated away by CsdB when this latter protein is overexpressed and is directed to perform rhamnan glucosylation.

csdAB genes from NZ9000 were then introduced on plasmid pNZ44::*csdAB* in strain 3107, which lacks functional homologs, to evaluate their role and significance for this strain. The composition of the purified rhamnan component of the CWPS was shown to exhibit a very substantial increase of Glc/Rha ratio (around 1:2), whereas only traces of Glc were found in the rhamnan peak of control *L. lactis* 3107 pNZ44. MALDI-TOF MS analysis confirmed a clear mass increase for the rhamnan extracted from *L. lactis* 3107 pNZ44::*csdAB* compared with the control *L. lactis* 3107 pNZ44 and WT strains (Fig. S5). When either *csdA* or *csdB* was expressed individually in *L. lactis* 3107, no difference in rhamnan composition or MS spectrum was observed (data not shown), indicating that *csdA* and *csdB* are both required for rhamnan glucosylation in strain 3107. Together, these results imply that CsdA and CsdB are functional when heterologously expressed in *L. lactis* 3107.

CsdEF is involved in LTA galactosylation

No modification of the rhamnan or PSP was detected in *L. lactis* NZ9000-*csdEF* or in *L. lactis* NZ9000 pNZ44::*csdEF* (data not shown). These results suggest that CsdE and CsdF are involved in the glycosylation of an alternative cell wall glycopolymer, such as WTA or LTA. Of note, *L. lactis* has previously been shown to synthesize LTA, which consists of glycerol-phosphate chains, with Gal as a sugar substituent (15). To assess whether LTA glycosylation depends on the products of *csdEF*, LTA was extracted from *L. lactis* NZ9000 and its mutant NZ9000-*csdEF* with hot phenol (16). The degree of LTA galactosylation was assessed based on relative quantities of glycerol, Gal, and terminal Gal (t-Gal) in two preparations. Our results demonstrated a 10-fold decrease in Gal levels at constant glycerol levels in the *L. lactis* NZ9000-*csdEF* mutant preparation (Fig. 5A). In agreement with these results, the amount of t-Gal decreased dramatically in this sample, compared with the preparation from *L. lactis* NZ9000 (Fig. 5B). These data clearly indicate that mutating both *csdE* and *csdF* negatively affects galactosylation of poly(glycerophosphate) chains of LTA in *L. lactis* NZ9000.

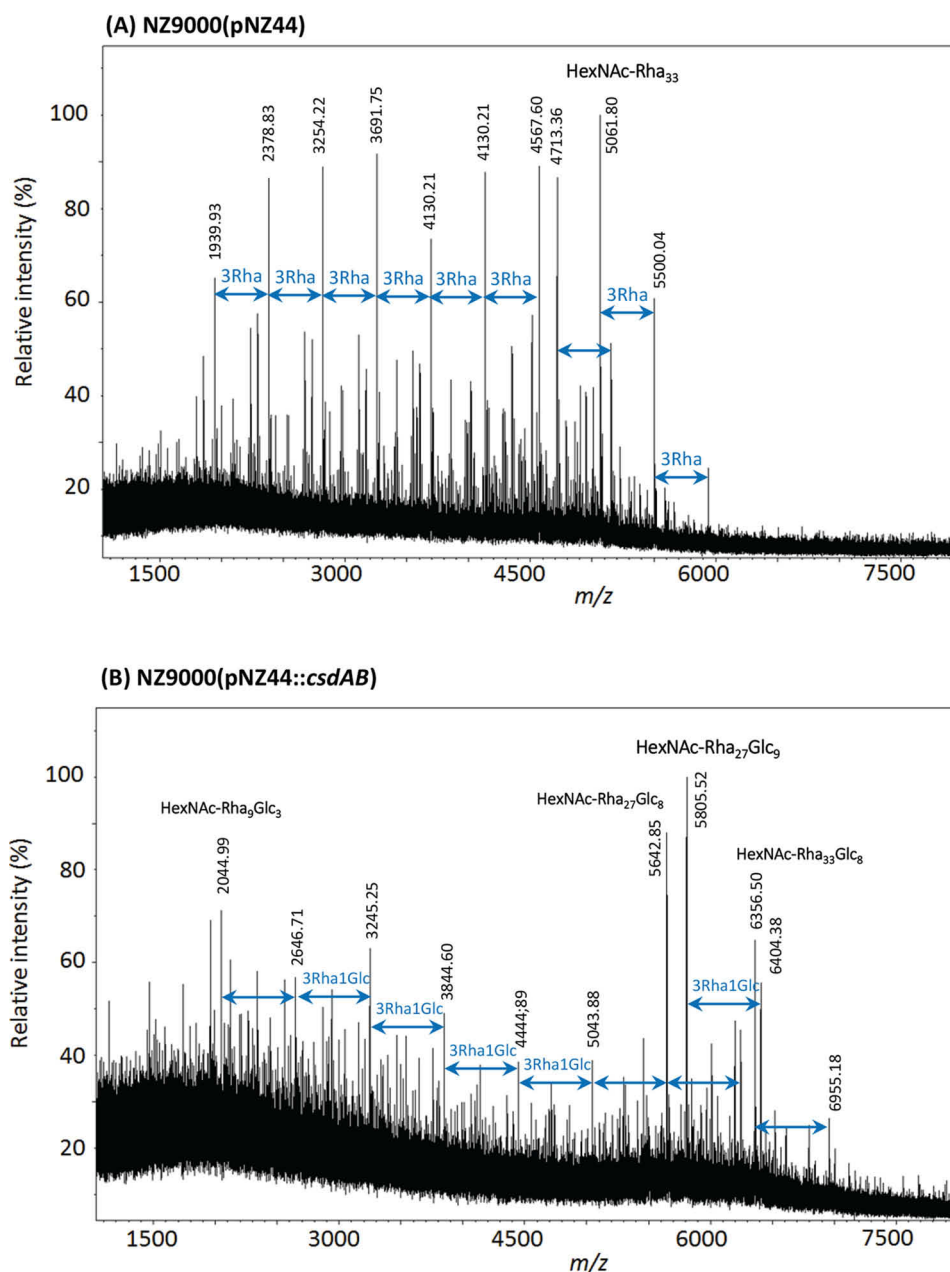


Figure 4. MALDI-TOF MS spectra of rhamnan purified from *L. lactis* NZ9000(pNZ44) (A) and *L. lactis* NZ9000(pNZ44::csdAB) (B). All m/z values correspond to $[M + Na]^+$ adducts.

The *cflA* gene encoding a putative flippase is involved in glucosylation of CWPS components as well as LTA galactosylation

As previously proposed (5), a protein with flippase activity should be involved in the TGS pathway. A gene (*cflA*) encoding a putative flippase with sequence identity to the *L. monocytogenes* GtC flippase (7) was identified in the *L. lactis* NZ9000 genome. Analysis of the PSP oligosaccharides purified from an *L. lactis* NZ9000-*cflA* mutant revealed a decreased level of Glc in PSP compared with the WT, suggesting incomplete addition of Glc side chain onto PSP subunits (Table 2). MS analysis confirmed this observation with a modified spectrum of the mutant strain versus the WT, in agreement with the presence of PSP oligosaccharides devoid of side-chain Glc (Fig. 3C). Comple-

mentation of the *cflA* mutant allowed the phenotypic restoration of the WT PSP structure as shown by MALDI-TOF MS analysis of PSP oligosaccharides (Fig. 3D).

To investigate the role of *cflA* in Glc substituent addition onto rhamnan, the *csdAB* genes were overexpressed (employing plasmid pNZ44str::csdAB) in the *L. lactis* NZ9000-*cflA* mutant. Whereas, as described above, overexpression of *csdAB* in *L. lactis* NZ9000 led to a very high level of Glc grafted to rhamnan as observed by composition analysis (Glc/Rha ratio around 1:4), NZ9000-*cflA* pNZ44str::csdAB showed no (or very little) Glc substitution of the rhamnan. This effect could also be revealed by MALDI-TOF MS analysis. Whereas in the NZ9000 background, overexpression of *csdAB* led to a significant increase of the rhamnan mass as described above, in the *cflA*

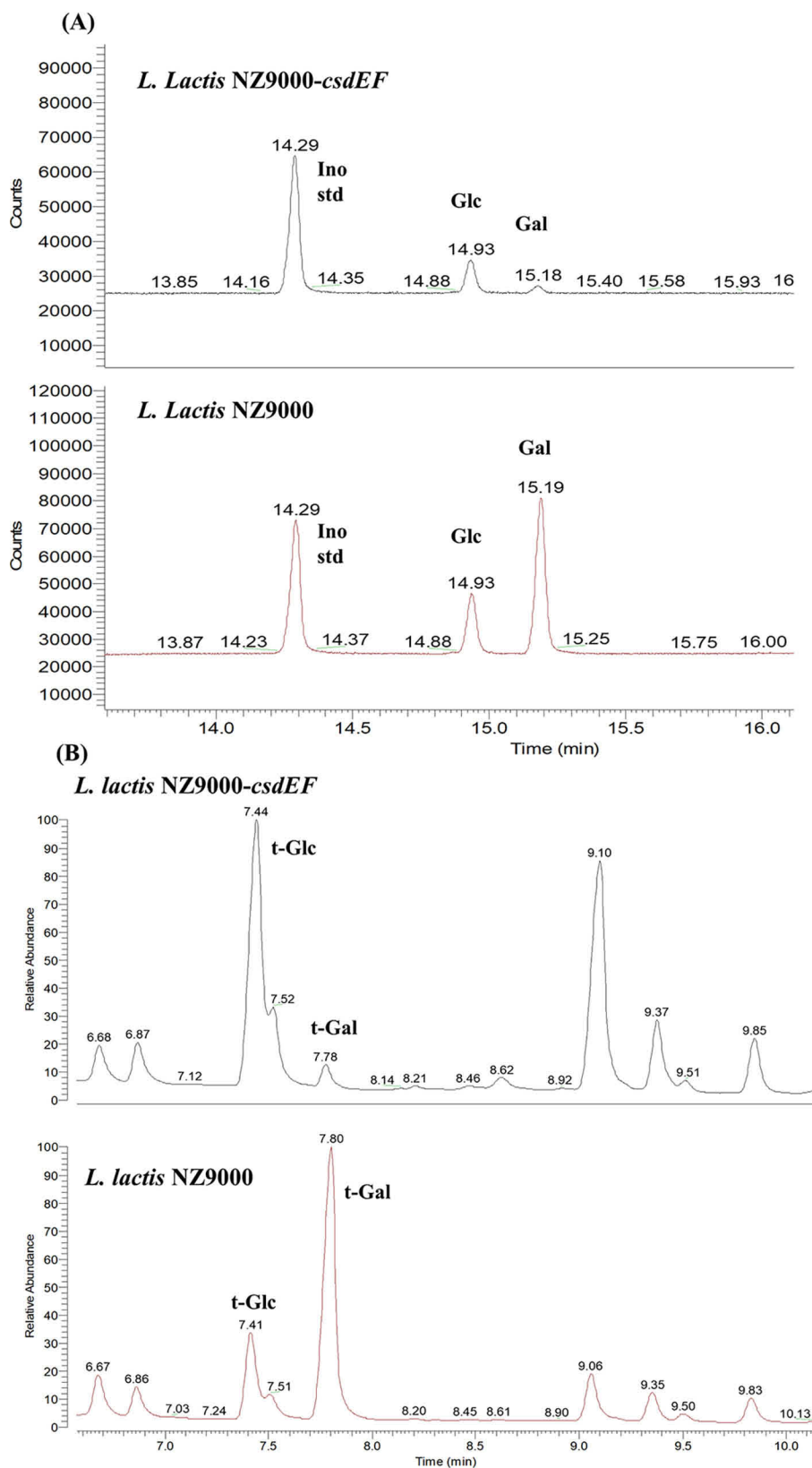


Figure 5. A, monosaccharide analysis of crude phenol extracts from *L. lactis* NZ9000 and NZ9000-*csdEF*. Each extract (5 mg), along with Ino (90 μ g) as an internal standard, were treated with HF, hydrolyzed, converted into alditol acetates, and analyzed by GC with flame-ionization detection. B, methylation analysis of crude phenol extracts from *L. lactis* NZ9000 and NZ9000-*csdEF*. Samples were analyzed by GC-MS. The figures were zoomed onto the region corresponding to hexoses.

background, overexpression of *csdAB* had no effect (Fig. S4, E and F). These results clearly show that CflA is required for glucosylation of rhamnan. Our results therefore identify CsdA, CsdB, and CflA as a three-component rhamnan glucosylation system in *L. lactis*.

The effect of *cflA* inactivation was also evaluated on LTA galactosylation. Monosaccharide analysis of dephosphorylated phenol extracts of the *L. lactis* NZ9000-*cflA* mutant, compared with the WT strain showed the absence of any detectable Gal. Methylation analysis of this preparation did not reveal any terminal Gal, confirming that the LTA of this strain lacked Gal substitution. Complementation of the *cflA* mutant restores the level of Gal and t-Gal (Fig. S6). These results indicate that the flippase CflA acts in concert with CsdE and CsdF in the galactosylation process of LTA chains.

Derivative strain fitness and bacteriophage interactions

Phenotypic effects due to the mutation or overexpression of *csdAB*, *csdCD*, and *csdEF* were investigated. All derived *L. lactis* NZ9000 strains were grown under optimal as well as various stress conditions by incorporating growth-limiting concentrations of NaCl, ethanol, and EDTA and reduced pH. It was observed that under optimal growth conditions (in M17Glc broth), nonsense mutations and overexpression of these three sets of genes did not exert any detrimental effect on growth rate (data not shown). With the exception of the antimicrobial peptide nisin, the stressors appeared to similarly affect both the WT and the derived strains. Overexpression of the *csdEF* provided increased resistance to nisin, whereas the opposite effect was observed for the nonsense mutant (Fig. 6). Interestingly, when the effect of a nonsense mutation in *cflA* was investigated, it was observed that the mutant strain exhibited a reduced growth rate compared with the control strain even under optimal conditions (Fig. S7).

Lactococcal PSP is required for adsorption by certain phages, and mutations that impair or alter PSP biosynthesis may lead to altered phage sensitivities (12–14). These findings combined with our observation that CsdCD is responsible for PSP glucosylation prompted us to determine whether this Glc substitution alters phage-host interactions. The mutant strain *L. lactis* NZ9000-*csdCD* was assayed for its sensitivity to the phages (936-type phages) listed in Table 1. The presence of the double knockout mutation did not significantly affect the infective capabilities of the phages under standard plaquing conditions (data not shown). Several environmental conditions that more accurately reflect the suboptimal environments in which phage-host interactions may be taking place, particularly those conditions associated with dairy fermentations, were also tested (see “Experimental procedures”). Among these conditions, significantly altered phage sensitivities between *L. lactis* NZ9000 and *L. lactis* NZ9000-*csdCD* were observed under high-salt conditions. Fig. 7 indicates the much higher efficiency (3.7 log) by which phage p2 infects the derivative strain *L. lactis* NZ9000-*csdCD* when compared with p2 infection of the *L. lactis* NZ9000 in the presence of salt, a difference that is not observed under standard conditions. A similar but much more subtle effect is observed for phage sk1. In addition, phage adsorption assays revealed that p2

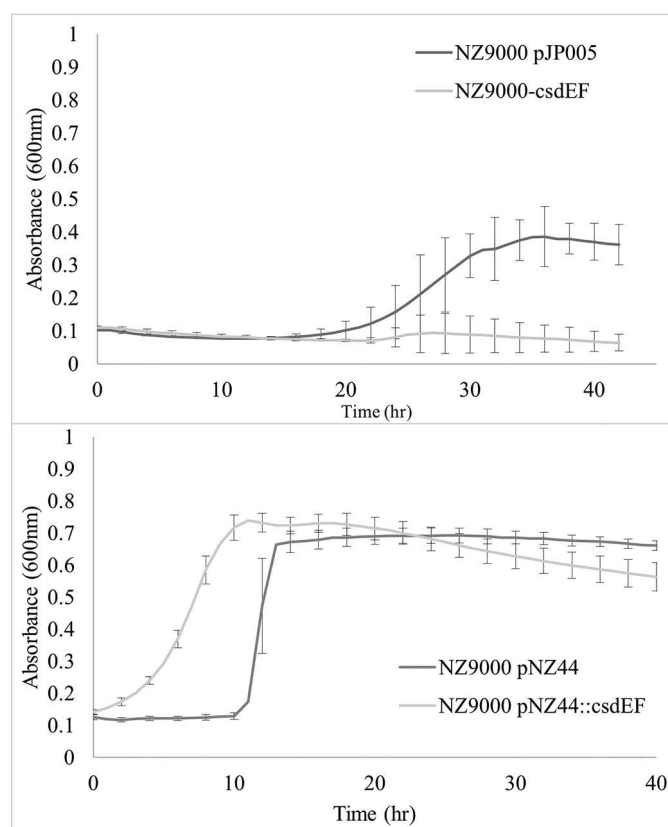


Figure 6. Growth behavior of *L. lactis* NZ9000 controls and *csdEF* derivatives in M17 Glc and 15 µg/ml nisin. Error bars, S.D.

exhibits enhanced reversible and irreversible adsorption to *L. lactis* NZ9000-*csdCD* relative to *L. lactis* NZ9000 under standard laboratory conditions (Fig. S8).

The structural modifications incurred by the changes in expression of *csdAB* and *csdEF* and their effect on phage/host interactions were also examined. *L. lactis* NZ9000-*csdAB*, NZ9000-*csdEF*, NZ9000 pNZ44::*csdAB*, NZ9000 pNZ44::*csdEF*, and *L. lactis* 3107 pNZ44::*csdEF* did not exhibit altered phage sensitivity profiles (four 936-type phages with *L. lactis* NZ9000 as a host and six 936-type and seven P335-type phages with *L. lactis* 3107 as a host; Table 1). In contrast, overexpression of *csdAB* in *L. lactis* 3107 caused reduced sensitivities against phages that belong to either the P335 or 936 group (Fig. S9A). Similar results were observed when *csdAB* was overexpressed in *L. lactis* VES5751, a PSP-deficient derivative of *L. lactis* MG1363 that is resistant to infection by most phages commonly infecting the WT strain (11). Two phages, jj50* and MCC1, derivatives of phage jj50 (NC_00837) and sk1 (NC_001835.1), respectively, are unaffected by the PSP-deficient phenotype of *L. lactis* VES5751 and can maintain their ability to infect it. However, upon overexpression of *csdAB* in *L. lactis* VES5751, the infectivity of jj50* is reduced approximately 100-fold compared with the control strains, whereas MCC1 appears to be unaffected by *csdAB* overexpression (Fig. S9B).

Finally, the effect of mutation of the flippase gene, *cflA*, on bacteriophage interaction was investigated. Surprisingly, despite the effect that the mutation has on the growth of the strain (Fig. S7), the tested phages were equally efficient at infecting the control and mutant *L. lactis* NZ9000-*cflA* strains,

Glycosylation of *L. lactis* glycopolymers

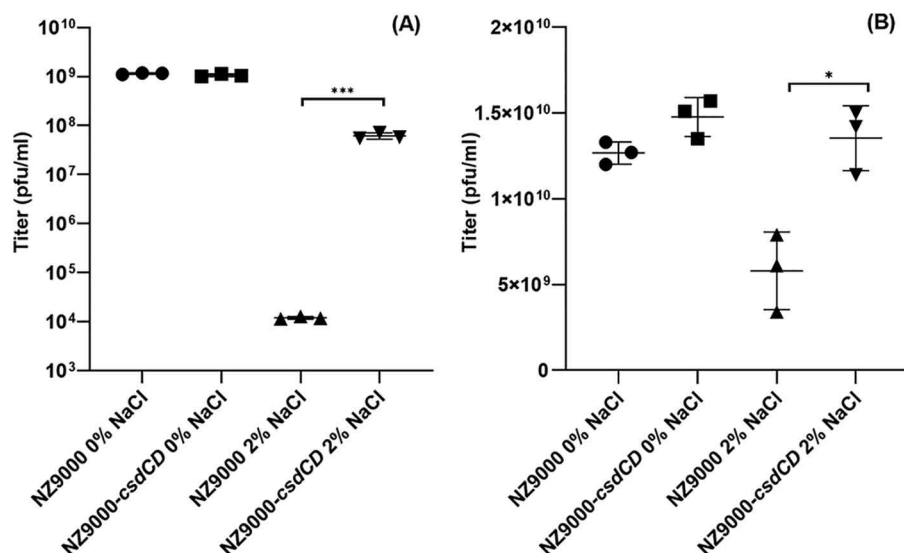


Figure 7. Titer of phages p2 (A) and sk1 (B) against *L. lactis* NZ9000 and *L. lactis* NZ9000-csdCD in 0 and 2% NaCl environment. Each data point represents a biological replicate. *p* values are indicated by asterisks. ***, *p* < 0.0001; *, *p* < 0.05. Error bars, S.D.

possibly owing to the maintenance of the Glc side chain in the PSP of *L. lactis* NZ9000-*cflA*, albeit at a reduced frequency compared with the WT strain.

Discussion

In the current work, we identified three gene pairs in *L. lactis* located outside the *cwps* gene cluster, each encoding two predicted glycosyltransferases, one GT able to transfer sugar from NDP-sugar to Und-P and the second a PolM GT, which possesses the characteristics of GTs involved in extracytoplasmic modification of bacterial glycoconjugates. We successfully assigned a function for these three gene pairs, showing that each is involved in the glycosylation of a distinct glycopolymer of the lactococcal cell wall. The *csdEF* gene pair is the most commonly encountered set of the three, whereas the complete absence of any intact *csd* gene (as seen in *L. lactis* JM1/JM2) hints at the accessory function of the gene pair products. In addition, our results clearly indicate that the three glycosylation systems share a common flippase involved in transferring Und-P-Glc as well as Und-P-Gal from the inner side to the outer side of the cytoplasmic membrane.

Original work involving the pathogenic microorganism *Shigella flexneri* first described the mechanism by which the O-antigen of the lipopolysaccharide is glycosylated and how this modified the strain's virulence and resistance to innate immune killing. This glycosylation process was shown to be mediated by the function of three contiguously located genes, *gtrABV* (17, 18). Similar TGSs (5) have been implicated in the serovar conversion of pathogenic species such as *Listeria monocytogenes* (19) as well as many *Salmonellae* (20). Our results show that the *csdCD* gene pair in *L. lactis* forms part of such a TGS and is involved in the attachment of the Glc side chain on PSP subunits. In addition, these genes are also functional when expressed in *L. lactis* 3107 (which lacks a functional homolog of *csdC*). We propose that CsdC is involved in the transfer of Glc from UDP-Glc to Und-P at the inner face of the cytoplasmic membrane, whereas CsdD is presumed to catalyze Glc transfer

onto PSP outside the cytoplasmic membrane (Fig. 8). Interestingly, CsdD is absolutely required for PSP glycosylation. In contrast, in the absence of CsdC, partial glycosylation of PSP was still observed, indicating that Und-P-Glc substrate can be provided to CsdD by another GT possessing the same activity as CsdC. CsdA described above was also shown to possess Und-P-Glc synthase activity and is thus a likely candidate to fulfill this role. Thus, our results indicate a certain level of functional redundancy and cross-talk among different systems dedicated to glycosylation of different cell wall glycopolymers, mediated by the availability of the Und-P sugar intermediate. We also show that the products of *csdAB* are responsible for the glycosylation of the rhamnan subunit and that the enzymatic activities of both proteins are required for this addition (Fig. 8). According to methylation analysis, Glc is connected to the 3-Rha of the [-2- α -Rha-2- α -Rha-3- α -Rha-] subunit. Overexpression of *csdB*, which encodes a PolM GT in NZ9000, allowed grafting of one Glc side chain on almost every 3-Rha subunit, as was observed upon overexpression of *csdAB*. These results suggest that CsdB can utilize the Und-P-Glc substrate produced by another Und-P-Glc synthase apart from that produced by its cognate partner CsdA, similar to our observations for the CsdCD pair. These observations confirm that CsdB provides specificity of the glycosylation reaction toward rhamnan. Finally, the gene pair *csdEF* was shown to be required for LTA galactosylation (Fig. 8). TGSs have previously been shown to glycosylate polymers derived from both a Wzy-dependent and ATP-binding cassette (ABC) transporter assembly pathway (5, 21). However, the existence of three TGSs to modify three distinct glycopolymers within a single bacterial species has not previously been observed.

With respect to gene organization, a hallmark of many TGSs is the close genomic proximity of the genes involved in glycosylation (18, 21). Interestingly, a gene encoding a putative flippase was identified on the *L. lactis* NZ9000 genome (*cflA*) that is in a noncontiguous position with respect to the gene pairs high-

Glycosylation of *L. lactis* glycopolymers

from the genomic variability of the central *cwps* gene cluster yet are now also seen to be influenced by additional genetic components beyond the *cwps* cluster. However, further analysis and investigation should be performed to evaluate the phenotypic implications of such modifications of cell wall glycopolymers.

Experimental procedures

Bacterial strains, phages, and growth conditions

Bacterial strains, plasmids, and phages used in this study are listed in Table 1. Strains were grown at 30 °C overnight in M17 broth and/or M17 agar (Oxoid Ltd.) supplemented with 5 g/liter glucose. Chloramphenicol (5 µg/ml), erythromycin (5 µg/ml), and tetracycline (10 µg/ml) (Sigma–Aldrich) were added to the media where appropriate. Induction of genes placed under the nisin-controlled promoter, P^{nis} , was achieved through supplementation of the media with nisaplin (40 ng/ml) (DuPont).

Bacteriophage assays

Phages used in this study are listed in Table 1. Propagation of phages on their respective host strains was performed as described previously (29). Similarly, both spot/plaque assays (30) and adsorption assays (31) were performed as described previously with sodium chloride added to a final concentration of 1–2% (w/v), where indicated.

Bioinformatic analysis

Candidate genes encoding proteins responsible for glucose side-chain decoration of the PSP of *L. lactis* MG1363 and *L. lactis* SMQ-388 were selected based on amino acid homologies to proteins previously investigated in *L. monocytogenes* (7). To this end, the proteins indicated in Fig. 1 were compared using BLASTP against the compiled coding sequences (CDS) of the *L. lactis* MG1363 genome (GenBankTM accession number NC_009004.1). Genes in the *L. lactis* MG1363 genome, which are not associated with the *cwps* gene cluster, were selected for further investigation based on their corresponding amino acid similarity to the following *L. monocytogenes* proteins: GtcA; GtcB and GtlA; and GtcC and GtlB (6, 7). Selected gene sequences from the *L. lactis* MG1363 genome were used to search for homologues in the genomes of *L. lactis* 3107 and *L. lactis* SMQ-388⁶ using BLASTN. Intrinsic properties of protein sequences were assessed using HHPred (32) and TMHMM online predictive tools (33, 34).

Selected genes were analyzed based on sequence identity against all publicly available *L. lactis* chromosomal and plasmid sequences, whereas protein sequence comparisons were performed using all-against-all bidirectional BLAST alignments (35) (cut-off: *E*-value 0.0001, with at least 50% identity across at least 50% of either protein sequence). A heat-map matrix, with the presence/absence/nonfunctionality of each of the retrieved candidate genes from all assessed *L. lactis* strains, was created and run in MeV suite (version 4.9) (36).

Molecular cloning

All recombinant plasmids (Table 1) were generated in *L. lactis* NZ9000, and primers used can be seen in Table S1 (Eurofins

MWG). The gene pairs of interest, *csdAB*, *csdCD*, and *csdEF*, were each amplified and cloned in the high-copy number constitutive expression vector pNZ44 (37). Due to the presence of pJP005 in *L. lactis* NZ9000-*cflA* that carries a chloramphenicol resistance marker, *csdAB* was also separately cloned into pNZ44str, a pNZ44-derivative that carries a streptomycin resistance marker instead of the chloramphenicol resistance marker, using the same oligonucleotides. Likewise, each of the genes from the two pairs, *csdAB* and *csdEF*, was cloned independently into the pNZ44 vector.

Recombineering and CRISPR-assisted recombineering

Recombineering was performed as described previously (38–40) with modifications to the assay to allow use with *L. lactis* NZ9000 on the selected genes. CRISPR-Cas9-assisted recombineering was adapted from a previous publication (41) and was used to create a knockout mutant of the *cflA* gene because the above-mentioned recombineering protocol proved to be unsuccessful, possibly due to decreased fitness of such mutants. A novel plasmid, named pCNR, containing the replication genes *repA*, *repD*, and *repE* from the backbone of the plasmid pPTPi (42) along with the chloramphenicol resistance gene (*cm^r*), P^{nisA} promoter, and the ssDNA-binding protein-encoding gene, *recT*, from plasmid pJP005 (39), was constructed. Subsequently, the single-step approach to CRISPR-Cas9-assisted recombineering was employed as outlined previously (42).

CWPS and LTA extraction, purification, and analysis

CWPS was extracted from cell envelope fractions prepared from *L. lactis* cells and analyzed as described previously (9). Briefly, cell walls were prepared and treated with HF to extract CWPS. Rhamnan and PSP oligosaccharides present in the HF extract were further separated by SEC-HPLC with two columns in tandem (Shodex Sugar KS-804 and KS-803 columns, Showa Denko, Japan) with a refractometer (2414 Refractive Index Detector, Waters) and/or UV detector at 206 nm. Fractions corresponding to peaks containing rhamnan and PSP oligosaccharides were collected and dried under vacuum. Monosaccharide composition was determined by high-performance anion-exchange chromatography coupled with pulse-amperometric detection (ICS5000 system, Thermo Fisher Scientific). Purified fractions were analyzed by MALDI-TOF MS using 2,5-dihydroxybenzoic acid matrix with an UltrafleXtreme instrument (Bruker Daltonics, Bremen, Germany). MS data are accessible on the Glycopost website (54), ID: GPST000056.

Crude LTA was extracted essentially as described previously (16). Identical amounts (2–5 mg) of LTA extracts of the WT strain *L. lactis* NZ9000 and mutant *L. lactis* NZ9000-*csdEF* were treated with HF (48%, 4 °C, 24 h) and subjected to comparative analysis for glycerol and monosaccharides, as well as methylation analysis. *m*-Inositol (Ino) was used as an internal standard to assess glycerol content. The use of phenol for the isolation of LTAs should guarantee relative purity of the sample, although the possibility of cross-contamination from other TA structures, such as WTA, cannot be excluded.

Methylation analysis was performed using the Ciucanu–Kerek procedure (43) and modified by Read *et al.* (44). The

⁶ S. Moineau, personal communication.

product was hydrolyzed with 4 M TFA (110 °C, 3 h), dried, reduced with NaBD₄, converted into the alditol acetates by conventional methods, and analyzed by GC-MS (45).

Strain fitness, viability test, and biofilm formation

Viability/survival and fitness of strains derived from *L. lactis* NZ9000 were investigated following growth under various stress-inducing conditions. Initially, the level of halotolerance (5.5% (w/v) NaCl), ethanol tolerance (8% (v/v) EtOH), tolerance to low pH conditions (pH 4.8), chelating agents (750 μM EDTA), and the bacteriocin nisin (15 μg/ml) was determined for *L. lactis* NZ9000. Derived mutant strains (Table 1) were also similarly tested. *L. lactis* NZ9000-*cflA* (Table 1) was evaluated to compare its growth behavior with that of the control strain *L. lactis* pVPL3004/pCRISPR.

Data availability

All data created, with the exception of MS data, can be found within the document. MS data have been deposited and are available on the Glycopost depository (54) with the ID GPST000056.

Author contributions—I. T., P. C., J. M., M.-P. C.-C., and D. v. S. conceptualization; I. T., P. C., I. S., S. P., F. F., and M.-P. C.-C. data curation; I. T., P. C., I. S., S. P., F. F., and M.-P. C.-C. formal analysis; I. T., P. C., and M.-P. C.-C. investigation; I. T., P. C., I. S., and M.-P. C.-C. methodology; I. T. writing-original draft; I. T., P. C., I. S., S. P., F. F., J. M., M.-P. C.-C., and D. v. S. writing-review and editing; J. M., M.-P. C.-C., and D. v. S. supervision; J. M. and D. v. S. funding acquisition; M.-P. C.-C. and D. v. S. project administration.

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