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

**Ilias Theodorou**\(^1\)*, Pascal Courtin\(^2\)*, Irina Sadovskaya\(^3\), Simon Palussière\(^2\), François Fenaille\(^4\), Jennifer Mahony\(^1,5\), Marie-Pierre Chapot-Chartier\(^3#\), Douwe van Sinderen\(^1,5#\)

From the \(^1\)School of Microbiology, University College Cork, Cork, Ireland;

\(^2\)Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France;

\(^3\)Équipe BPA, Université du Littoral Côte d’Opale, Institut Régional Charles Violette EA 7394, USC Anses-ULCO, Boulogne-sur-Mer, France;

\(^4\)Université Paris-Saclay, CEA, INRAE, Médicaments et Technologies pour la Santé (MTS), MetaboHUB, 91191 Gif-sur-Yvette, France

\(^5\)APC Microbiome Ireland, University College Cork, Cork, Ireland.

**Running Title:** *Glycosylation of Lactococcus lactis* glycopolymers

# To whom correspondence should be addressed:
Douwe van Sinderen, School of Microbiology, University College Cork, Western Road, Cork, Ireland, d.vansinderen@ucc.ie, Tel: +353214901365
Marie-Pierre Chapot-Chartier, Micalis Institute, INRA, 78350 Jouy-en-Josas, France, Marie-Pierre.Chapot-Chartier@inra.fr, Tel: +33134652268

*I.T. and P.C. contributed equally to this work.

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Extra-cytoplasmic 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 (*llnz_02975, cflA*) and confirmed that it participates in the glycosylation of the three cell wall glycopolymer rhamnan, PSP, and LTA, thus indicating that its function is shared by the three TGSs. Finally, we observed that glucosylation of both rhamnan and PSP can increase resistance to bacteriophage predation and that LTA galactosylation alters *L. lactis* resistance to bacteriocin.
Introduction

The cell wall of Gram-positive bacteria plays a protective role in maintaining cell integrity, yet also makes the bacterial cell vulnerable since 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 (LTA) (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 glyco-conjugates, 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 re-orients 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 [PoIM] GT) were shown to be involved in the galactosylation of WTA in Ls. monocytogenes serotype 4nonb (7). Additionally, GtIA (Und-P GT) and GtIB (PoIM GT) were shown to attach galactose (Gal) onto the LTA moiety of Ls. 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) (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), while 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 Ls. 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 Ls. 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 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 are 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), while all three strains also harbour 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**), **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 (Figure 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 [aa] identity), while the putative PolM GT proteins do not exhibit such a high degree of identity (Figure 1). CsdA, CsdC and CsdE belong to the GT2 family (CAZY; [http://www.cazy.org](http://www.cazy.org)) that contains enzymes transferring sugar from NDP-sugar to various substrates including Und-P. They contain two TMHs at their C-terminus and the typical DXD conserved motif (D92VD94 in CsdA, D95AD97 in CsdC and D102AD104 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. Besides, 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 aa) integral membrane protein containing a total of four TMHs as found previously in polyisoprenyl-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 (}
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 Figure 1. Sequence comparisons at protein level indicated the almost ubiquitous presence of *csdEF* in *L. lactis* genomes (29 out of 33 strains), whereas *csdAB* and *csdCD* are much less widely distributed (9 and 10 strains out of a total of 33 examined strains, respectively) (Figure 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 non-functional 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. (Table 1). In parallel, the *csdCD* gene pair was expressed in *L. lactis* strain 3107 which lacks functional *csdCD* homologs (Table 1).

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, while they were also analyzed by matrix-assisted laser desorption/ionization mass-spectrometry (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 to 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 wild type *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) (Figure 3A), whereas this peak was absent in the spectrum of 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 3B). Methylation analysis showed that terminal Glc (t-Glc) detected in the wild-type 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 structure of PSP purified from the single *csdC* and *csdD* mutants was also analysed. Monosaccharide composition analysis revealed a clear decrease of Glc/Gal ratio in both mutants compared to the PSP of *L. lactis* NZ9000 (Table 2). Maldi-Tof MS analysis and methylation analysis of the PSP obtained from the corresponding *csdD* mutant revealed the complete loss of the side chain Glc, similar to the effect observed in the double *csdCD* mutant (Supplementary Figure 1). 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 (Supplementary Figure 1). These results suggest that another enzyme with 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 to the value in the PSP of wild-type 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 to WT 3107 (Figure 3E & 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 to WT (data not shown). Since 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 to NZ9000 (WT) (Figure 4). In the spectrum of WT rhamnan, a major family of signals were separated by 438-m.u. (mass units) (Figure 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-m.u. increments that can be assigned to a difference of 3 Rha plus 1 Hex, corresponding to one rhamnan subunit bearing a Glc side chain (Figure 4B). Methylation analysis confirmed a substantial increase of t-Glc in the rhamnan purified from L. lactis NZ9000 (pNZ44::csdAB), while a 2,3-disubstituted Rha appeared with a concomitant decrease in the level of 3-Rha, compared to the control NZ9000 rhamnan (Supplementary Figure 2). 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: \( (1\rightarrow2)\)-α-Rha-(1\rightarrow2)-α-Rha-(1→3)-[Glc-(1→2)]-α-Rha-. No effect of csdAB overexpression was detected on PSP structure either by composition or MS analysis (Supplementary Figure 3).

Overexpression of csdA alone in NZ9000 did not modify the rhamnan or PSP structures (Supplementary Figures 3B and 4B). 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 (Supplementary Figure 4C) 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 (Supplementary Figure 3C). 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 directed to perform rhamnan glucosylation.
csdAB genes from NZ9000 were then introduced on plasmid pNZ44:csdAB in strain 3107 that 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 to the control L. lactis 3107 pNZ44 and wild-type strains (Supplementary Figure 5). 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 glycolpolymers such as WTA or LTA. Of note, L. lactis has previously been shown to synthesize LTA that consists of glycerol-phosphate chains, with Gal as a sugar substituent (15). To assess if LTA glycosylation is dependent 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 ten-fold decrease in Gal levels at constant glycerol levels in the L. lactis NZ9000-csdEF mutant preparation (Figure 5A). In agreement with these results, the amount of t-Gal decreased dramatically in this sample, compared to the preparation from L. lactis NZ9000 (Figure 5B). These data clearly indicate that mutating both csdE and csdF negatively affects galactosylation of poly(glycerophosphate) chains of LTA in L. lactis NZ9000.

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 Ls. monocytogenes GtcA flippase (7) was identified in the L. lactis NZ9000 genome. Analysis of the PSP oligosaccharides purified from a L. lactis NZ9000-cflA mutant revealed a decreased level of Glc in PSP compared to 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 (Figure 3C). Complementation of the cflA mutant allowed the phenotypic restoration of the WT PSP structure as shown by Maldi-ToF MS analysis of PSP oligosaccharides (Figure 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 lead to a significant increase of the rhamnan mass as described above, in the cflA background, overexpression of csdAB had no effect (Supplementary Figure 4E, 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-cfIA mutant, compared to 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 cfIA mutant restores the level of Gal and t-Gal (Supplementary Figure 6). These results indicate that the flippase CfIA 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, 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, EDTA, and reduced pH. It was observed that under optimal growth conditions (in M17Glc broth) non-sense 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 wild-type and the derived strains. Overexpression of the csdEF provided increased resistance to nisin, while the opposite effect was observed for the non-sense mutant (Figure 6). Interestingly, when the effect of a non-sense mutation in cfIA was investigated, it was observed that the mutant strain exhibited a reduced growth rate compared to the control strain even under optimal conditions (Supplementary Figure 7).

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 if 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 knock-out 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 sub-optimal 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. Figure 7 indicates the much higher efficiency (3.7 log) by which phage p2 infects the derivative strain *L. lactis* NZ9000-csdCD when compared to p2 infection of the *L. lactis* NZ9000 in the presence of salt, a difference which 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 exhibits enhanced reversible and irreversible adsorption to *L. lactis* NZ9000-csdCD relative to *L. lactis* NZ9000, under standard laboratory conditions (Supplementary Figure 8).

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 groups (Supplementary Figure 9A). 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 wild-type 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* VESS5751 and can maintain their ability to infect it. However, upon overexpression of csdAB in *L. lactis* VESS5751 the infectivity of jj50* is reduced approximately hundred-fold compared to the control strains,
while MCC1 appears to be unaffected by csdAB overexpression (Supplementary Figure 9B).

Finally, the effect of mutation of the flippase gene, cfIA, on bacteriophage interaction was investigated. Surprisingly, despite the effect that the mutation has on the growth of the strain (Supplementary Figure 7), the tested phages were equally efficient at infecting the control and mutant L. lactis NZ9000-cfIA strains, possibly owing to the maintenance of the Glc side-chain in the PSP of L. lactis NZ9000-cfIA, albeit at a reduced frequency compared to the wild-type 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, the second a polytopic transmembrane GT (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, while 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, while CsdD is presumed to catalyse Glc transfer onto PSP outside the cytoplasmic membrane (Figure 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 (Figure 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 polytopic membrane glycosyltransferase (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 CsbB can utilise the undecaprenyl-phosphate-glucose (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 CsbB provides specificity of the glycosylation reaction towards rhamnan. Finally, the gene pair csdEF was shown to be required for LTA galactosylation (Figure 8). TGSs have previously been shown to glycosylate polymers derived from both a Wzy-dependent and ATP-binding cassette (ABC) transporter assembly pathways (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 highlighted above. Inactivation of cflA was shown to completely block glycosylation of the rhamnan, as well as galactosylation of LTA and, though partially, to block PSP glycosylation. From these results, it is clear that CflA is shared by the three TGSs with CsdAB, CsdCD and CsdEF (Figure 8). Partial substitution of PSP subunits was still present in the cflA mutant suggesting the presence of another flippase endowed with the same activity as CflA, although a sequence homology search did not return any likely candidate. Interestingly, unlike the mutants of csdAB or csdCD, the cflA mutant strain exhibits a distinctly reduced growth rate which may result from an indirect effect of the accumulation of Und-P-Glc intermediates preventing recycling of Und-P (required for essential processes such as peptidoglycan synthesis).

It has previously been established that the receptor for both 936 (10,13,22-24) and P335 group phages (10,12,25) is saccharidic in nature. It was thus expected that modifications of the lactococcal CWPS affect phage infectivity. We observed that phage p2 preferentially infected the csdCD mutant derivative under saline conditions. Such environmental conditions seemingly destabilize the already weak protein-saccharide interactions characteristic between the 936-type receptor binding proteins (RBPs) and their CWPS receptors. Since phage p2 infects and also adsorbs more efficiently to the derivative lacking the PSP glucose side-chain, we hypothesize that its RBP has evolved to preferentially bind to the primary pentasaccharide unit of the PSP. The existence of the Glc-side chain on PSP may thus provide a degree of phage insensitivity to the strain. Similar effects from the decoration of cell wall polysaccharide components such as WTA, have previously been reported for phage adsorption on various Gram positive bacterial strains (3). The overexpression of csdAB and subsequent increase of Glc side chain on rhamnan, was shown to also act as a phage predation deterrent both in L. lactis 3107 (PSP(+) and rhamnan unexposed) and L. lactis VES5751 (PSP(-) and rhamnan exposed).

In addition to phage/host interactions, cell wall glycopolymers and their substitutions may contribute to increased antibiotic resistance (15,26,27). Here, we investigated the effect of LTA glycosylation on various phenotypic responses in L. lactis. Interestingly, our results support previous evidence for the mechanism of nisin resistance in L. lactis through increased TA substitution and increased septal density (15). In detail, altering the levels of galactosylation of the teichoic acids in L. lactis NZ9000 led to equivalent changes in nisin resistance, i.e. increased glycosylation caused increased nisin resistance and vice versa.

Three-component glycosylation systems are commonly associated with prophage elements in Shigella (18) and Salmonella species (28). They have also been shown to directly interfere with phage/host interactions in Ls. monocytogenes (7,19). Interestingly, we observed that the genomic vicinity of csdCD is rich in mobile elements including transposases and prophage components. This observation combined with the effect on phage-host interactions upon modulation of the gene pair expression strongly suggests that such mobile gene cassettes provide a swift response to phage predation by providing non-essential decorations onto an already existing polysaccharide structure. We speculate that genes encoding such CWPS modulating functions have been “hijacked” by active prophages to further enhance any pre-existing superinfection exclusion mechanisms and activities. Our research reiterates the structural complexity as well the dynamic nature of the CWPS in L. lactis. Such characteristics were previously thought to stem 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/L 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\textsuperscript{nis}, was achieved through supplementation of the media with nisaplin (40 ng/ml) (DuPont, USA).

Bacteriophage assays.

Phages used in this study are listed in Table 1. Propagation of phages on their respective host strains was performed as previously described (29). Similarly, both spot/plaque assays (30) and adsorption assays (31) were performed as previously described 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 Ls. monocytogenes (7). To this end, the proteins indicated in Figure 1 were compared using BLASTP against the compiled coding sequences (CDS) of the L. lactis MG1363 genome (Genbank 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 Ls. monocytogenes proteins: GtcA; GtcB and GtlA; 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-388 (Sylvain Moineau, personal communication) using BLASTN. Intrinsic properties of protein sequences were assessed using HHpred (32) and TMHMM online predictive tools (33,34).

Selected genes were analysed based on sequence identity against all publicly available L. lactis chromosomal and plasmid sequences, while protein sequence comparisons were performed using all-against-all bi-directional 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/non-functionality of each of the retrieved candidate genes from all assessed L. lactis strains, was created and run in MeV suite (V4.9) (36).

Molecular cloning.

All recombinant plasmids (Table 1) were generated in L. lactis NZ9000 and primers used can be seen in Supplementary Table 1 (Eurofins MWG). The gene pairs of interest, csd\textsubscript{AB}, csd\textsubscript{CD}, and csd\textsubscript{EF}, 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, csd\textsubscript{AB} 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, csd\textsubscript{AB} and csd\textsubscript{EF} was cloned independently into the pNZ44 vector.

Recombineering and CRISPR-assisted-recombineering.

Recombineering was performed as previously described (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 cfl\textsubscript{A} gene since 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 rep\textsubscript{A}, rep\textsubscript{D}, rep\textsubscript{E} from the backbone of the plasmid pPTPi (42) along with the chloramphenicol resistance gene (cm\textsuperscript{R}), P\textsuperscript{nis}\textsuperscript{A} promoter, and the ss-DNA
binding protein encoding gene, recT, from plasmid pJP005 (39), was constructed. Subsequently, the single-step approach to CRISPR-Cas9-assisted recombineering was employed as previously outlined (42).

**CWPS and LTA extraction, purification and analysis.**

CWPS was extracted from cell envelope fractions prepared from L. lactis cells and analysed 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 (HPAEC-PAD) (ICS5000 system, ThermoFisher scientific). Purified fractions were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Maldi-Tof MS) using 2,5-dihydroxy-benzoic acid (DHB) matrix with an UltraFleXtreme instrument (Bruker Daltonics, Bremen, Germany). MS data are accessible on the Glycopost website (https://glycopost.glycosmos.org/) ID: GPST000056.

Crude LTA was extracted essentially as previously described (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 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 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 behaviour 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 repository (https://glycopost.glycosmos.org) with the ID GPST000056.

**Conflict of interest**

The authors declare that they have no conflict of interest with the contents of this article.
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FOOTNOTES
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The abbreviations used are: CWPS, cell wall polysaccharide; LTA, lipoteichoic acid; 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.
### Table 1. Strains, plasmids, and phages used 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 | (46) |
| L. lactis subsp. cremoris 3107 | Host to phages LC3, TP901-1, 66901, 66902, 66903, 62604, 62605, and 62601 | (47) |
| L. lactis subsp. cremoris VES5751 | *L. lactis* MG1363 derivative exhibiting a deficient PSP phenotype due to a mutation in *llmg_0226* | (11) |
| L. lactis subsp. cremoris NZ9000-csdAB | NZ9000 with GAATTCG insert in *llnz_00690* (*csdA*) and *llnz_00695* (*csdB*), resulting in a TGA stop codon insertion in each gene. | This work |
| L. lactis subsp. cremoris NZ9000-csdC | NZ9000 with GAATTCG insert in *llnz_03080* (*csdC*) resulting in a TGA stop codon in *csdC* | This work |
| L. lactis subsp. cremoris NZ9000-csdD | NZ9000 with GAATTCG insert in *llnz_03075* (*csdD*) resulting in a TGA stop codon in *csdD* | This work |
| L. lactis subsp. cremoris NZ9000-csdCD | NZ9000 with GAATTCG insert in *llnz_03075* (*csdD*) and *llnz_03080* (*csdC*) resulting in a TGA stop codon insertion in both genes. | This work |
| L. lactis subsp. cremoris NZ9000-csdEF | NZ9000 with GATAACCC insert in *llnz_07820* (*csdE*) and *llnz_07825* (*csdF*), resulting in a TGA and TAA stop codon insertion, respectively. | This work |
| L. lactis subsp. cremoris NZ9000-cflA | NZ9000 with TAATAGGGG insert in *llnz_02975* (*cflA*) resulting in a TAA and TAG double stop codon in *cflA* | This work |
| **Plasmids**              |            |        |
| pJP005                    | Recombineering-facilitating vector containing *recT*, P^rec^, Cm^r^ | (39) |
| pNZ44                     | High-copy expression vector, contains P44 constitutive promotor, Cm^r^ | (37) |
| pNZ44str                  | Derivative vector of pNZ44, Str^r^ | Developed by Andrea Garzon, UCC Collection |
| pCNR                      | Recombineering-facilitating vector containing *recT*, P^rec^, Cm^r^ derived from the low-copy vector pJP005 | This work |
| pVPL3004                  | Low-copy vector expressing cas9 along with tracRNA, Ery^r^ | (41) |
| pCRISPR                   | High-copy vector carrying CRISPR repeats and used for inserting target spacer sequences, Tet^r^ | (41) |
| pCRISPR::cflA | pCRISPR plasmid carrying a CRISPR repeat targeting the recombineered sequence of gene cflA, Tet<sup>+</sup> | This work |
|---------------|------------------------------------------------------------------------------------------------|----------|
| pNZ44::csdCD  | pNZ44 containing genes csdC and csdD | This work |
| pNZ44::csdA   | pNZ44 containing gene csdA | This work |
| pNZ44::csdB   | pNZ44 containing gene csdB | This work |
| pNZ44::csdAB  | pNZ44 containing genes csdA and csdB | This work |
| pNZ44str::csdAB | pNZ44str containing genes csdA and csdB | This work |
| pNZ44::csdE   | pNZ44 containing gene csdE | This work |
| pNZ44::csdF   | pNZ44 containing gene csdF | This work |
| pNZ44::csdEF  | pNZ44 containing genes csdE and csdF | This work |

**Bacteriophages**

| jj50*         | Spontaneously acquired derivative of jj50 (NC_008371.1), propagated on NZ9000 | This work |
|---------------|--------------------------------------------------------------------------------|----------|
| p2            | 936 species, propagated on NZ9000 | (48) |
| sk1           | 936 species, propagated on NZ9000 | (49) |
| MCC1          | Derivative of sk1, propagated on NZ9000 | (8) |
| LC3           | P335 species, propagated on 3107 | (50) |
| TP901-1       | P335 species, propagated on 3107 | (47) |
| 66901         | 936 species, propagated on 3107 | (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 | (51) |
| 62605         | 936 species, propagated on 3107 | (51) |
| 62601         | 936 species, propagated on 3107 | (51) |
| 63301         | P335 species, propagated on 3107 | (52) |
| 50101         | P335 species, propagated on 3107 | (52) |
| 07501         | P335 species, propagated on 3107 | (53) |
| 58601         | P335 species, propagated on 3107 | (53) |
| 86501         | P335 species, propagated on 3107 | (52) |
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\(^1\) | Glc |
|-------------------------|-----|--------|-----------|-----|
| *L. lactis* NZ9000      | 0.8 | 2.6    | 1         | 2.4 |
| *L. lactis* NZ9000-csdCD| 0.6 | 2.1    | 1         | 1   |
| *L. lactis* NZ9000-csdC | 0.5 | 2.1    | 1         | 1.1 |
| *L. lactis* NZ9000-csdD | 0.6 | 2.5    | 1         | 1.1 |
| *L. lactis* NZ9000-cflA | 0.7 | 2      | 1         | 1.3 |
| *L. lactis* 3107 pNZ44  | 0   | 1.1    | 1         | 0.4 |
| *L. lactis* 3107 pNZ44::csdCD | 0.3* | 1.0   | 1         | 0.9 |

Footnotes: \(^1\)Values are standardized relative to Gal, \(*\)Presumed contamination of PSP by short rhamnan chains during HP-SEC purification
Figure 1. Schematic representation of 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 colour coded according to predicted functional domains: green, Und-P GT, blue, PolM GT, yellow, flippase., Percentage similarity based of BLASTP alignment (of the encoded protein products) is also indicated. Finally, sequences representing predicted transmembrane helices (TMHs) (predicted with TMHMM Server v. 2.0) are indicated by red rectangular bands. Solid lines = adjoining genes, Dashed lines = Order/orientation of genes altered for visualization purposes.
Figure 2. Heatmap representing the presence (light green), absence (black), or non-functionality (dark green) of the 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 heatmap 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.
Figure 3. Maldi-ToF MS spectra obtained on purified PSP oligosaccharides extracted by HF treatment from cell walls of (A) wild-type *L. lactis* NZ9000; (B) *L. lactis* NZ9000-csdCD mutant; (C) *L. lactis* NZ9000-cflA(pNZ44str) flippase mutant; (D) *L. lactis* NZ9000-cflA(pNZ44str::cflA) complemented flippase mutant (E) *L. lactis* 3107 with empty pNZ44; (F) *L. lactis* 3107(pNZ44::csdCD). 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 Galβ 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 Galβ 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 Supplementary Table 2. 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.
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.
Figure 5. (A) Monosaccharide analysis of crude phenol extracts from *L. lactis* NZ9000 and NZ9000-csdEF. Each extract (5 mg), along with Inositol (Ino, 90 µg) as an internal standard, were treated with HF, hydrolysed, converted into alditol acetates and analysed by GC-FID. (B) Methylation analysis of crude phenol extracts from *L. lactis* NZ9000 and NZ9000-csdEF. Samples were analysed by GC-MS. t-Glc = Terminal glucose; t-Gal = Terminal galactose; Ino = m-Inositol Standard. The figures were zoomed onto the region corresponding to hexoses.
**Figure 6.** Growth behaviour of *L. lactis* NZ9000 controls and csdEF derivatives in M17 Glc and 15 μg/ml of nisin.
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 stars; ***p<0.0001, *p<0.05.
Figure 8. Schematic representation of the suggested functions of the proteins encoded by each of the three identified gene pairs (csdAB, csdCD, csdEF) and the flippase-encoding gene cflA.
Three distinct glycosylation pathways are involved in the decoration of Lactococcus lactis cell wall glycopolymers

Ilias Theodorou, Pascal Courtin, Irina Sadovskaya, Simon Palussière, François Fenaille, Jennifer Mahony, Marie-Pierre Chapot-Chartier and Douwe van Sinderen

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