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GtfA and GtfB Are Both Required for Protein *O*-Glycosylation in *Lactobacillus plantarum*

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Acm2, the major autolysin of *Lactobacillus plantarum* WCFS1, was recently found to be *O*-glycosylated with *N*-acetylhexosamine, likely *N*-acetylglucosamine (GlcNAc). In this study, we set out to identify the glycosylation machinery by employing a comparative genomics approach to identify Gtf1 homologues, which are involved in fimbria-associated protein 1 (Fap1) glycosylation in *Streptococcus parasanguinis*. This *in silico* approach resulted in the identification of 6 candidate *L. plantarum* WCFS1 genes with significant homology to Gtf1, namely, *tagE1* to *tagE6*. These candidate genes were targeted by systematic gene deletion, followed by assessment of the consequences on glycosylation of Acm2. We observed a changed mobility of Acm2 on SDS-PAGE in the *tagE5E6* deletion strain, while deletion of other *tagE* genes resulted in Acm2 mobility comparable to that of the wild type. Subsequent mass spectrometry analysis of excised and in-gel-digested Acm2 confirmed the loss of glycosylation on Acm2 in the *tagE5E6* deletion mutant, whereas a lectin blot using GlcNAc-specific succinylated wheat germ agglutinin (sWGA) revealed that besides Acm2, *tagE5E6* deletion also abolished all but one other sWGA-reactive, protease-sensitive signal. Only complementation of both *tagE5* and *tagE6* restored those sWGA lectin signals, establishing that TagE5 and TagE6 are both required for the glycosylation of Acm2 as well as the vast majority of other sWGA-reactive proteins. Finally, sWGA lectin blotting experiments using a panel of 8 other *L. plantarum* strains revealed that protein glycosylation is a common feature in *L. plantarum* strains. With the establishment of these enzymes as protein glycosyltransferases, we propose to rename TagE5 and TagE6 as GtfA and GtfB, respectively.

Probiotics, of which the majority belong to the genera *Lactobacillus* and *Bifidobacterium* (1–3), have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (4). One mechanism by which these health benefits are mediated is through molecular interactions between probiotic bacteria and host cells, in which bacterial surface molecules appear to play a pivotal role (1–3). These surface effector molecules include canonical polymers such as wall teichoic acids (WTA) and lipoteichoic acids (LTA), peptidoglycan, and capsular polysaccharides, but also proteinaceous molecules (2, 5–7).

Many proteinaceous molecules have established functions associated with adhesion to intestinal mucus, such as the mucin-binding proteins (Mub) of *Lactobacillus acidophilus* NCFM (8) and *Lactobacillus reuteri* 1063 (9), a mucus adhesion-promoting protein (MapA) of *L. reuteri* 104R (10), and the mannose-specific adhesin (Msa) of *Lactobacillus plantarum* WCFS1 (11). Examples of proteins involved in adhesion to epithelial cells include the surface layer proteins of *Lactobacillus brevis* ATCC 8287 (12), *Lactobacillus crispatus* JCM 5810 (13), and *Lactobacillus helveticus* R0052 (14). In addition to their role in the capacity for adhesion to mucus and epithelial cells, some *Lactobacillus* surface proteins are able to bind with extracellular matrix (ECM), which is a complex structure surrounding epithelial cells and composed of various proteins, including laminin, collagen, and fibronectin. Reported examples include the collagen-binding protein of *L. reuteri* NCIB11951 (15) and fibronectin-binding protein A of *L. acidophilus* NCFM (8).

Other surface proteins have an impact on probiotic-host interactions via their immunomodulating capacity, for example, Msp1 and Msp2, two peptidoglycan hydrolases of *Lactobacillus rhamnosus* GG which promote epithelial homeostasis (16, 17). Recombinant Msp2 was also shown to prevent and ameliorate experimental colitis in mice by an epidermal growth factor receptor-dependent mechanism (18). Furthermore, surface layer protein A (SlpA) of *L. acidophilus* NCFM was documented to be recognized by the dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor and as a consequence modulates human DCs and T cell functions, leading to regulatory T cell differentiation through increased interleukin 10 (IL-10) and reduced IL-12p70 production (19). A serine- and threonine-rich peptide (STp) harbored by protein D1 that is secreted by *Lactobacillus plantarum* BMCM12 represents another example of a proteinaceous effector molecule, as it was recently demonstrated to stimulate regulatory responses in human intestinal DCs (20).

The most common modification found in proteinaceous molecules is glycosylation, in which glycans can be attached to the amide nitrogen of asparagine, i.e., *N*-glycosylation, or to the hydroxyl oxygen of serine or threonine, i.e., *O*-glycosylation (21). Although protein glycosylation was initially studied exclusively

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for eukaryotes, bacterial protein glycosylation has recently received increasing attention, and it is now clear that bacteria can also modify proteins with diverse *N*-linked and *O*-linked glycan moieties (22–26). So far, most studies on bacterial protein glycosylation have focused on pathogenic organisms (23, 27–29), resulting in the identification of general glycosylation pathways (26), including an *N*-glycosylation pathway in *Campylobacter jejuni* (30, 31) and *O*-linked glycosylation systems in *Neisseria gonorrhoeae* (32, 33). Specific machineries responsible for *O*-glycosylation of abundant surface proteins such as flagellin and pilin have also been described for various pathogenic bacteria (34–36). Moreover, fimbria-associated protein 1 (Fap1), a serine-rich adhesin of *Streptococcus parasanguinis*, has been demonstrated to be heavily glycosylated with *N*-acetylglucosamine (GlcNAc) and glucose (37, 38). This glycosylation requires the concerted activities of two putative glycosyltransferases; Gtf1 and Gtf2 (37). More recent studies pinpointed that protein glycosylation also occurs in certain human intestine commensals, including several *Bacteroides* species (39, 40), and probiotic species such as *L. plantarum* WCFS1 (41, 42) and *L. rhamnosus* GG (43). More specifically, Msp1 of *L. rhamnosus* GG is *O*-glycosylated at serine residues 106 and 107, and its glycan moieties are recognized by the concanavalin A (ConA) lectin, which is specific for mannose and/or glucose moieties (43). Similarly, the major autolysin of *L. plantarum* WCFS1, Acm2, was shown to be *O*-glycosylated in its N-terminal alanine-, serine-, and threonine-rich region (AST domain), which could be selectively detected by using the GlcNAc-specific biotinylated succinylated wheat germ agglutinin (sWGA) lectin (41, 42). Intriguingly, AST domains are present in several other proteins encoded in the *L. plantarum* WCFS1 genome, including several other peptidoglycan hydrolases (41) and Lp_2145 (44), suggesting that these proteins could also be subjected to glycosylation (41). Indeed, a recent study found 10 novel glycoproteins in *L. plantarum* WCFS1, including 2 AST domain-containing peptidoglycan hydrolases (Lp_2162 and Lp_3421), 4 cytoplasmic proteins (DnaK, ELp_2152, FtsY, and FtsK1), and the secreted proteins Lp_2260 and Lp_1643 (45).

To date, no protein glycosylation machinery has been described for *Lactobacillus* species (41, 43). In this study, we employed a comparative genomics approach to identify Gtf1 homologues in the genome of *L. plantarum* WCFS1, resulting in the identification of 6 candidate genes [previously annotated as poly(glycerolphosphate) α-glucosyltransferases, i.e., *tagE1* to *tagE6*] that might encode protein glycosyltransferases (46). These candidate genes were targeted by a gene deletion and complementation approach, after which we assessed the consequences of these genetic modifications for the presence of glycan moieties in proteins by employing the GlcNAc-specific lectin sWGA in blotting experiments. Moreover, we specifically assessed the impact of *tagE5E6* deletion on the previously established glycosylation of Acm2 (41, 42) by mass spectrometry analysis (MS). These experiments revealed that TagE5 and TagE6 are both required for the glycosylation of proteins, including Acm2, in *L. plantarum* WCFS1. Moreover, expansion of our lectin blotting experiments to a panel of other *L. plantarum* strains revealed that protein glycosylation is widespread in this species. To the best of our knowledge, these results represent the first example of protein glycosylation machinery in a *Lactobacillus* species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus plantarum* strains were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strain TOP10 (Invitrogen, Bleiswijk, the Netherlands) was grown at 37°C in tryptone yeast broth (47) with aeration (48). Solid media were prepared by adding 1.5% (wt/vol) agar to the broths. Where appropriate, antibiotics were added for *L. plantarum* and *E. coli* at 10 μg/ml of chloramphenicol and 30 and 200 μg/ml of erythromycin, respectively.

DNA manipulations. Primers used are listed in Table 2 and were synthesized by Sigma-Aldrich (Zwijndrecht, the Netherlands). Standard procedures were used for DNA manipulations in *E. coli* (48). Plasmid DNA was isolated from *E. coli* using a JETSTAR kit (Genomed GmbH, Bad Oberhausen, Germany). *L. plantarum* DNA was isolated and transformed as described previously (49). PCR amplifications were performed using hot-start KOD polymerase (Novagen, Madison, WI). Amplicons were purified using the WizardSV Gel and PCR Clean-Up System (Promega, Leiden, the Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB Spin PCRapace (Invitek GmbH, Berlin, Germany), PCR master mix (Promega), and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

Construction of *tagE* deletion mutants. The *tagE* deletion mutants were constructed as described previously (50), using a double-crossover strategy to replace the target *tagE* genes with a chloramphenicol resistance cassette (*lox66*-P_{32}*cat-lox71*) (50). In this study, a derivative of the mutagenesis vector pNZ5319 (50), designated pNZ5319TAG (P. A. Bron et al., unpublished data), was used to introduce a unique DNA tag into the chromosome during gene deletion, which can be used for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of each *tagE* gene set (*tagE1*, *tagE2E3*, *tagE4*, and *tagE5E6*) were amplified by PCR using tagEs-Up-F/R and tagEs-Down-F/R primers, respectively (Table 2). Each amplicon generated was subsequently joined by a second PCR to *tag-lox66*-P_{32}*cat-lox71* by a splicing by overlap extension strategy (51), using tagE-Up-F/tagE-Down-R primer pairs (Table 2). The resulting PCR products were digested with SwaI and Ecl136II and cloned into similarly digested pNZ5319TAG. The obtained mutagenesis plasmids were transformed into *L. plantarum* WCFS1 as described previously (49). The resulting integrants were assessed for a double-crossover integration event by using tagE-out-F/R primers (Table 2). For each of the mutant constructions a single colony displaying the anticipated genotype was selected, yielding the mutants NZ3540Cm (Δ*tagE1*), NZ3541Cm (Δ*tagE2E3*), NZ3542Cm (Δ*tagE4*), and NZ3543Cm (Δ*tagE5E6*).

Complementation of Δ*tagE5E6*. The genomic organization of *tagE5* and *tagE6* (*lp_2843-2844*) is shown in Fig. 1A. The *tagE5E6* genes and the individual *tagE6* gene of *L. plantarum* WCFS1 were amplified, including their native promoter (P_{tagE6}, upstream of *tagE6*), using primers IC013/IC014 and IC013/IC015, respectively. Since *tagE5* is also transcribed from the *tagE6* promoter, the P_{tagE6} promoter and *tagE5* were joined by splicing using an overlap extension strategy (51). The promoter was amplified by using primers IC013/IC016; primer IC016 contains the initial 23 nucleotides of *tagE5*. The *tagE5* gene was amplified using primer IC017, which contains the terminal 21 nucleotides of the promoter region, and primer IC014. The two PCR products were mixed in a molar ratio of 1:1 and amplified using primers IC013/IC014 to join the promoter and *tagE5*. A SacI site was introduced by primer IC014 and IC015 downstream of *tagE6* and *tagE5*, respectively. pMEC10 was digested by SacI and SfoI, whereas PCR products of *tagE5E6*, *tagE6*, and *tagE5* were digested with SacI. Digested fragments were ligated using T4 DNA ligase. Subsequently, the ligation mixtures were transformed into *E. coli* TOP10; positive clones were selected by colony PCR (52) using primers IC013/IC015 for *tagE6*, IC014/IC017 for *tagE5*, and IS260/IS247 for *tagE5E6*. Resulting plasmids were designated pNZ8204, pNZ8205, and pNZ8206 for the complementation plasmid of *tagE5E6*, *tagE6*, and *tagE5*, respectively. Integrity of nu-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source[b]
Strains		
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	46
ATCC 14917	Isolate from pickled cabbage	ATCC
ATCC 8014	Isolate from maize ensilage	ATCC
CIP104440	Isolate from human stool	CIP
CIP104450	Isolate from human stool	CIP
NC8	Isolate from grass silage	77
NCIMB12120	Isolate from Ogi, Nigeria	NCIMB
LP80	Isolate from silage	78
LP85-2	Origin from silage, France	57
Δ*acm2* mutant	NZ3557Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat-lox71-tagH9* replacement of *acm2* (*acm2*::*lox66*-P_{32}-*cat-lox71-tagH9*)	41
Δ*tagE1* mutant	NZ3540Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat-lox71*-tag8.5 replacement of *tagE1* (*tagE1*:: *lox66*-P_{32}-*cat-lox71-tag8.5*)	This study
Δ*tagE2E3* mutant	NZ3541Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat-lox71-tagF10* replacement of *tagE2E3* (*tagE2E3*::*lox66*-P_{32}-*cat-lox71-tagF10*)	This study
Δ*tagE4* mutant	NZ3542Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat-lox71-tagG1* replacement of *tagE4* (*tagE4*:: *lox66*-P_{32}-*cat-lox71-tagG1*)	This study
Δ*tagE5E6* mutant	NZ3543Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat-lox71-tagG7* replacement of *tagE5E6* (*tagE5E6*::*lox66*-P_{32}-*cat-lox71-tagG7*)	This study
tagE5E6 complementation mutant	NZ8204CmEm; Cm^r Em^r; derivative of NZ3543Cm containing chromosomally integrated pNZ8204 at $tRNA^{Ser}$ site	This study
tagE6 complementation mutant	NZ8205CmEm; Cm^r Em^r; derivative of NZ3543Cm containing chromosomally integrated pNZ8205 at $tRNA^{Ser}$ site	This study
tagE5 complementation mutant	NZ8206CmEm; Cm^r Em^r; derivative of NZ3543Cm containing chromosomally integrated pNZ8206 at $tRNA^{Ser}$ site	This study
E. coli		
TOP 10	Cloning host; F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pNZ5319	Cm^r Em^r; mutagenesis vector for gene replacements in *L. plantarum*	50
pNZ3540	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE1*	This study
pNZ3541	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE2-E3*	This study
pNZ3542	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE4*	This study
pNZ3543	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE5E6*	This study
pMEC10	Em^r; integration plasmid	79
pNZ8204	Em^r; pMEC10 derivative harboring *tagE5E6*	This study
pNZ8205	Em^r; pMEC10 derivative harboring *tagE6*	This study
pNZ8206	Em^r; pMEC10 derivative harboring *tagE5*	This study

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Str^r, streptomycin resistant.
[b] ATCC, American Type Culture Collection, Manassas, VA; CIP, Collection de l'Institut Pasteur, Paris, France; NCIMB, National Collections of Industrial, Food and Marine Bacteria, Aberdeen, Scotland.

cleotide sequences for each construct was confirmed by sequence analysis. Subsequently, the complementation plasmids were introduced into the Δ*tagE5E6* strain by electroporation as described previously (49). Transformants were screened for chloramphenicol and erythromycin resistance, followed by PCR amplifications to confirm the chromosomal integration of introduced plasmid using primers tRNA/IC021 for NZ8204 and NZ8205 and primers tRNA/IC020 for NZ8206.

Preparation of surface proteins and whole-cell extracts and proteinase K treatment. Overnight cultures of *L. plantarum* strains were diluted in fresh MRS broth to an optical density at 600 nm (OD_{600}) of 0.1. After 5 h of incubation at 37°C (OD_{600} of approximately 1.0), the exact OD_{600}s of the cultures were determined and cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C. For surface protein isolation, a procedure adapted from that of Fredriksen et al. (41) was used. Briefly, harvested cells were washed once with phosphate-buffered saline (PBS) to remove residual medium and resuspended in 1 ml of cold PBS. Surface proteins were extracted by gentle agitation at 600 rpm for 30 min using an Eppendorf thermomixer (Eppendorf, Hamburg, Germany). The supernatants were collected after centrifugation at 5,000 × *g* for 10 min. The surface proteins were precipitated from supernatants by addition of trichloroacetic acid (TCA) to a final concentration of 16% and an overnight incubation at 4°C, followed by centrifugation at 16,000 × *g* for 15 min. The precipitated proteins were washed with 200 μl of acetone and then air dried with open lids at 50°C. Dried protein pellets were solubilized in

TABLE 2 Primers used in this study

Primer	Sequence[a]	Reference or source
is128 tag-lox66-F3	5′-AAATCTACCGTTCGTATAATGTATG-3′	80
is129 tag-lox71-R3	5′-CTCATGCCCGGGCTGTAACCG-3′	80
87	5′-GCCGACTGTACTTTCGGATCC-3′	50
CreF	5′-CGATACCGTTTACGAAATTGG-3′	50
CreR	5′-CTTGCTCATAAGTAACGGTAC-3′	50
EryintF	5′-TCAAATACAGCTTTTAGAACTGG-3′	50
EryintR	5′-ATCACAAACAGAATGATGTACC-3′	50
tagE1-Up-F	5′-GCCGCAACAACCATACTGGG-3′	This study
tagE1-Up-R	5′-GCATACATTATACGAACGGTAGATTTAAAATAATACATCACCTAGCCG-3′	This study
tagE1-Down-F	5′-CGGTTACAGCCCGGGCATGAGATAGCAGCACTTTAAGAACTGG-3′	This study
tagE1-Down-R	5′-GCGATTACATCGCCTTGGCG-3′	This study
tagE1-out-F	5′-GCTAGTCATGTCACGGATGC-3′	This study
tagE1-out-R	5′-TCACTCACAATAAATTCCCCC-3′	This study
tagE2E3-Up-F	5′-GCAATTACAATGTTGTGCGGC-3′	This study
tagE2E3-Up-R	5′-GCATACATTATACGAACGGTAGATTTGAAGTAAAACATACAGTCACCC-3′	This study
tagE2E3-Down-F	5′-CGGTTACAGCCCGGGCATGAGACGGCTTAAGTAGATTTGACGG-3′	This study
tagE2E3-Down-R	5′-AAGTGCGCGTTTTAGTACGC-3′	This study
tagE2E3-out-F	5′-TACGGTTATTTTCCGGCTCG-3′	This study
tagE2E3-out-R	5′-ATCGGTGGCCTTTTACTTGG-3′	This study
tagE4-Up-F	5′-CGTATCGATTGTTGACAGCG-3′	This study
tagE4-Up-R	5′-GCATACATTATACGAACGGTAGATTTATCGGCTAAACAACCACATGC-3′	This study
tagE4-Down-F	5′-CGGTTACAGCCCGGGCATGAGGAAATACATTTGCTACGCCCC-3′	This study
tagE4-Down-R	5′-CGAAGTGACGACTGCAAACG-3′	This study
tagE4-out-F	5′-CTTTCGTAGCCAAAATCGACG-3′	This study
tagE4-out-R	5′-CAAGAACAAGTCACAGCCGC-3′	This study
tagE5E6-Up-F	5′-ATTGGAAACGTTCTGTGCGG-3′	This study
tagE5E6-Up-R	5′-GCATACATTATACGAACGGTAGATTTGTTGTTCAGTGAATATCAAAAATGG-3′	This study
tagE5E6-Down-F	5′-CGGTTACAGCCCGGGCATGAGATAATACATTATTACTCGCTCCC-3′	This study
tagE5E6-Down-R	5′-AGTTGTTGATGAACTGCTGC-3′	This study
tagE5E6-out-F	5′-AAATAATAGTTAGGGGTGAACAC-3′	This study
tagE5E6-out-R	5′-CTTCAGCACTACTTGATGTGC-3′	This study
tRNA	5′-GCGAACCGGCTAATACCGGC-3′	81
IC013	5′-AGCTAACAGACCGGTAGCTGCCAATGAAG-3′	This study
IC014	5′-AACCAGAGCTCCTGGCTGCTACGTGAACCTAATTCC-3′	This study
IC015	5′-TTTCCGAGCTCGCGTTACTAGTTTAGCCGGTGCCTG-3′	This study
IC016	5′-TATTGGTTCACAAAAAAATTCATTATTACTCGCTCCCTTACACGA-3′	This study
IC017	5′-CGTGTAAGGGAGCGAGTAATAATGAATTTTTTTGTGAACCAATATT-3′	This study
IC021	5′-ACGCCACATGCAGTCGATCC-3′	This study
IS169	5′-TTATCATATCCCGAGGACCG-3′	82
IS247	5′-AGATTGTACTGAGAGTGCACC-3′	This study
IS260	5′-GTTGAAAGAACCTGTACTCTCC-3′	This study

[a] Underlined nucleotides indicate parts of the primers that are complementary to the is128-lox66-F3 and is129-lox71-R3 primers.

NuPAGE loading buffer and reducing agent (both from Invitrogen). The NuPAGE buffer volumes were normalized by OD_{600} measurement of original cultures to ensure that the samples represent the surface proteins from similar amounts of cells, and these samples were subsequently used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie brilliant blue staining (48).

For whole-cell extract samples, harvested cells were washed once with 50 mM sodium phosphate buffer, pH 7, to remove residual medium and subsequently resuspended in 50 mM sodium phosphate buffer, pH 7, to a final OD_{600} equivalent of 2.5. Cell suspensions of 1 ml were added to a screw-cap 2-ml tube containing 1-g zirconium beads. Cells were disrupted by three rounds of bead beating (30 s at speed 4.0) using a Fastprep cell disrupter (QBiogene Inc., Cedex, France), interspaced with cooling intervals on ice. The tubes were left for 5 min to allow zirconium bead sedimentation. The resulting supernatants were collected as whole-cell extracts and used in sWGA lectin blot experiments.

For proteinase K treatment, the whole-cell extract samples were treated with proteinase K (Qiagen GmbH, Hilden, Germany; final concentration of 50 μg/ml) for 10, 30, or 60 min at 37°C.

SDS-PAGE and lectin blot analyses. SDS-PAGE and wet blotting were performed using the NuPAGE electrophoresis system (Invitrogen) and XCell II blot module (Invitrogen), respectively, as described in the user manuals. Whole-cell extracts were mixed with NuPAGE sample buffer and were separated under denaturing conditions on NuPAGENovex 4 to 12% bis-Tris gels with morpholinepropanesulfonic acid (MOPS) SDS running buffer (Invitrogen).

For visualization of surface proteins by Coomassie brilliant blue, the standard procedure was used (48).

For lectin blotting, the gels were transferred to nitrocellulose membranes (Thermo Scientific, Bremen, Germany) using a wet blotting method described in the NuPAGE manual (Invitrogen). The membranes were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBST) for 1 h at room temperature. The membranes were then incubated with biotinylated sWGA (Vector Laboratories, Bur-

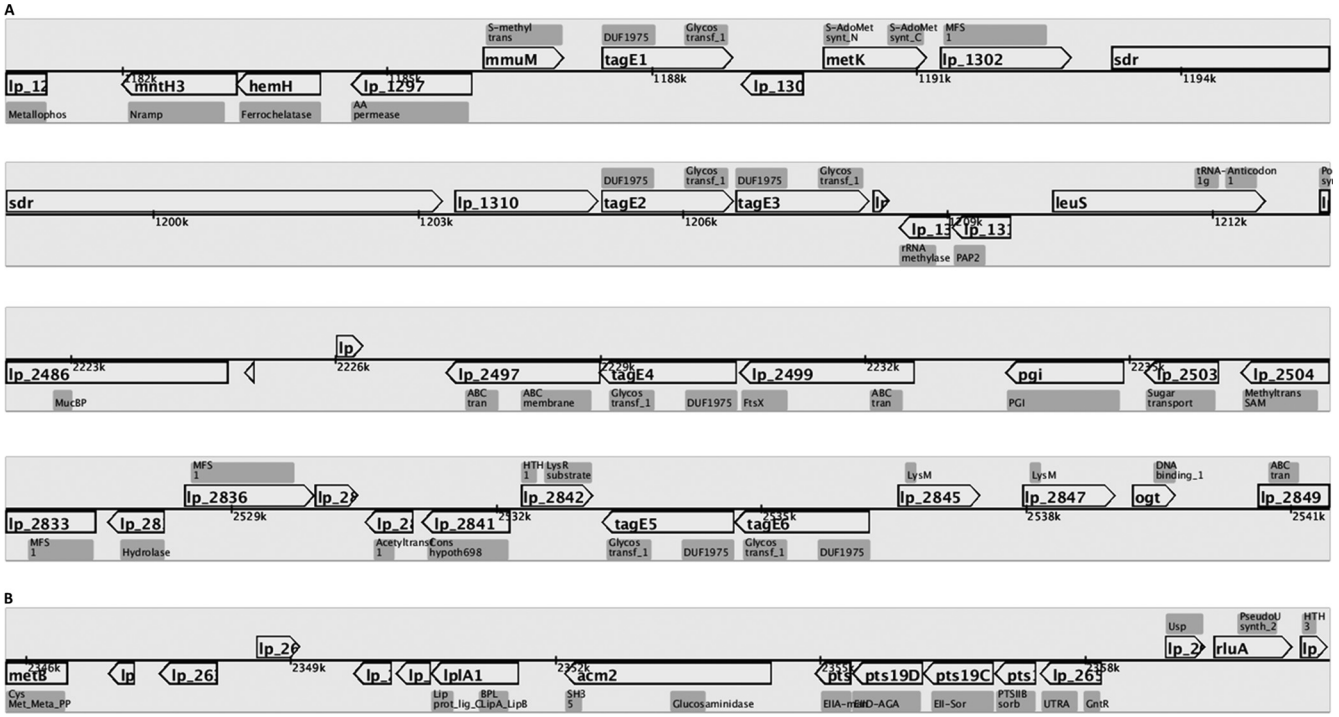

FIG 1 Genetic organization views of *tagE1-E6* and their neighboring genes (A) and *acm2* (B) generated by Microbial Genomic context Viewer (MGcV) (76). The annotated Pfam domains of each gene are shown in gray.

lingame, CA; final concentration of 14.3 μg/ml), *Dolichos biflorus* lectin (Sigma-Aldrich, Zwijndrecht, the Netherlands; final concentration of 14.3 μg/ml), or *Lens culinaris* lectin (EY Labs Inc., San Mateo, CA; final concentration of 5 μg/ml) in the blocking solution, followed by incubation with 0.1 nl/ml (1:10,000 dilution) of streptavidin poly-horseradish peroxidase (poly-HRP; ImmunoTools GmbH, Friesoythe, Germany). In between the incubations, the membranes were washed three times with PBST for 15 min. Precision Plus protein dual color standards (Bio-Rad, Richmond, CA) were used as a reference of molecular size. RNase B (New England BioLabs, Ipswich, MA) was used as a positive control for sWGA blotting. After the membranes were washed, they were developed by using Super Signal West Pico chemiluminescent substrate (Thermo Scientific) and Kodak BioMax Light film (Kodak, Rochester, NY).

Mass spectrometry. The protein bands apparent around 100 kDa were excised from a Coomassie blue-stained gel (see above), followed by characterization of the glycosylation pattern using the same method as described by Rolain et al. (42). Briefly, the protein was in-gel digested with trypsin (Promega) for 16 h at 37°C. Digested peptides were recovered and vacuum dried (Speedvac SC200; Savant). Peptides were then dissolved in 0.025% (vol/vol) trifluoroacetic acid (TFA) and 5% (vol/vol) acetonitrile (ACN), desalted using a C_{18} Pep Map 100 precolumn (10 mm, 5-μm inside diameter [i.d.], 100 Å), and subsequently subjected to reverse-phase chromatography using Ultimate 3000 chromatography chain (LC Packings) with a C_{18} Pep Map 100 analytical column (150 mm, 3-μm i.d., 100 Å). Peptides were back-flushed onto the analytical column with a flow rate of 300 nl/min using a 180-min linear gradient from 8 to 76% (vol/vol) ACN in water containing 0.1% (vol/vol) TFA in 4% ACN–0.1% TFA and 0.085% (vol/vol) TFA in 80% ACN–0.1% TFA. The eluted peptides were mixed with α-cyano-4-hydrocinnamic acid (4 mg/ml in 70% ACN-0.1% TFA) and spotted directly onto a matrix-assisted laser desorption ionization (MALDI) target using a Probot system (LC Packings). The spotted plates were analyzed in reflector mode on an Applied Biosystems 4800 MALDI–time of flight (TOF)/TOF analyzer using a 200-Hz solid-state laser operating at 355 nm. MS spectra were obtained using a laser intensity of 3,600 and 2,000 laser shots per spot in the *m/z* range of 800 to 4,000, while MS/MS spectra were obtained by automatic selection of the 20 most intense precursor ions per spot using a laser intensity of 4,000 and 2,000 laser shots per precursor. Collision-induced dissociation was performed with an energy of 1 kV with air gas at a pressure of 1×10^6 torr. Data were collected using Applied Biosystems 4000 series Explorer software. LC-MS/MS data were processed using Applied Biosystems GPS Explorer 3.6 software.

For peptide identification, a local database containing Acm2 sequence was used, with the tolerance set to 200 ppm on the precursors and 0.3 Da on the fragments. One trypsin miscleavage was authorized. For modifications, methionine oxidation and *N*-acetylhexosamine (HexNAc) glycosylation (203.08 Da) on Ser, Thr, and Asn were selected. HexNAc-modified peptides were checked by manual *de novo* sequencing on the MS/MS fragmentation spectra. The data presented for the wild type (WT) were combined from 2 independent analyses, while those for the *tagE5E6* deletion mutant were combined from 3 independent analyses.

RESULTS

Comparative genomics and mutagenesis of candidate protein glycosyltransferases. Acm2 of *Lactobacillus plantarum* WCFS1 was previously established to be *O*-glycosylated with *N*-acetylhexosamines, most likely GlcNAc, at multiple positions in its AST domain (41, 42). Glycosylation with GlcNAc was also found in flagellin of *Listeria monocytogenes* (35) and Fap1 of *S. parasanguinis* (37, 38). The glycosylation with GlcNAc moieties in the latter species requires two genetically coupled functions, Gtf1 and Gtf2 (37). Bu et al. and Wu and Wu suggested that Gtf1 catalyzes GlcNAc glycosylation via its C-terminal glycosyltransferase domain, while Gtf2 might act as a chaperone to maintain correct folding of Gtf1 and to promote efficient glycosylation (37, 53). Based on these previous findings, we performed a BLASTP analy-

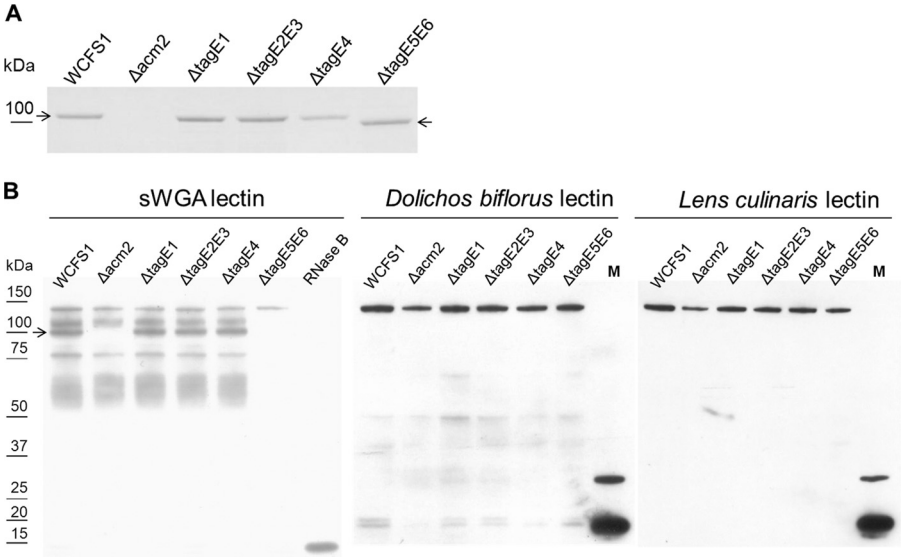

FIG 2 (A) Coomassie brilliant blue-stained SDS-PAGE gel of surface proteins extracted from the *tagE* deletion mutants as well as from the wild type and *acm2* deletion mutant to detect Acm2 (indicated by the arrow). (B) sWGA, *Dolichos biflorus* lectin, and *Lens culinaris* lectin blots of whole-cell extracts derived from the *tagE* deletion mutants, *Lactobacillus plantarum* WCFS1 (wild type), and the *acm2* deletion mutant to assess glycan moieties. On the left side of the blot the protein sizes are indicated based on the Precision Plus protein dual color standard (Bio-Rad) molecular marker (data not shown). M, CandyCane glycoprotein molecular size marker. The arrow indicates Acm2.

sis (54, 55) using the Gtf1 sequence to identify candidate protein glycosyltransferases in the *L. plantarum* WCFS1 genome. Six genes (*tagE1* to *tagE6*) which are annotated as poly(glycerolphosphate) α-glucosyltransferases, and consequently are thought to be involved in teichoic acid glycosylation (46), appeared the closest homologues of the Gtf1 protein, and all share more than 20% sequence identity with Gtf1. Two pairs of *tagE* genes are genetically coupled in the *L. plantarum* chromosome (*tagE2-tagE3* and *tagE5-tagE6*) (Fig. 1). Therefore, all 6 *tagE* genes identified were targeted by gene deletion, with the notion that the genetically coupled *tagE* pairs were deleted jointly. This genetic engineering approach yielded four *L. plantarum* WCFS1 derivatives, NZ3540Cm (Δ*tagE1*), NZ3541Cm (Δ*tagE2E3*), NZ3542Cm (Δ*tagE4*), and NZ3543Cm (Δ*tagE5E6*).

Deletion of *tagE5* and *tagE6* abolishes protein glycosylation in *L. plantarum* WCFS1. Surface proteins derived from the *tagE* deletion mutants, as well as the wild type and *acm2* deletion mutant (41), were analyzed by SDS-PAGE. One protein band detected in the wild-type extract appeared to be absent in the sample derived from the *acm2* deletion derivative, suggesting that this protein band represents Acm2 (Fig. 2A). To substantiate this suggestion, the band representing Acm2 was excised and in-gel digested with trypsin, and the resulting peptides were extracted and subjected to liquid chromatography coupled to mass spectrometry analysis (LC-MS/MS), which generated MS spectra that represented 75% and 69% coverages of the mature Acm2 protein sequence and its N-terminal glycosylated AST domain, respectively (see Fig. S1A in the supplemental material). Importantly, the MS spectra detected 5 different glycopeptides (designated glycos I, II, IV, V, and VI, according to nomenclature introduced by Rolain et al. [42] [Table 3]) that appeared all located in the AST domain and to be 1-, 2-, or 3-fold substituted with a molecule of an approximate mass of 203 Da, which corresponds to the previously suggested glycosylation with GlcNAc (41, 42, 45) (Table 3; see also Table S1 in the supplemental material). This observation is in agreement with the apparent molecular mass of wild-type Acm2, which was estimated to be approximately 100 kDa (Fig. 2), higher than the molecular mass of 78.9 kDa predicted on basis of the mature protein sequence. Moreover, we were able to identified some of the glycosylated residues (Table 3; see also Table S1), but not all. These glycosylated residues were all also found in the study of Rolain et al. (42). Interestingly, different glycosylated forms were found in glyco II (one or two HexNAc) and glyco VI (one, two, or three HexNAc), which might imply a dynamic level of glycosylation in Acm2. In the Δ*tagE1*, Δ*tagE2E3*, and Δ*tagE4* mutants, the mobility of the Acm2 protein appeared to be unaffected compared to that of the wild-type strain. In contrast, the Acm2 protein present in the wild type was absent in the Δ*tagE5E6* deletion strain, but a protein band of higher mobility (lower apparent molecular mass) appeared in the gel (Fig. 2A). These observations provide a first clue that TagE5 and/or TagE6 is involved in the glycosylation of Acm2. Indeed, the loss of glycosylation of Acm2 in the Δ*tagE5E6* deletion strain could also be confirmed by LC-MS/MS, as the Acm2 protein band extracted from gel was used to generate MS spectra that enabled 52% and 59% coverages of the mature Acm2 protein sequence and its AST domain, respectively (Fig. S1B). Notably, 6 distinct peptides of the AST domain that contained proposed HexNAc glycosylations (42) (designated glycos II to VII [Table 3]) that were detected in the wild-type Acm2 protein spectra (glycos II, IV, V, and VI in this study and glyco VII in the work of Rolain et al. [42]) were also detected in the Acm2 protein spectra derived from the *tagE5E6* deletion strain, although in the latter strain, these peptides consistently lacked the substitutions seen in the wild type (Table 3; see also Table S1). In addition, three peptides (glycos III, IV, and V) were detected exclusively in their nonglycosylated form in Acm2 isolated from the Δ*tagE5E6* mutant (Table 3). These data reveal that the HexNAc-glycosylated peptides derived from Acm2 are detected only in their nonglyco-

TABLE 3 Numbers of HexNAc on trypsinized Acm2 peptides isolated from the wild type or the TagE5E6 deletion mutant (Δ*tagE5E6*)

			WT			Δ*tagE5E6* mutant		
Name[a]	Peptide sequence[b]	Calculated $[M + H]^{+c}$	No. of HexNAc[d]	Observed *m/z*, WT	Δm (Da), WT[e]	No. of HexNAc	Observed *m/z*, WT	Δm (Da), Δ*tagE5E6* mutant[e]
Glyco I	GNSAASAASQQVTLSAGSQTETTAA GATDQSVASDGAK	3,495.62	2	3,901.25	405.63	ND		
Glyco II	TDDQAESTSTTTATTSATSR	2,030.89	0	2,031.62	0.73	0	2,031.67	0.78
Glyco II	TDDQAESTSTTTATTSATSR	2,030.89	1	2,234.68	203.79			
Glyco II	TDDQAESTSTTTATTSATSR	2,030.89	2	2,437.74	406.85			
Glyco III	ADSTGPQSQSSASEAAK	1,620.72	ND			0	1,621.55	0.83
Glyco IV	DNAATSATADSTTSAVDQLDK	2,080.94	2	2,487.75	406.81	ND		
Glyco V	ASAATSQASHSTTNETAK	1,761.81	2	2,168.75	406.94	0	1,762.63	0.82
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2,576.22	0	2,576.86	0.64	0	2,576.9	0.68
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2,576.22	1	2,779.89	203.67			
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2,576.22	2	2,982.94	406.72			
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2,576.22	3	3,186	609.78			

[a] Glycopeptide number as reported previously (42).
[b] The glycosylated amino acids that could be identified are underlined.
[c] Calculated $[M + H]^+$ values correspond to nonglycosylated peptides.
[d] Number of HexNAc detected or not (ND, not detected) of peptides by LC-MS/MS from secreted Acm2 digested by trypsin.
[e] Δm, difference between calculated and observed *m/z* values.

sylated form in the Δ*tagE5E6* mutant (Table 3), supporting the role of TagE5E6 in the Acm2 glycosylation that is observed in the wild-type strain.

To further investigate this, we employed a lectin-based detection of glycan moieties using biotinylated sWGA, specific for GlcNAc, in a Western blot-like setup. This approach showed that an sWGA-recognized protein of approximately 100 kDa derived from the wild type was absent in the *acm2* deletion strain, reconfirming the glycosylation of Acm2. Moreover, the glycans linked to these proteins react only with the GlcNAc-specific lectin sWGA and not with *Dolichos biflorus* lectin (specific for α-GalNAc) or with *Lens culinaris* lectin (specific for α-mannose) (Fig. 2B), implying that the glycan is most likely GlcNAc. Interestingly, the sWGA blot revealed signals other than Acm2 that appeared to be glycosylated and were detected in both wild-type- and *acm2* mutant-derived whole-cell extracts. All these signals were lost when sWGA blotting experiments were performed using samples that were proteinase K treated, indicating that all glycan signals in the lectin blotting experiment were derived from proteinaceous molecules (Fig. 3). In addition, the sWGA blot revealed that deletion of *tagE1*, *tagE2E3*, or *tagE4* did not affect protein glycosylation, since these mutants displayed the same banding pattern as was observed for the wild-type strain. In contrast, deletion of *tagE5* and *tagE6* abolished almost all detectable sWGA-specific signals, including that of Acm2, indicating that TagE5 and TagE6 play a critical role in the glycosylation of Acm2 and the additional proteins detected. Intriguingly, a single band with an apparent molecular mass of approximately 125 kDa not only appeared to be unaffected by the Δ*tagE5E6* mutation but also was recognized by *Dolichos biflorus* and *Lens culinaris* lectins (Fig. 2B), implying that another, TagE5/E6-independent mechanism of glycosylation may be active for the glycosylation of this particular protein. Taken together, these results evidence the essential role of TagE5 and/or TagE6 for protein glycosylation in *L. plantarum* WCFS1.

Both TagE5 and TagE6 are required for protein glycosylation in *L. plantarum* WCFS1. To investigate whether TagE5, TagE6, or their concerted action is required for protein glycosylation in *L. plantarum* WCFS1, the Δ*tagE5E6* mutant was complemented with *tagE5*, *tagE6*, or both genes. Complementations were achieved by integrating a single copy of the original gene(s) at a specific chromosomal site located downstream of the $tRNA^{Ser}$ locus, under the control of the native *tagE6* promoter. Whole-cell extracts from the different complemented Δ*tagE5E6* derivatives were analyzed by SDS-PAGE and sWGA blotting and compared to extracts derived from the wild type, as well as the Δ*acm2* and Δ*tagE5E6* mutants. Complementation with either *tagE5* or *tagE6* did not restore protein glycosylation and generated the same banding patterns as observed for the Δ*tagE5E6* strain (Fig. 4). However, complementation with the complete locus, encompassing both *tagE5* and *tagE6*, restored the glycosylation not only of Acm2 but also of all other proteins that were detected in the wild-type banding pattern. These results indicate that glycosylation of proteins in *L. plantarum* WCFS1 requires both TagE5 and TagE6 activities.

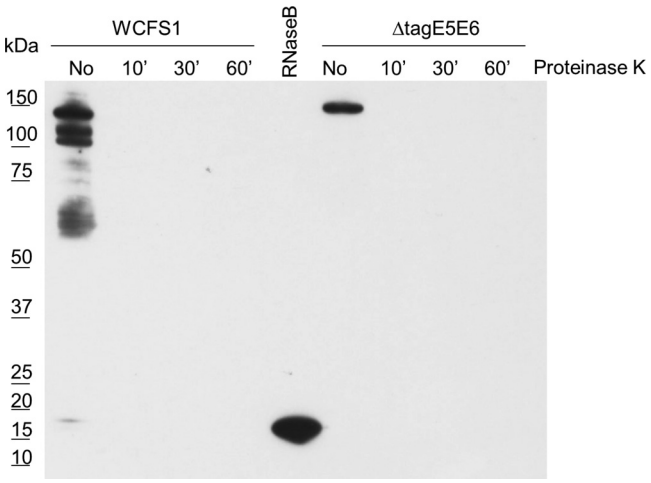

FIG 3 sWGA blot of whole-cell extracts derived from the wild type and *tagE5E6* deletion mutant with or without proteinase K treatment for 10, 30, or 60 min. On the left side of the blot the protein sizes are indicated based on the Precision Plus protein dual color standard (Bio-Rad) molecular marker.

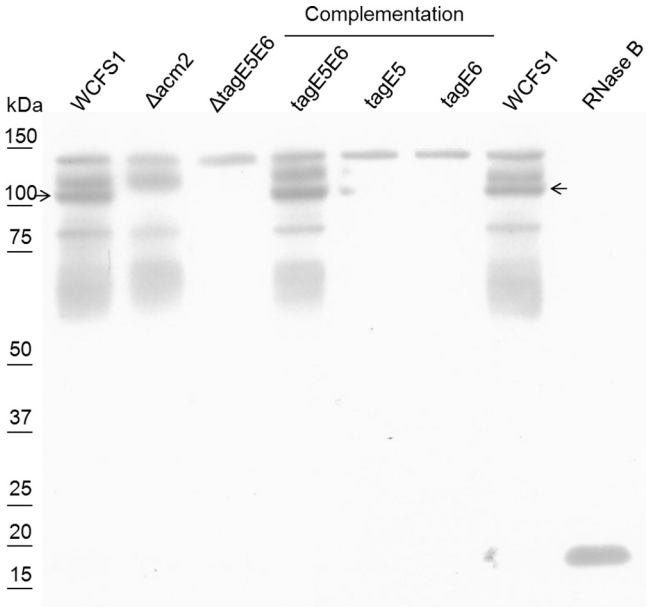

FIG 4 sWGA blot of the wild type, *acm2* and *tagE5E6* deletion mutants, and a panel of complemented mutants. On the left side of the blot the protein sizes are indicated based on the Precision Plus protein dual color standard (Bio-Rad) molecular marker (data not shown). The arrow indicates Acm2.

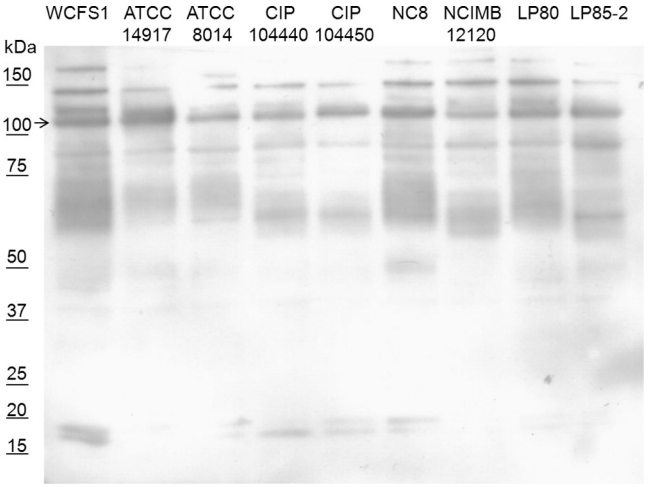

FIG 5 Assessment of 9 *Lactobacillus plantarum* strains for protein glycosylation, using whole-cell extract SDS-PAGE and sWGA lectin-based detection. On the left side of the blot the protein sizes are indicated based on the Precision Plus protein dual color standard (Bio-Rad) molecular marker (data not shown). The arrow indicates Acm2 of strain WCFS1.

Protein glycosylation is a common feature in *L. plantarum* strains. Using previously generated comparative genome hybridization (CGH) data for 42 *L. plantarum* strains (56, 57), we concluded that the 6 *tagE* genes recognized in the genome of *L. plantarum* WCFS1 appear to be conserved among all these 42 strains, with the notable exception of strain NCIMB12120, which appeared to lack genes that hybridize to the *L. plantarum* WCFS1 *tagE4*, *tagE5*, and *tagE6* probes. To evaluate glycosylation of proteins in other *L. plantarum* strains, 9 of the 42 mentioned strains were selected, including NCIMB12120 and LP85-2 from *Lactobacillus plantarum* subsp. *argentoratensis*, for analysis of whole-cell extracts by SDS-PAGE and GlcNAc-specific sWGA blotting. Notably, these 9 strains were selected to maximize the coverage of the phylogenetic tree based on the whole-genome comparative genome hybridization data sets (56), as well as to include strains isolated from diverse niches (Table 1). All selected strains, including NCIMB12120, displayed sWGA-recognized glycosylated proteins that showed similar banding patterns on SDS-PAGE gels (Fig. 5). This result strongly suggests that glycosylation of proteins is a common feature in the species *L. plantarum*.

DISCUSSION

Existing information on the protein glycosyltransferase Gtf1 in *S. parasanguinis* (37, 38, 41) enabled us to employ a comparative genomics approach, resulting in the identification of the 6 TagE orthologues as candidate protein glycosyltransferases in *L. plantarum* WCFS1. All 6 TagE proteins contain a GT1_gtfA-like domain designated cd04949 (58) at their C-terminal ends (46). This domain is named after *gtfA* in *Streptococcus gordonii*, in which it plays a role in the *O*-linked glycosylation, and this family containing this domain is most closely related to the GT1 family of glycosyltransferases (58). Limiting the amount of glycosyltransferases (50 annotated in the *L. plantarum* WCFS1 genome [46]) to the six TagE glycosyltransferases as most likely candidates for protein glycosylation enabled us to perform a systematic gene deletion and complementation strategy, followed by assessment of the consequences for protein glycosylation. This approach revealed that the concerted activities of TagE5 and TagE6 are required for the previously established glycosylation of Acm2 (41, 42), as well as other proteins. To the best of our knowledge, these results represent the first example of protein glycosylation machinery in a *Lactobacillus* species. The TagE proteins of *L. plantarum* WCFS1 are annotated according to their originally predicted function in teichoic acid glycosylation [poly(glycerolphosphate)-α-glucosyltransferases]. The glucose substitution levels in lipoteichoic acids (LTA) of *L. plantarum* WCFS1 are very low (59, 60), while glucose is a backbone constituent into the repeating unit of wall teichoic acids (WTA) that does not have additional glucose substitutions in this strain (60, 61). Neither LTA nor WTA of *L. plantarum* WCFS1 contain *N*-acetylhexosamine (59, 61), the glycan transferred by TagE5 and TagE6. Since the glucose substitution level is very low in LTA from *L. plantarum* WCFS1, we have isolated deacylated and dealanylated LTA (dd-LTA) to obtain better nuclear magnetic resonance (NMR) spectral resolution for signals from anomeric protons of sugar residues. The one-dimensional (1D) ^{1}H NMR spectra revealed that dd-LTA isolated from Δ*tagE5E6* mutants has the same level of glycosylation as LTA isolated from the WT (see Fig. S2A in the supplemental material). Moreover, 2D NMR spectra also showed that the glycosylation position of LTA is unaltered in the *tagE5E6* deletion mutant (Fig. S2B). Therefore, with the establishment of TagE5 and TagE6 as dedicated protein glycosyltransferases, we propose to rename these enzymes (and genes) to GtfA (*gtfA*) and GtfB (*gtfB*), respectively.

Currently established bacterial *O*-linked glycosylation pathways employ either block or sequential transfer pathways for the addition of sugars to proteins (26). The block transfer pathway is exemplified by the glycosylation of *Neisseria* sp. pilin. This pathway assembles an oligosaccharide using nucleotide-activated sugars on a lipid anchor at the cytoplasmic side of the inner membrane. The assembled oligosaccharide is subsequently translo-

cated across the inner membrane by a flippase to the periplasm, where the lipid-linked oligosaccharide is transferred to Ser/Thr residues of proteins (26, 33). On the other hand, the sequential transfer pathway, for example, employed in flagellar glycosylation of *Campylobacter jejuni*, transfers nucleotide-activated sugars individually onto Ser/Thr residues of proteins at the cytoplasm-inner membrane interface (26). Acm2 of *L. plantarum* WCFS1 undergoes cytoplasmic *O*-glycosylation with single *N*-acetylhexosamine moieties, likely GlcNAc, at multiple sites of its AST domain (41, 42). The fact that this glycosylation occurs in the cytoplasm might imply that the machinery responsible for Acm2 glycosylation is more similar to the sequential transfer pathway. Moreover, the glycosylation nature of Acm2 is similar to that of the glycosylation found in flagellin from *L. monocytogenes*, which is glycosylated with single GlcNAc at 3 to 6 sites (35), and in Fap1 fimbrial adhesin from *S. parasanguinis*, of which all the oligosaccharides are primed with GlcNAc (37). Interestingly, since the glycosyltransferases responsible for their glycosylations (GmaR for listerial flagellin [62] and Gtf1/Gtf2 for Fap1 [37, 53]) are predicted to be cytoplasmic proteins, the glycosylations of flagellin from *L. monocytogenes* and Fap1 from *S. parasanguinis* are also thought to occur in the cytoplasm. Notably, another example of a *Lactobacillus* glycoprotein, Msp1 of *L. rhamnosus* GG, was found to be glycosylated in the supernatant but not in the cytosolic fraction (43), hinting at species-specific *O*-glycosylation pathways in *Lactobacillus* species.

We have successfully identified the glycosyltransferases GtfA/B for the glycosylation of Acm2 based on the similar glycan moieties found in Fap1. However, the protein property and function are completely different between Acm2 and Fap1. Fap1 belongs to serine-rich repeat proteins (SRRPs), which are a family of surface-exposed adhesion-mediated proteins predominately found within the oral *Streptococcus* species (63). Currently, seven SRRPs have been researched, including Fap1 of *S. parasanguinis*, Has and GspB of *S. gordonii*, PsrP of *Streptococcus pneumoniae*, Srr-1 and Srr-2 of *Streptococcus agalactiae*, and SraP of *Staphylococcus aureus* (53). Each SRRP locus locates at a close proximity with a highly conserved core region, consisting of accessory secretory components and two essential glycosyltransferases (38). In this study, we found that GtfA and GtfB are required for the glycosylation of Acm2 as well as unidentified proteins other than SRRPs. Moreover, *acm2* (*lp_2645*) locates in a distinct region of the chromosome, not linked to the *gtfA/B* (*lp_2843/lp_2844*) genes (Fig. 1), which is also distinct from *Streptococcus* SRRPs. Recently, the two glycosyltransferases, Gtf1 and Gtf2, of *S. parasanguinis* have been investigated, and it was found that the glycosylation of Fap1 requires the glycosyltransferase activity from Gtf1 together with the chaperone function of Gtf2 to maintain the correct folding of Gtf1 (53). However, GtfA and GtfB in *L. plantarum* are both homologues of Gtf1 in *S. parasanguinis* and display much lower similarity with the chaperone Gtf2. Although we have not experimentally excluded the possibility that the coexpression of GtfA or GtfB of *L. plantarum* WCFS1 is required for the correct folding of GtfB or GtfA, respectively, it does not seem likely that either GtfA or GtfB acts as a chaperone.

Comparative genome hybridization (CGH) data suggest that 6 orthologues of *tagE* genes are typically present in *L. plantarum* strains (56), with the notable exception of strain NCIMB12120, which appears to lack *tagE4* and *gtfA-gtfB*. However, this strain still contains sWGA-recognized, glycosylated proteins, similar to the other 7 strains tested (Fig. 5). NCIMB12120 belongs to a subspecies (*L. plantarum* subsp. *argentoratensis*) different from reference strain WCFS1. Strains in this subspecies commonly have a smaller genome size (64) and appear to lack homologues of approximately 20% of the genes present in WCFS1 (57). Despite the apparent absence of *tagE4* and *gtfA-gtfB* in NCIMB12120, the glycosylation of proteins apparently still occurs, suggesting that this strain (subspecies) contains genes with the same function that are of low homology and therefore were missed in the CGH analysis. Taken together, our data suggest that glycosylation and the presence of *tagE* genes are common features in *L. plantarum* strains. Moreover, the sequence and length of Acm2 are highly similar in all sequenced *L. plantarum* strains, e.g., WCFS1 (46), ST-III (65), JDM1 (66), and NC8 (67) (785 residues) and ATCC 14917 (781 residues). This suggests that the Acm2 proteins of different *L. plantarum* strains may all have similar sizes as well as similar degrees of glycosylation and are represented by the universal abundant protein band around 100 kDa (Fig. 5).

Other *Lactobacillus* species also harbor genetically coupled *gtfA-gtfB* homologues, for example, *tagE2-tagE3* of *Lactobacillus casei* BL23, *lsei_0891-lsei_0892* of *L. casei* ATCC 334 (68), and *yohH-yohJ* of *L. rhamnosus* GG (69). However, the genomes of other species, including *Lactobacillus acidophilus* NCFM (70), do not appear to contain *gtfA-gtfB* homologues, while *Lactobacillus johnsonii* NCC533 (71) and *Lactobacillus delbrueckii* subsp. *bulgaricus* ND02 (72) harbor a single gene displaying similarity with *gtfA-gtfB*. Although we successfully identified the role of GtfA-GtfB in glycosylation of proteins based on their sequence homology with Gtf1 of *S. parasanguinis*, sequence similarity alone did not provide a direct identification of this specific glycosyltransferase function, since all 6 TagE proteins display similar degrees of sequence homology with Gtf1. The role of the other 4 TagE glycosyltransferases in *L. plantarum* WCFS1 is currently unestablished but might involve the transfer of other glycan moieties to proteins or *N*-glycosylation. Indeed, among recently found glycoproteins in *L. plantarum* WCFS1, glycosylation of hexoses was also found in Lp_2162, Lp_3421, and DnaK, besides the HexNAc substitutions already established for Acm2 (45). Moreover, some lectin-based studies suggested the presence of glycoproteins modified with glycans other than GlcNAc, such as glycoproteins of *L. acidophilus* JCM1132^{T} (recognized by β-galctoside-specific lectin) (73), SlpA of *L. acidophilus* NCFM (recognized by fucose- and mannose-specific lectins) (19, 74), and Msp1 of *L. rhamnosus* GG (recognized by glucose- and mannose-specific ConA lectin) (43). Furthermore, many *Lactobacillus* genomes contain the genes to produce multiple nucleotide-activated sugars, including UDP-glucose, UDP-galactose, sialic acid, and dTDP-rhamnose (6), suggesting the potential capacity to glycosylate proteins with diverse sugar moieties. Alternatively, WTA of *L. plantarum* WCFS1 contains glucose in its backbone (61), and biosynthesis of this structure could require the activity of specific TagE proteins, as predicted by the current annotation.

We have conclusively shown that protein glycosylation is a common feature in *L. plantarum* strains and does not target a single protein but modifies a much broader range of proteinaceous compounds. One important question remains unanswered: what is the biological role of protein glycosylation in lactobacilli? Earlier studies with pathogens showed that glycoproteins are often involved in adherence, pathogenicity, flagellum assembly, and protein stability (23). A more recent example illus-

trated that the glycans attached on surface layer proteins of *Tannerella forsythia*, which is implicated in periodontitis, modulate the function of DCs and suppress T-helper 17 responses (75). In light of this, it is intriguing that glycosylation of Msp1 of *L. rhamnosus* GG is not essential for its peptidoglycan hydrolyase activity (17, 43), nor for activating Akt signaling in Caco-2 cells (43), but does influence Msp1 protein stability and protein localization (43). Moreover, Lebeer et al. suggested the possibility of an indirect modulating role of the Msp1 glycan moieties in Akt activation via shielding bacteria and host interaction (43). Furthermore, the ConA and *Aleuria aurantia* (AAL)-reactive glycans on SlpA of *L. acidophilus* NCFM are essential for the modulation of T cell function and lead to more IL-4 production (19). Importantly, it was recently established that *O*-glycosylation of Acm2 in *L. plantarum* functions as a major negative modulator of Acm2 peptidoglycan hydrolase activity (42), which is the first evidence that glycosylation regulates the bacterial enzyme activity. In fact, we observed different glycoforms of glyco II and glyco VI (Table 3), which might imply a kinetic modulation of Acm2 hydrolase activity via *O*-glycosylation (42). Our future work will focus on recognizing the biological roles of glycosylation of other proteins in *L. plantarum*, especially in relation to its possible consequences for host-microbe interactions in the gastrointestinal tract.

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